



# Pneumococcal Vaccines



Edited by

George R. Siber

Keith P. Klugman

P. Helena Mäkelä

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**The Impact of  
Conjugate Vaccine**

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## The Impact of Conjugate Vaccine

Edited by

**George R. Siber**

*Wyeth Vaccines Research (retired),  
Pearl River, New York*

**Keith P. Klugman**

*Rollins School of Public Health,  
Emory University, Atlanta, Georgia,  
and University of the Witwatersrand,  
Johannesburg, South Africa*

**P. Helena Mäkelä**

*National Public Health Institute,  
Helsinki, Finland*



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## *Section Editors*

**JOHN W. BOSLEGO**

PATH, 1800 K Street, N.W., Suite 800, Washington, DC 20006

**CARL E. FRASCH**

Frasch Biologics Consulting, P. O. Box 986, Martinsburg, WV 25402

**HELENA KÄYHTY**

National Public Health Institute, Mannerheimintie 166, 00300 Helsinki,  
Finland

**KEITH P. KLUGMAN**

Rollins School of Public Health, Emory University, Atlanta, GA 30322

**P. HELENA MÄKELÄ**

National Public Health Institute, 00300 Helsinki, Finland

**STEPHEN I. PELTON**

Boston University Schools of Medicine and Public Health, Boston Medical  
Center, Boston, MA 02118

**GEORGE R. SIBER**

Wyeth Vaccines Research (retired), Pearl River, NY 10965

**CYNTHIA G. WHITNEY**

Centers for Disease Control and Prevention, 1600 Clifton Road NE,  
Mailstop C23, Atlanta, GA 30333

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# *Contributors*

RICHARD A. ADEGBOLA

Bacterial Diseases Programme, Medical Research Council (UK) Laboratories,  
Atlantic Boulevard, Fajara, The Gambia

TRACY ASSARI

Institute of Child Health, University College London, 30 Guilford St., London,  
WC1N 1EH, United Kingdom

STEPHEN D. BENTLEY

Sanger Institute, Wellcome Trust Sanger Institute, Hinxton, Cambridge, United  
Kingdom

STEVEN BLACK

Dept. of Pediatric Infectious Diseases, Stanford University, Palo Alto, CA 94304

MILAN S. BLAKE

Division of Bacterial, Parasitic and Allergenic Products, Office of Vaccine  
Research and Review/Center for Biologics Evaluation and Research, U.S. Food  
and Drug Administration, 29 Lincoln Dr., Bethesda, MD 20892

JOHN W. BOSLEGO

PATH, 1800 K St., N.W., Suite 800, Washington, DC 20006

DAVID R. BOULWARE

Infectious Disease & International Medicine, Dept. of Medicine, University of  
Minnesota, MMC 250, 420 Delaware St. SE, Minneapolis, MN 55455

DAVID E. BRILES

Dept. of Microbiology and Dept. of Pediatrics, 658 Bevill Biomedical Sciences  
Building, University of Alabama at Birmingham, Birmingham, AL 35294

**ANGELA B. BRUEGEMANN**

Dept. of Zoology, University of Oxford, Oxford, OX1 3PS, United Kingdom

**JAY C. BUTLER**

Alaska Division of Public Health, Anchorage, AK 99508

**FELICITY CUTTS**

Medical Research Council (UK) Laboratories, Atlantic Boulevard, Fajara,  
The Gambia

**RON DAGAN**

Pediatric Infectious Disease Unit, Soroka University Medical Center, and the  
Faculty of Health Sciences, Ben-Gurion University of the Negev, P.O. Box 151,  
Beer-Sheva 84101, Israel

**CARL E. FRASCH**

Frasch Biologics Consulting, P.O. Box 986, Martinsburg, WV 25402

**NEIL FRENCH**

Infectious Disease Epidemiology Unit, London School of Hygiene & Tropical  
Medicine, London, United Kingdom, and Karonga Prevention Study, Box 46,  
Chilumba, Malawi

**DAVID GOLDBLATT**

Institute of Child Health, University College London, 30 Guilford St., London,  
WC1N 1EH, United Kingdom

**BARRY M. GRAY**

Pediatric Infectious Diseases, University of Illinois College of Medicine at  
Peoria, 530 NE Glen Oak Ave., Peoria, IL 61637

**BRIAN GREENWOOD**

London School of Hygiene & Tropical Medicine, Keppel Street, London WC1E  
7HT, United Kingdom

**MARION F. GRUBER**

Office of Vaccines Research and Review, Center for Biologics Evaluation and  
Research, Food and Drug Administration, 1401 Rockville Pike, WOC I, 360  
North, Rockville, MD 20852

**MANFRED HAASE**

Division of Bacteriology, Paul-Ehrlich-Institute, P.O. Box 1740, D-63207  
Langen, Germany

**JILL G. HACKELL**

New York, NY 10956

**WILLIAM P. HAUSDORFF**

GlaxoSmithKline Biologicals, Rue de l'Institut, 89, B-1330 Rixensart, Belgium

**JOHN P. HENNESSEY, JR.**

Bioprocess Research & Development, Merck Research Laboratories, West  
Point, PA 19486

**SUSAN K. HOLLINGSHEAD**

Dept. of Microbiology, 654 Bevill Biomedical Sciences Building, University of  
Alabama at Birmingham, Birmingham, AL 35294

**MARGARET K. HOSTETTER**

Dept. of Pediatrics, Yale School of Medicine, 333 Cedar St., P.O. Box 208064,  
New Haven, CT 06520-8064

**LISA A. JACKSON**

Group Health Center for Health Studies, 1730 Minor Ave., Suite 1600,  
Seattle, WA 98101

**EDWARD N. JANOFF**

Division of Infectious Diseases, Colorado Center for AIDS Research, University  
of Colorado at Denver and Health Sciences Center, Denver Veterans Affairs  
Medical Center, Denver, CO 80220

**JUKKA JOKINEN**

Dept. of Vaccines, National Public Health Institute, FIN-00300 Helsinki,  
Finland

**INGILEIF JONSDOTTIR**

Landspítali University Hospital and Faculty of Medicine, University of Iceland,  
Dept. of Immunology, Hringbraut, 101 Reykjavik, Iceland

**HELENA KÄYHTY**

National Public Health Institute, Mannerheimintie 166, 00300 Helsinki,  
Finland

**TERHI KILPI**

National Public Health Institute, FIN-00300 Helsinki, Finland

**KEITH P. KLUGMAN**

Hubert Dept. of Global Health, Rollins School of Public Health, Emory  
University, Atlanta, GA 30322, and Medical Research Council, University  
of the Witwatersrand Respiratory and Meningeal Pathogens Research Unit,  
Johannesburg, South Africa

**ROBERT C. KOHBERGER**

Blair and Company, Greenwich, CT 06831

**ANDREW LEES**

Fina BioSolutions LLC, 9610 Medical Center Dr., Suite 200, Rockville, MD  
20850

**ORIN S. LEVINE**

GAVI's Pneumococcal Vaccines Accelerated Development and Introduction Plan  
(PneumoADIP), and Dept. of International Health, Johns Hopkins Bloomberg  
School of Public Health, Baltimore, MD 21205

**STEPHEN LOCKHART**

Wyeth Vaccine Research, Taplow, Maidenhead, Berkshire SL6 0PH, United  
Kingdom

**SHABIR A. MADHI**

Respiratory and Meningeal Pathogens Research Unit, Medical Research  
Council/University of the Witwatersrand, and Department of Science and  
Technology/National Research Foundation: Vaccine Preventable Diseases,  
Bertsham, Gauteng, South Africa

DACE V. MADORE  
1 Schoen Rd., Pittsford, NY 14534-1125

P. HELENA MÄKELÄ  
Dept. of Vaccines, National Public Health Institute, FIN-00300 Helsinki,  
Finland

RICHARD MALLEY  
Division of Infectious Diseases, Children's Hospital, 300 Longwood Ave.,  
Boston, MA 02115

MATTHEW R. MOORE  
Centers for Disease Control and Prevention, 1600 Clifton Rd. N.E.,  
Atlanta, GA 30333

DANIEL M. MUSHER  
Infectious Disease Section, Veterans Affairs Medical Center, 2002 Holcombe  
Blvd., Houston, TX 77030

SHARON NACHMAN  
Dept. of Pediatrics, SUNY Health Science Center at Stony Brook,  
Stony Brook, NY 11794-8111

MOON H. NAHM  
Depts. of Pathology and Microbiology, University of Alabama at Birmingham,  
845 19th St. South (BBRB 614), Birmingham, AL 35294-2170

KATHERINE L. O'BRIEN  
Center for American Indian Health, Dept. of International Health,  
Johns Hopkins Bloomberg School of Public Health, 621 N. Washington St.,  
Baltimore, MD 21205

JAMES C. PATON  
School of Molecular and Biomedical Science, University of Adelaide,  
Adelaide, S.A., 5005, Australia

STEPHEN I. PELTON  
Pediatrics and Epidemiology, Boston University Schools of Medicine and Public  
Health, Maxwell Finland Laboratory for Infectious Diseases, Boston, MA  
02118

DOUGLAS PRATT  
Office of Vaccines Research and Review, Center for Biologics Evaluation and  
Research, Food and Drug Administration, 1401 Rockville Pike, WOC I, 308  
North, Rockville, MD 20852

VELUPILLAI PUVANESARAJAH  
Sanofi-Aventis, Swiftwater, PA 18370

SALLY A. QUATAERT  
University of Rochester Medical Center, 601 Elmwood Ave., Rochester, NY  
14642

G. THOMAS RAY  
Division of Research, Kaiser Permanente, 2000 Broadway, Oakland, CA 94612

**SANDRA ROMERO-STEINER**

Division of Bacterial Diseases, Centers for Disease Control and Prevention,  
Bldg. 18, Room B105, MS A-36, 1600 Clifton Rd., Atlanta, GA 30333

**JEFFREY B. RUBINS**

Division of Pulmonary Medicine, Veterans Affairs Medical Center, and  
University of Minnesota, Minneapolis, MN 55417

**MATHURAM SANTOSHAM**

Center for American Indian Health, Dept. of International Health, Johns  
Hopkins Bloomberg School of Public Health, Baltimore, MD 21205

**LODE SCHUERMAN**

GlaxoSmithKline Biologicals, Rue de l'Institut 89, 1330 Rixensart, Belgium

**J. ANTHONY G. SCOTT**

Wellcome Trust/KEMRI Centre for Geographic Medicine, Research,  
Coast, Kilifi, Kenya, and Nuffield Dept. of Medicine, University of Oxford,  
John Radcliffe Hospital, Headington, Oxford OX3 9DU, United Kingdom

**HENRY SHINEFIELD**

Dept. of Pediatrics, University of California-San Francisco,  
San Francisco, CA 94143

**GEORGE R. SIBER**

Wyeth Vaccines Research (retired), Pearl River, NY 10965

**ANUSHUA SINHA**

Dept. of Preventive Medicine and Community Health, New Jersey Medical  
School-UMDNJ, 185 South Orange Ave., Newark, NJ 07103

**CLIFFORD SNAPPER**

Dept. of Pathology, Uniformed Services University of the Health Sciences,  
Bethesda, MD 20814

**JONATHAN A. C. STERNE**

Dept. of Social Medicine, University of Bristol, Canyng Hall, Whiteladies  
Road, Clifton, Bristol, BS8 2PR, United Kingdom

**MERJA VAKEVAINEN**

Dept. of Vaccines, National Public Health Institute, Mannerheimintie 166,  
FIN-00300 Helsinki, Finland

**JEFFREY N. WEISER**

University of Pennsylvania, 402A Johnson Pavilion,  
Philadelphia, PA 19104-6076

**CYNTHIA G. WHITNEY**

Centers for Disease Control and Prevention, 1600 Clifton Rd. N.E., Mailstop  
C23, Atlanta, GA 30333

**JANET YOTHER**

Dept. of Microbiology, University of Alabama at Birmingham,  
Birmingham, AL 35242

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## Dedication

Robert Austrian, M.D., died on March 25, 2007, just before his ninety-first birthday. Since it was largely through his efforts that pneumococcal disease was recognized to be a continuing problem in the antibiotic era, and that the first licensed pneumococcal vaccine was developed, it seems fitting that this book be dedicated in his honor. Dr. Austrian had a remarkable career. His nearly 7-decade assault on the pneumococcus was best summarized by the words of Lewis Thomas, M.D., in the forward to Dr. Austrian's book, *Life with the Pneumococcus*.

The major figures in American biomedical research come in several quite different classes. There are those who shift swiftly from problem to problem, sometimes leaping freely from one biological discipline to another and then back again, lighting finally on a soluble problem as though by accident. There are others who meditate on a single puzzle for years at a time, scarcely moving, and then, obsessed overnight by the idea of a lifetime, swoop down like nightowls on the single answer.

And there are those who pick out the one problem that will preoccupy them for an entire career of hard work and then just keep at it, year after year. This may seem the safest way to live a life in science, but it is actually, in real life, the chanciest of all gambles, like putting all your chips on a single number, play after play, until all your money runs out.

Robert Austrian's career has been this last kind. He became fascinated by a single microorganism, *Streptococcus pneumoniae*, long ago, and simply stuck with it. As the years went by, some of his colleagues came to believe that he was simply stuck with it. Finally, not as a result of good luck or any nocturnal revelation or unforeseen laboratory accident, but as the uncommon reward for steady, meticulous, logical experimentation, he got what he was after: a polyvalent vaccine against pneumococcal infection.

The papers in this book are a nice historical record of how science goes when it is going slowly but going well. They are also a lesson in what most savvy investigators take on faith: if you can learn enough new things about living things at a fundamental level, sooner or later you may have the chance, as Austrian has had, to turn basic science onto applied science and, at last, into a useful product.

In fact, after this passage was written and the book appeared in 1985, Dr. Austrian spent another 22 years, until the day prior to his death, monitoring the evolution of pneumococcal types. It was my pleasure to work with him at the same institution over the last 15 of these years while he continued his work as an emeritus professor. While there are many treasured memories and lessons gained from interacting with this grand gentleman of medicine, one seems especially fitting for this book. Dr. Austrian's career spanned the use of serum therapy, chemotherapy, antibiotics, and two generations of vaccines, each of which was initially believed to offer a final solution to the problem. The pneumococcus, however, has proven to be a particularly elusive and adaptable foe. Dr. Austrian would caution us to neither underestimate it nor be overly confident that the quest is complete.

JEFFREY N. WEISER, M.D.  
University of Pennsylvania

# *Preface*

The first pneumococcal conjugate vaccine (PCV) was licensed 7 years ago in the United States and has now been introduced into general use in many countries in Europe and the rest of the world. Its dramatic impact on the target population was anticipated by the results of efficacy trials. The magnitude of herd immunity provided to unimmunized individuals of all ages was not, and has enormously enhanced the public health impact of the vaccine.

The introduction of this exciting vaccine has invigorated the field and stimulated a great many studies in multiple areas including animal models; immunologic mechanisms; conjugation methods; epidemiology of pneumococcal disease; serotype distributions and antibiotic resistance in many geographic areas; diagnostic methods; antibody response measurements; PCV immunogenicity in healthy and high-risk individuals; impact on colonization, invasive disease, otitis media, and pneumonia; and effectiveness studies of direct and indirect protective effects.

This book seeks to summarize, for professionals in academia, public health, government, or industry, the current state of the art of pneumococcal vaccines, with particular emphasis on the years after introduction of the conjugate vaccine.

GEORGE R. SIBER  
KEITH P. KLUGMAN  
P. HELENA MÄKELÄ

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# *History*

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Barry M. Gray  
Daniel M. Musher

1

# The History of Pneumococcal Disease

The pneumococcus came to light in the golden era of microbiology that began in the last quarter of the 19th century. *Streptococcus pneumoniae*, a major cause of human disease, was one of the first pathogens to be isolated and characterized. It is not surprising that the pneumococcus has played an important role in the development of modern bacteriology, genetics, immunology, antimicrobial therapy, and vaccine immunity (10, 125). In this chapter, we review some of the major events in the history of this remarkable microorganism—events that have led to our modern understanding of the pathogenesis, diagnosis, treatment, and prevention of pneumococcal disease. Our intention is to place the pneumococcus in the scientific and social context of the times. The interested reader is encouraged to consult Roderick Heffron's monumental work, *Pneumonia, with Special Reference to Pneumococcus Lobar Pneumonia*, published in 1939 (61), and Benjamin White's *The Biology of the Pneumococcus*, published the previous year (127), both of which have been reprinted and are readily available. We also recommend a number of our favorite historical accounts by participants in the pneumococcal story: Harry Dowling's *Fighting Infection: Conquests of*

*the Twentieth Century* (33); *Life with the Pneumococcus*, a collection of articles and talks by Robert Austrian (5); *Penicillin: Meeting the Challenge*, Gladys Hobby's definitive account of individual discovery coupled with international cooperation and achievement (64); *The Professor, the Institute, and DNA*, the biography of Oswald Avery by his student and colleague René Dubos (37); and Maclyn McCarty's *The Transforming Principle: Discovering that Genes Are Made of DNA* (80).

## FROM DISCOVERY TO DISEASE

Edwin Klebs, in 1875, was probably the first to recognize pneumococci in infected sputum and lung tissue, some years before they were isolated and identified. The pathogenic role of the pneumococcus was not yet appreciated by George M. Sternberg or Louis Pasteur when they independently isolated the organism for the first time in 1880. Sternberg was in New Orleans studying malarial fever, thought to be caused by the so-called *Bacillus malariae* of Klebs. To provide a control substance for one of his experiments, Sternberg injected his own saliva subcutaneously into a rabbit, which quickly

sickened and died. The blood of this and similarly infected rabbits all had an "immense number of micrococci, usually joined in pairs and having a diameter of  $0.5\mu$ ." Working in Paris around the same time, Pasteur inoculated a rabbit with saliva from a child who had died of rabies. The rabbit died quickly of septicemia, exhibiting a disease course clearly unlike the usual slow progression to rabies. Pasteur was able to pass his "virus" (Latin for poison) from infected to normal rabbits and was able to recover the organism in bouillon and other media, which could then be used to infect other rabbits. In describing the organism he saw under the microscope, he noted that "each of these little particles is surrounded at a certain focus with a sort of aureole which corresponds perhaps to a material substance"—in fact, the capsule that proved to be central to pathogenesis and immunity (10, 98, 115, 127). The organisms that both Sternberg and Pasteur had isolated were pneumococci from asymptomatic carriers.

Pasteur called his isolate the "microbe septicémique du saliva," and Sternberg graciously called his *Micrococcus pasteurii*. M. Mátray applied the term "pneumoniekokken" to this organism in 1883, and Albert Fraenkel gave us the familiar name "pneumokokkus" in 1886 (127). That same year, Anton Weichselbaum suggested the name *Diplococcus pneumoniae*, which became official until the organism was reclassified as *Streptococcus pneumoniae* in 1974 on the basis of its growth in chains in liquid media.

In 1883, Hans Christian Gram, a young Danish physician, came to Berlin to work with Carl Friedländer. Gram was trying to develop a stain that afforded better visualization of microbes within tissue samples, but he soon found himself in the thick of controversy. Friedländer had observed diplococci and chains of cocci in lung tissue from most of his first 50 pneumonia patients in 1881. Shortly thereafter, he isolated and studied what became known as Friedländer's bacillus (*Klebsiella pneumoniae*) from perhaps only a single patient who died late in the course of pneumonia. Because of some misinterpretation of his various reports, it was widely assumed that Friedländer was advancing the bacillus as the sole cause of pneumonia, whereas in fact, he was suggesting that there might be several different causative organisms, among which the pneumococcus predominated. Fraenkel took exception to the idea of more than one causative agent, claimed to have seen the pneumonia cocci first himself, and continued to foment a controversy lasting several years (47, 127). Meanwhile, Gram found that gentian violet (introduced as a stain by Paul Ehrlich) was retained in certain cocci after precipitation with iodine and washing with alcohol.

Other organisms and tissue proteins were decolorized. He used his newly developed staining technique on a variety of clinical specimens, including lung tissue from 20 patients who died of lobar pneumonia. Nineteen samples had gram-positive cocci, but the organisms in one, from which Friedländer had isolated his bacillus, were gram negative. Publicly, Gram tried to stay out of the fray by stating, in the conclusion of his paper, "Hopefully the method will prove useful in the hands of other investigators." And so it did. Privately, in letters to a colleague, Gram noted something "fishy" about Friedländer's bacilli, which were all derived from one patient whose illness appeared to have been quite different from the ordinary pneumonias (5, 56, 67).

The contentious Fraenkel nevertheless produced the first complete descriptions of the pneumococcus. He established the pneumococcus as the cause of lobar pneumonia and identified the organism and those described by Sternberg and Pasteur as one and the same. During this same period, 1881 to 1886, Weichselbaum repeated and expanded upon the work of Friedländer and Fraenkel in careful animal studies and examinations of human pathologic specimens. His landmark paper confirmed that the pneumococcus was observed microscopically in the lungs of 94 of 129 patients with pneumonia and recovered from 54 cultures, while Friedländer's bacillus was found in samples from only 9 patients (5, 126, 127). By 1887, there had been numerous sightings of the pneumococcus, the organism had been grown by at least a dozen different investigators, and Koch's postulates were confirmed by the results of animal experiments by Fraenkel and others. The acrimony over the etiology of lobar pneumonia was subsiding, and Gram's staining method, the description of which was finally published in 1884, was being used by increasing numbers of investigators. It was becoming apparent that a variety of organisms, including streptococci, staphylococci, and haemophili, as well as Friedländer's bacillus, could be implicated as the cause of pneumonia, but the pneumococcus was clearly the most common and most consistent pathogen.

In the preantibiotic era, pneumonia was exceedingly common, generally taking third place after heart disease and cancer as a cause of death in the 1930s. No population-based data from this period are available, and most statistics on pneumonia included influenzal disease. The mortality from all forms of pneumonia during the years 1924 to 1933 averaged 88 per 100,000 persons in the United States. Pneumonia was responsible for about 7% of all deaths in the United States and in Europe. About half of U.S. cases were classified as lobar pneumonia, accounting for 4.3% of deaths over the

decade ending in 1929 (61). The annual incidence of pneumonia (all forms) was estimated at 558 per 100,000 persons in a 1935 U.S. Public Health Service survey (61). Rates were highest in children, pregnant women, and older adults. More than 60% of all cases of pneumonia were caused by *S. pneumoniae* (61). The mortality from bacteremic pneumococcal pneumonia rose from about 10% in healthy young adults to nearly 100% in adults over the age of 60 (16, 117). Infants under 2 years of age had the highest mortality, especially those with bronchopneumonia or bacteremia, which was often due to *S. pneumoniae* type 14 or 19. Lobar pneumonia, which occurred more frequently in older children, was less common and less often fatal in children than in adults (16, 92, 122). During the 1930s, pneumonia in pregnancy accounted for 1 death in every 5,000 deliveries and was the cause of about half of maternal deaths due to nonobstetric causes. Infants survived about 40% of the time (45).

Fraenkel was one of the first to recognize pneumococcal infections of other body sites in association with pneumonia. In 1886, he described pneumococci in exudate in the pia mater of a patient who died of meningitis, but he could not determine whether the meningitis was primary or whether it was secondary to the pneumonia. C. Nauwerk in the same year reported acute nephritis in 13 of 550 patients with croupous pneumonia and found cocci in the kidneys, presumably carried there by the blood. Within a few years, investigators documented other complications of pneumonia, including arthritis, endocarditis, pericarditis, and peritonitis, as well as infections of the orbit and eye. Soon, the full spectrum of disease caused by this versatile pathogen became apparent. Manifestations of primary infection involved the respiratory tract, including pneumonia, acute purulent tracheobronchitis, otitis media, and acute purulent sinusitis. Empyema associated with pneumonia could result from direct extension or from hematogenous spread. Meningitis, brain abscess, osteomyelitis, septic arthritis, peritonitis, abscess of the liver or spleen, and bacteremia without a recognized focus all indicated hematogenous infection.

Primary peritonitis was one of the first conditions noted to have a predisposing risk factor, namely, alcoholic cirrhosis, as reported by A. Charrin and A. Veillon in 1893 (24). It took another five decades before primary peritonitis was associated with nephrosis (96). Although the importance of the spleen in resistance to infection was noted by D. Morris and F. D. Bullock in 1919 (86), postsplenectomy infection was not widely recognized until 1952, when H. King and H. B. Shumaker, Jr., reported five cases of severe sepsis among in-

fants who had undergone splenectomy for spherocytosis (69). That same year, Ogden Bruton described the first patients with X-linked agammaglobulinemia, who also presented with severe pneumococcal infections. Sickle-cell disease was not noted as a risk factor until 1966 (106). It is now well known that any condition associated with poor synthesis of immunoglobulins, including congenital or acquired hypogammaglobulinemia, multiple myeloma, lymphoma and leukemia, and human immunodeficiency virus infection, puts the host at risk for pneumococcal infection. Defects in complement production and diseases of polymorphonuclear leukocytes, such as cyclic neutropenia, also predispose to infection. A long list of common conditions and events, such as cirrhosis, hepatitis, renal insufficiency, diabetes mellitus, glucocorticosteroid administration, and alcoholism, exerts multifactorial effects on host resistance to pneumococcal infection, as do excessive physical stress and fatigue (88). Factors involving the lung, including cigarette smoking, chronic obstructive lung disease, and asthma, are important local predisposing factors. It is interesting that Heffron commented on most of these factors in his 1938 monograph but never mentioned cigarette smoking!

Otitis media is of interest because of its enormous frequency and its pivotal role in the development of contemporary pneumococcal conjugate vaccines. First described by E. Zaful in 1887, otitis media went essentially unnoticed as a childhood infection except as an occasional complication of pneumonia (9, 102). Until early in the antibiotic era, *Streptococcus pyogenes* received the greatest attention for its role in this infection. It was not until the 1960s that the etiologies of otitis media in children were defined in modern terms by J. Coffey (26) and others. Despite the availability of an increasing array of antibiotic choices, otitis media stubbornly remained the most common bacterial infection treated by pediatricians. This prevalence was complicated by the rise in resistance, first by *Haemophilus influenzae* and then by pneumococci in the 1980s. Several trials of pneumococcal carbohydrate vaccines showed little or no efficacy in young children. The problem was summarized succinctly in the 1954 edition of *Holt's Pediatrics*: "Specific active immunization of children against pneumococci is impractical because of the large number of types and the poor antigenic response of infants—the group most in need of protection" (130). What changed everything was the spectacular success of the conjugate vaccines against *H. influenzae* type b. With the express purpose of re-evaluating the prospects for prevention, a group of clinicians and scientists from academia, government, and

industry met in Pittsburgh in the fall of 1988. The consensus was that the prevention of otitis media was an important goal and that the first step should be to develop a multivalent conjugate pneumococcal vaccine for children, beginning with the types of pneumococci that most commonly affected children, types 6A or 6B, 14, 19F, and 23F (91).

## SCIENTIFIC ADVANCES

Pneumococcal pneumonia was a driving force behind clinical and microbiologic research. Until the 1880s and 1890s, pneumonia had been regarded as a respiratory affliction rather than an infectious disease, with no specific therapy other than supportive care and various ineffectual potions and poultices. The identification of a particular causative agent of pneumonia eventually opened the way to new approaches to therapy and prevention. It nevertheless took several decades before scientific concepts were sufficiently developed to support meaningful research into the epidemiology, immunology, and treatment of pneumococcal disease.

Although Fraenkel, in 1886, and Pio Foa and Guido Bordoni-Uffreduzzi, as early as 1884, observed that animals developed resistance to reinfection with a given pneumococcal strain, it was the brothers Felix and Georg Klemperer who used insights gained from studying the pneumococcus to develop the concept of humoral immunity (127). They introduced the intravenous route of immunization and repeatedly injected heat-killed pneumococci, filtered broth cultures, and bacterium-free pleural exudates from recovering pneumonia patients into rabbits. They were successful in immunizing the rabbits against challenge with live pneumococci, and they also observed that the offspring of immunized mother rabbits were usually protected, suggesting the passive transfer of immunity. They then demonstrated that naïve rabbits could be protected by an infusion of sera from immunized rabbits and also by sera from patients recovering from pneumonia. Since serum was the quintessential humoral substance, this immunity was called humoral immunity. After testing the rabbit anti-sera for safety by infusing them into themselves, they treated six patients with pneumonia, and all appeared to show some kind of improvement. Of course, they did not know the nature of their vaccine. In the context of important work by Pasteur, Friedrich Loeffler, Emile Roux, and Alexandre Yersin on fowl cholera, anthrax, and diphtheria, they thought that they were vaccinating against a pneumococcal toxin. Their chemical techniques, however, would not have distinguished between

a protein toxin and the capsular material released from replicating pneumococci.

Elie Metchnikoff, in 1891, was the first to observe the clumping of pneumococci in the presence of immune rabbit serum (83). Although working with phagocytes, he did not make the connection between clumping and opsonization, a term he coined from the Greek word for preparing food. A few years later, F. Bezançon and V. Griffon gave a formal description of agglutination with immune serum and noted that “there exist several races of pneumococci, which behave as though different microbes” (13, 127). More than a decade went by before these phenomena were revisited in 1902 by F. Neufeld, who developed the Quellung or capsular swelling test (93), but distinct pneumococcal serotypes were not recognized by these techniques until the description by Neufeld and L. Haendel of types I and II in 1910. A short time later, A. R. Dochez and L. J. Gillespie identified the three common types, called I, II, and III, and a grab bag of other types known as group IV (31). Although these methods opened the way for rational experimentation with serum therapy, routine typing was not available until the early 1930s, when Albert B. Sabin popularized the microagglutination test and R. R. Armstrong standardized the Neufeld capsular swelling test (3, 16, 108). Many more years of work were needed to identify the 90 or more serotypes we now recognize in the American type system, developed by Georgia Cooper and colleagues at the New York State Health Department Laboratory, and in the more familiar Danish system, developed by Erna Lund and coworkers at the Statens Serum Institut in Copenhagen (27, 76). (In this paper, Roman numerals are used to discuss the serotypes in their historical context, and Arabic numerals identify the serotypes as we now know them in the Danish system.)

The first vaccines were used in animal experiments to study pathology and immunity in rabbits, guinea pigs, chickens, cows, horses, donkeys, dogs, and monkeys. Following the Klemperers' lead, later investigators used chickens, horses, and finally rabbits for production of sera for therapeutic use in humans (54, 127). The first whole-cell vaccines underwent human trials in South African gold miners in 1911 under the direction of Sir Almroth Wright (7). His protégé, F. S. Lister, continued to study pneumococcal disease and its prevention (73, 90). Of particular note was Lister's recognition of the possibility of “herd immunity,” that immunizing half of a closed population might confer protection on the unimmunized subjects (95). R. L. Cecil and J. H. Austin carried out vaccine studies in the U.S. Army during the First World War. In all, nearly two dozen investigations of whole-cell pneumococcal vaccines administered to

over 400,000 people were conducted between 1911 and 1936 (127). Despite some apparent success, all of these studies suffered from design flaws, principally, the lack of randomization and proper controls, and all revealed at least some serious untoward reactions to the vaccines.

In 1917, Dochez and O. T. Avery described what they called a soluble specific substance that was elaborated by the pneumococcus and appeared in the urine of patients with pneumococcal pneumonia (29, 30). This substance had been observed by F. P. Gay and H. T. Chickering in precipitation studies a few years earlier (51). M. Heidelberger and Avery isolated the substance from type II pneumococci and identified it as a carbohydrate (62). L. D. Felton and G. H. Bailey soon purified the capsular polysaccharide themselves, confirmed the observation regarding the role of antibody in the precipitation reactions, and made the enormously important conclusion that antibody to the capsule was responsible for immunity to pneumococcal infection (43). Proof of concept for a polysaccharide vaccine was provided a year later by O. Schiemann and W. Casper, who demonstrated that protein-free capsular material provoked a type-specific antibody response in mice (109). Results from studies showing the human response to the purified polysaccharides followed quickly in reports by T. Francis, Jr., and W. S. Tillett, Maxwell Finland and W. D. Sutliff, and José Zozaya and Janet Clark (61). In one of the first practical applications, W. G. Smillie and coworkers abruptly terminated an outbreak of type I pneumococcal pneumonia at a state hospital by mass vaccination (114). Studies of purified polysaccharide vaccines culminated in controlled trials among U.S. military subjects in 1942 to 1944, under the direction of C. M. MacLeod and colleagues (77). They demonstrated that under epidemic conditions, vaccination with four capsular polysaccharides (types 1, 2, 5, and 7) protected healthy young adults against pneumococcal pneumonia due to the vaccine types.

It was known from the late 1880s that humoral immunity alone was not a sufficient defense against the pneumococcus. N. Gamaléia was one of the first to recognize the role of pulmonary phagocytes in the prevention of pneumonias in mice and humans (50). B. Issaeff, also at the Pasteur Institute and a student of Metchnikoff, demonstrated that immune serum did not actually possess direct antitoxin or antibacterial properties but did promote the phagocytosis of pneumococci (66). A decade later, A. E. Wright and S. R. Douglas reported some of the earliest quantitative studies on opsonization, demonstrating that phagocytosis required not only immune serum but also something in normal serum that was inactivated by heating (129). H. K. Ward and J. F.

Enders clarified these observations in 1933, when they defined the roles of antibody and complement in the opsonization and phagocytosis of pneumococci (124). Thus, by the 1930s the medical community had accumulated most of the basic information needed to render serum therapy practicable. These were also the basic concepts that laid the foundations for the development of polysaccharide vaccines.

An account of the central role of *S. pneumoniae* in the scientific advances of the 20th century is not complete without a mention of how studies of this organism led to the recognition that DNA is the basic unit of genetic material (37, 80). Early in the century, L. M. Stryker noted that serial cultures of pneumococci yielded colonies that appeared rough and essentially avirulent because the organisms had lost their capacity to generate capsular material (116). F. Griffith studied this phenomenon in the late 1920s. He discovered that when mice were injected with live, rough type II organisms mixed with dead but encapsulated type III organisms, the mice died, and live type III organisms were recovered from their tissues. He demonstrated this result with rough and smooth organisms of different serotypes and called the phenomenon transformation (56). Within a few months, Avery confirmed Griffith's findings and embarked on a quest that culminated in the 1944 report by Avery, MacLeod, and McCarty proving DNA—not a protein or carbohydrate—to be the "transforming principle" (12).

At the heart of the ability of the pneumococcus to adapt is its capacity for natural transformation. Following the lead of Griffith, Avery, MacLeod, and Rollin D. Hotchkiss (80), S. A. Lacks demonstrated that pneumococci could take in large sequences of single-stranded DNA (while degrading the other strand) and incorporate them into the genome (71). A. Tomasz, D. A. Morrison, J.-P. Claverys, and others have further explored the genetics of transformation and "competence," the transient physiological state that allows efficient DNA uptake (25, 101). These programmed mechanisms of genetic exchange explain such phenomena as capsular type switching and the horizontal transfer of penicillin binding protein genes in the development of penicillin resistance (121).

## VACCINE TREATMENT AND SUPPORTIVE CARE

Vaccine treatment, introduced in 1918 by E. C. Rosenow in Chicago, was the practice of giving whole-cell vaccines to patients already ill with pneumonia. Heffron noted that "the so-called rationale of vaccine therapy

is based on the assumption that the parenteral injection of devitalized organisms will accelerate the immune response." He reviewed nearly two dozen papers published between 1918 and 1933 but found little theoretical or clinical information in its favor (61).

Nonspecific treatments emphasized pain management and conserving the patient's strength (61). Numerous nostrums and remedies, including bleeding, digitalis, morphine, quinine, strychnine, and hydrotherapy, were tried and discarded. About alcohol, Jesse G. M. Bullowa commented that "the time honored tradition of aborting pneumonia by becoming drunk has in our experience only the support of wishful thinking" (16).

One area that saw considerable progress was oxygen therapy and mechanical ventilation. In 1919, J. S. Haldane defined the physiology of anoxemia and oxygen saturation of the blood (57, 58). These principles were applied to pneumonia by C. A. L. Binger, who confirmed that supplemental oxygen was beneficial. More notably, he found that patients who recovered generally attained oxygen saturation levels of at least 90% when breathing 40% oxygen, whereas most patients with fatal cases had low saturation levels and actually died of anoxemia (15). Oxygen was given through a variety of devices, such as rebreathing oxygen masks and nasal cannulas (16). In 1929, Philip Drinker and Louis Agassiz Shaw developed a practical negative pressure ventilator that was later perfected by John Haven Emerson: the iron lung (78).

Infection control issues were considered important (61). Patients were generally subject to semi-isolation during the early phases of disease, when the sputum contained large numbers of organisms. Paper handkerchiefs were often used for easy disposal, and sputum cups were boiled after use. Linens were washed and boiled, and disinfectants were used to clean contaminated rooms and surfaces. Strict isolation was generally recommended for patients with type I or type II infections, since it was known that these types were associated with hospital and family outbreaks. Types I and II were not commonly carried by healthy individuals, and carriage rates among family members were 10 times higher than those among health care workers caring for the patients (113).

## SERUM THERAPY AND PNEUMONIA CONTROL PROGRAMS

It took over 20 years for the work of the Klempers to become translated into a rational treatment for pneumonia. The practice model was serum treatment of diphtheria, which was subjected to the world's first con-

trolled clinical trial by Johannes Fibiger in 1897 (65). The New York City Health Department began making its own diphtheria antiserum in 1895. St. Louis did the same a few years later, but one batch of antitoxin became contaminated with tetanus bacilli, and several children died as a result. Outrage from health officials, doctors, and the public prompted the U.S. Congress to pass the Biologics Control Act of 1902, the first of several reforms that shaped the regulation of biologicals and pharmaceuticals over the 20th century (33). Prior to 1902, results with serum therapy for pneumonia were less than convincing, according to J. M. Anders, who reviewed more than 500 cases described in reports and small series published up to that time (2). However, within a year of Neufeld's identification of types I and II in 1910, the New York City Health Department, convinced that type-specific antiserum would be effective, began making antipneumococcal sera in horses.

Meanwhile, Simon Flexner and Rufus Cole of the newly established Rockefeller Institute and Hospital chose pneumococcal pneumonia as a major focus of research. They assembled a group of investigators, including Dochez, Avery, Dubos, Heidelberger, F. L. Horsfall, and others, to serve the institute's goal of becoming a model for the clinical application of scientific medicine. In 1913, Dochez and Avery used type I antiserum successfully, curing 10 of 11 patients with type I pneumonia (31). Soon the Rockefeller group developed practical methods and a handbook for the administration of serum. They became newsworthy during a pneumonia epidemic at the army mobilization camp in El Paso, Texas, in the winter of 1916 to 1917, when the Rockefeller group helped the camp's staff with medical guidance and 10 liters of type I antiserum. Only 5 (8%) of 63 treated soldiers died, compared to 7 (39%) of 18 in the untreated group (94, 99). Success, however, was seldom this impressive, as noted by A. K. Wadsworth, who reviewed this and 16 other trials conducted between 1915 and 1924 (123). His major criticism was that "no satisfactory control series of untreated cases is given in these reports." Regarding 11 trials in military camps, he commented, "The marvel is that the army organization was capable of undertaking such an advanced problem, and was able to accomplish as much as it did, in treating pneumonia by these exacting methods."

Up until this time, untreated horse serum was the primary source of passive antibodies, although chicken and rabbit sera had also been studied. Human convalescent-phase serum and sera from immunized healthy donors did not have sufficient antibody levels to be clinically useful (61). Using methods developed in 1915 by Gay and Chickering at the Rockefeller Institute, F. M. Huntoon

evolved the first practical “refined” antibody solution that removed most of the serum proteins suspected of causing thermal reactions and serum sickness. At Harvard, Felton perfected several methods for both purifying and concentrating the antibody product, and he further developed methods for the standardization of potency based on mouse protection tests (41, 42, 61). These antibody preparations made serum therapy practical and demonstrably effective, at least for type I pneumonia (21, 99). Results were favorable, though less convincing, for type II, but serum therapy had no effect on type III infections (22, 23, 61). Many studies reached statistical significance by retrospective analyses, with mortality in treated groups averaging about 20 percent compared to 30 percent in controls (20). The design of clinical trials and the use of statistics, however, evolved slowly (79, 99). Whereas Norman Plummer, Bullowa, and the Medical Research Council in Britain (82) strongly favored the alternate-patient method of assigning treatment or control subjects, other investigators rejected the use of controls on ethical and practical grounds (46, 99). It is worthy of note that Bullowa included a 7-page discourse on statistical analysis in his chapter on serum therapy (16). True randomization and treatment blinding was not done until streptomycin was studied for use against tuberculosis in 1948 (32), but the experience in conducting large cooperative studies of serum therapy set the stage for these and other developments.

Health departments and commercial producers of specific sera were required to demonstrate both the specificity and the potency of serum products, according to standards set in the United States by the National Institutes of Health and elsewhere by the League of Nations Permanent Commission on Biologic Standardization. A final advance in serum production was the development of high-potency rabbit antisera in the mid-1930s by K. Goodner, Horsfall, and Dubos (54). Rabbit antiserum, treated by heating and absorption with kaolin, was easier to produce and cost about one-fifth as much as concentrated horse serum. It also had a number of physiological advantages, including smaller antibody size with presumed better tissue penetration (rabbit immunoglobulin G is divalent, whereas horse immunoglobulin G is tetravalent). By 1940, sera for the common types I to V, VI, VII, and XIV were approved, and many for higher types were also available (44). Most antisera were made by commercial drug companies, among which Lederle had the distinction of having “the world’s largest rabbit warren” of 28,000 animals (99).

The mechanics of serum therapy were well worked out in the academic centers, especially in New York City at the Rockefeller Hospital, Bellevue, and Harlem Hos-

pital and in Boston at Harvard and Boston University. Several handbooks on serum therapy were available, most notably those by F. T. Lord and Heffron (75) and Bullowa (16). Patients selected for serum treatment first needed to have the correct diagnosis, usually made clinically and (about half the time) confirmed by a chest roentgenogram examination. For treatment to be effective, patients had to be in the early stages of illness, usually the first 2 or 3 days but not more than 4 days after the onset. As soon as possible, the serotype was determined from a sputum sample, sometimes by direct examination with the Neufeld reaction but more often by culture or mouse inoculation, as illustrated in Bullowa’s handbook (Fig. 1). A history was taken to check for sensitivity to horses (or rabbits), prior serum (e.g., diphtheria antitoxin) administration, asthma, urticaria, or other allergic disease. A direct eye or skin sensitivity test was required of all patients prior to the administration of the intravenous test dose of serum to be used for treatment. Epinephrine was at hand in case of anaphylaxis. The appropriate volume of serum or concentrate, based on the number of antibody units, was given in one or several doses. Dosage was entirely empirical until Felton and others developed potency standards. In 1933, Francis described the method of determining the adequacy of dosage by using a skin test in which purified capsular polysaccharide was injected subcutaneously. A wheal-and-flare reaction appeared when a sufficient antibody level was achieved (48, 128). Treatment results for individual patients were often depicted in creative graphic form, as shown in Fig. 2. According to Cecil and Plummer (21), “There is no more striking clinical effect in the whole domain of specific therapy than that which frequently follows the early administration of Felton’s serum in type I pneumonia. The temperature drops rapidly, very much as in a natural crisis, and all signs of toxemia frequently disappear within twenty-four hours of the initiation of treatment.”

The major barriers to the practical application of serum therapy were that it was complicated, technically demanding, and expensive. It required a skilled laboratory staff and the rapid availability of appropriate serum preparations. To provide serum treatment outside the academic medical centers, an ambitious new infrastructure of pneumonia control programs was developed with the intention of providing serum, usually at no cost, to treat the largest number of people possible (34, 99). In New York, the Metropolitan Life Insurance Company, which lost \$24 million in payouts during the influenza pandemic of 1918 to 1919, became an early supporter of pneumonia research and the statewide program initiated in 1937 (99, 107). The model for this

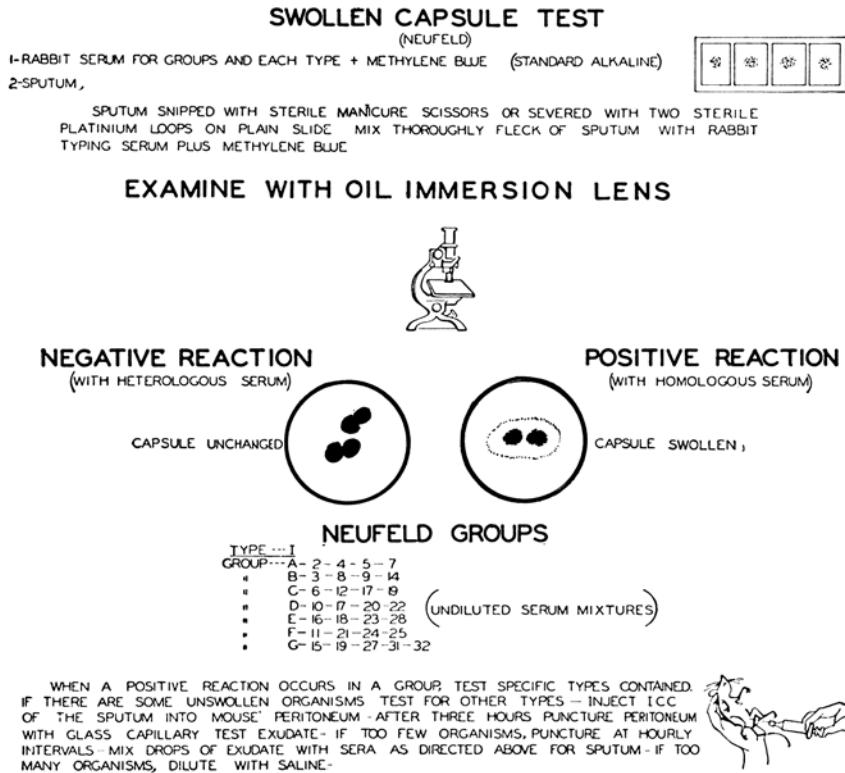


Figure 1 Part of Fig. 28, "Examination of sputum and cultures," from Bullowa's handbook (reference 16, p. 83). Reproduced with permission of Oxford University Press.

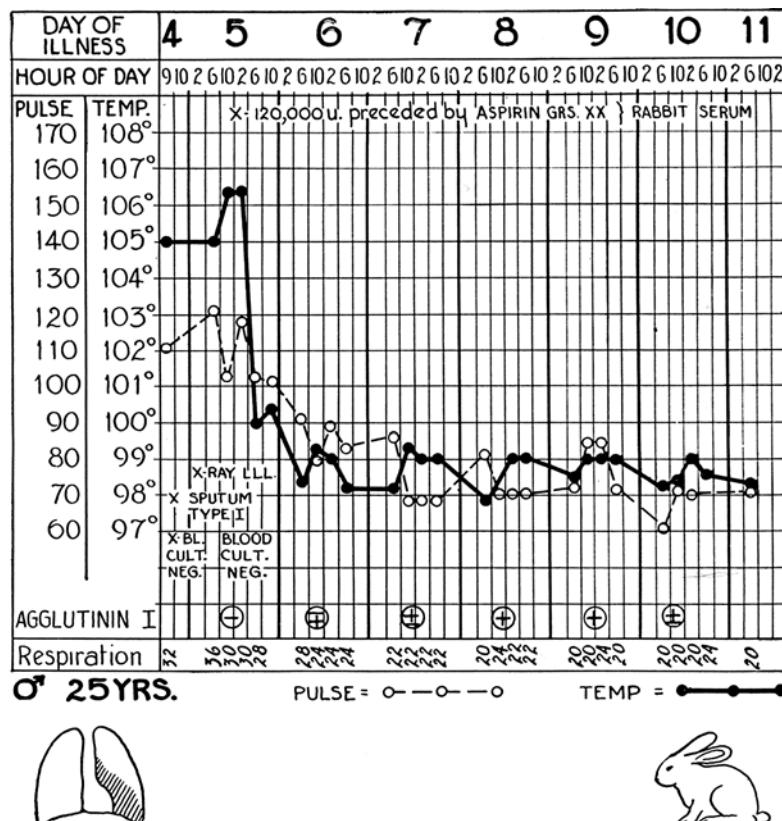
program was the Massachusetts Pneumonia Study and Continuing Program begun in 1931 with financial support from the Commonwealth Fund. It also had the co-operation of the state and local health departments, community and academic medical centers, and more than 1,000 physicians in all parts of the state (61). With the advent of federal funding in 1940, over 30 states initiated similar programs. But within a mere 5 years, serum therapy was supplanted by sulfapyridine and penicillin, and this peculiarly American social and public health experiment faded away.

## CHEMOTHERAPY AND ANTIBIOTICS

Antitoxins and antisera were, in René Dubos's words, "charmed bullets which strike only those objects for whose destruction they have been produced" (36). Paul Ehrlich believed that synthetic chemicals, like the azo dyes and antibodies, could have selective affinity for and action on bacterial cells. Mercurochrome, potassium permanganate, aspirin, and many other chemicals received indiscriminate trials for pneumonia treatment without any significant supervision. Partial success was obtained with the use of the quinine derivative ethylhydrocupreine (optochin) synthesized by Julius Morgan-

roth and Richard Levy in 1911. Pneumococci appeared to be uniformly susceptible to lysis by optochin, which is still used in the P disk test for their identification in the clinical laboratory. M. Morganroth and M. Kauffmann used this compound to abort pneumococcal disease in experimentally infected mice but found that pneumococci rapidly became resistant. Uncontrolled trials in humans were conducted without any understanding of the drug's pharmacology. The use of this drug was abandoned after 1918, when H. F. Moore and A. Chesney showed that it did not reliably kill the organism yet posed a significant risk of optic neuropathy to the patient (84, 85, 125, 127).

The discovery of Prontosil by Gerhard Domagk of I. G. Farbenindustrie in Germany opened the modern era of antimicrobial agents. Domagk focused on azo dyes because of their propensity for binding to proteins, and in 1932 he found that Prontosil, a water-soluble salt of sulfonamido chrysoidine, was effective against hemolytic streptococci in experiments with mice. Researchers at the Pasteur Institute showed that Prontosil was cleaved in vivo, releasing the active component, sulfa-nilamide, which by itself could inhibit the growth of pathogenic bacteria in vitro (33, 61). By 1937, more than a hundred companies were involved in the manu-



**Figure 2** Fever curve and treatment details from a patient, "Male, aged 25 years, 5th day single dose (120 cc.) (Processed Rabbit serum Pn. I) temperature normal in 6 hours." (Bullock's Fig. 114, p. 348 [16].) Note X-ray and culture results and tests for type I agglutinins in serum. Reproduced with permission of Oxford University Press.

facture and marketing of sulfanilamide. One company concocted a syrup made with ethylene glycol and distributed it under the name elixir sulfanilamide. It was only after 105 people died that the Food and Drug Administration (FDA) was given the job of investigating their cause of death (19). This event sparked a public outrage fierce enough finally to pass the U.S. Federal Food, Drug and Cosmetic Act of 1938, from which developed the regulatory process we know today (79).

Meanwhile, chemists at May and Baker had been synthesizing hundreds of sulfanilamide derivatives in search of more-effective, less-toxic compounds. Compound M&B 693, now known as sulfapyridine, was just another such derivative when it arrived at Lionel Whitby's laboratory for animal testing in November 1937. But because the lab was temporarily out of the standard streptococcus-infected mice, the lab assistant substituted pneumococcus-infected mice and found quite unexpectedly that sulfapyridine was a spectacular success (72). Animal and safety experiments quickly ensued, and the first human was given the drug in February 1938. The patient, who was thought to be near death

from lobar pneumonia, improved and went on to full recovery. In March 1938, G. M. Evans and W. F. Gaisford began a clinical trial of 100 patients with lobar pneumonia, but news of a "wonderful new drug" was already in the British press before their report was published in the *Lancet* on 2 July 1938 (39). While animal testing was still in progress, May and Baker made enough of the drug to market it in Britain in September 1938. Sulfapyridine was enthusiastically received by physicians and the public, but Whitby and others felt that widespread misuse of the drug had outpaced the knowledge of its proper use and potential toxicities. Yet public enthusiasm reached even higher levels in 1943, when Winston Churchill's miraculous cure from pneumonia was featured in banner headlines in the British press (72).

Considerably more caution was exercised in the United States under the newly reformed FDA. Sulfapyridine was released to only a limited number of "qualified investigators," pending additional research and safety testing. The FDA collected clinical data from manufacturers, surveyed a large number of physicians who had

research experience with the drug, and gave final approval in March 1939. The full range of toxicities was not appreciated, nor was it known if sulfapyridine would supplement or supplant serum therapy in the treatment of pneumonia (72, 79). Yet to come in 1943 on the heels of such an enormous success was the resourceful organism's response: sulfonamide resistance (118).

Penicillin had a longer and more arduous route to the pneumococcus. Its heroes, especially Sir Alexander Fleming and Howard Walter Florey, became the stuff of medical legend, but the development of this antibiotic involved a huge international collaboration of scientists and participants from academia, industry, and government (14, 64). None of Florey's first 10 patients had pneumococcal infections, because the initial focus was on the staphylococcus and pneumococci seemed to be less sensitive by *in vitro* testing (1). The first pneumococcal infections treated with penicillin were three cases of fulminating endocarditis in patients under the care of M. H. Dawson and Gladys Hobby in 1942 (28, 64). These cases were chosen because all these infections had become completely refractory to sulfonamide therapy and because pneumococci were known to be much more sensitive to penicillin than were viridans group streptococci. Although all patients died after 3 to 7 days of therapy, when supplies of penicillin ran out, two showed temporary improvement and the sterilization of blood cultures. It would take a large industrial process to produce enough penicillin to effect a cure for this uniformly fatal disease.

Until the summer of 1943, the supply of penicillin in the United States was so small that only 22 accredited investigators were selected to receive it, under the auspices of the National Research Council (NRC). Tillett and colleagues had begun to use penicillin for the treatment of pneumococcal pneumonia in 1942. Results from their first 76 patients appeared in a preliminary report on 500 patients (most with staphylococcal infections) compiled by C. S. Keefer, chairman of the NRC Committee on Chemotherapeutic and Other Agents, and coworkers (68). Tillett's full report and the account of his successful treatment of pneumococcal empyema by injecting penicillin directly into the pleural space were not published until 1945 (119, 120). Meanwhile, in July 1941 the first steps toward industrializing penicillin were begun when Florey and Norman Heatley arrived at the U.S. Department of Agriculture's Northern Regional Research Laboratory in Peoria, IL (14, 64). They worked with Charles Thom, who had identified Fleming's original *Penicillium notatum*, and with Percy Wells, Robert D. Coghill, and Andrew Moyer, who had years of experience in mold fermentation. After a

worldwide search for a more productive mold, the team found a strain of *Penicillium chrysogenum* growing on a famous moldy melon in a local Peoria market. This strain grew submerged in the medium instead of just on the surface and produced far more penicillin than the original mold, especially after UV irradiation and the repeated selection of mutants. By the end of 1943, about 21 billion U of penicillin had been produced by a consortium of pharmaceutical companies, all under the direction of the War Production Board. By the end of 1944, production was up to 1,600 billion U and the cost was coming down from \$200 to \$6 per million U. The control of allocations remained with the NRC until the end of 1945, by which time the program had collected data on 10,838 patients (64).

These events opened the way to further clinical research and to the treatment of conditions that had never been amenable to serum therapy. The pharmacology involved in treating meningitis was investigated, and that disease became amenable to treatment with "massive" doses of penicillin (35, 38). Septic arthritis could be effectively treated without the use of serum (17). Patients with pneumococcal pneumonia had fewer complications, and Austrian's syndrome of pneumococcal endocarditis, meningitis, and rupture of the aortic valve became a curiosity (8, 52). Penicillin fully deserves the title of miracle drug. With its availability in commercial quantities, diseases that were nasty (gonorrhea), potentially fatal (syphilis), or nearly 100 percent lethal (meningitis due to *S. pneumoniae*, puerperal fever due to *S. pyogenes*, and endocarditis due to viridans group streptococci) could now be controlled.

The remarkable rates of cure with penicillin led to a half-full, half-empty conundrum. While cures were readily achieved with what are now regarded as minuscule doses of the drug, there was significant residual mortality. The general impression was that penicillin had conquered the pneumococcus: it eliminated the need for serotyping, for serum therapy, and for the further development of vaccines. The reality was that the very young and the very old continued to have high rates of mortality. This theme was sounded loudly by H. A. Reimann through three editions of his monograph *The Pneumonias* (103–105). Austrian and J. Gold reviewed the dismal condition of bacteremic pneumonia in the early antibiotic era (11), and M. A. Mufson and colleagues found the situation no better a decade later (87), as illustrated in Fig. 3. Recent studies continue to emphasize a persistent mortality of 20% in patients with bacteremic pneumococcal pneumonia (89). Throughout this time, Austrian remained a stalwart in the cause for effective pneumococcal immunization (6) and was a major force in bridging the divide between the experi-

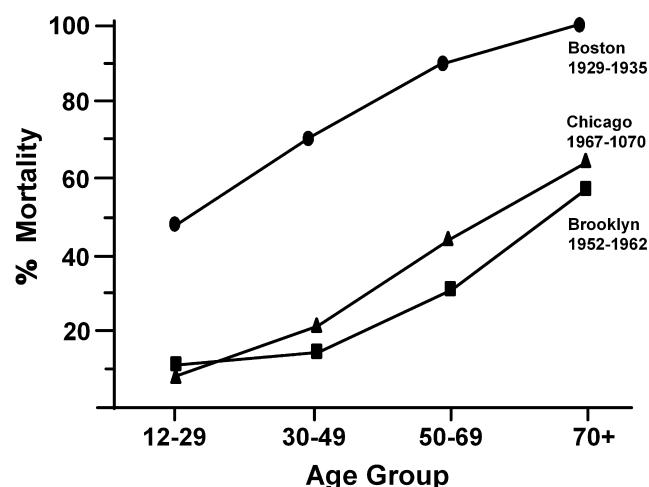


Figure 3 Mortality rates from pneumococcal pneumonia by age in the period before antibiotics (Boston 1929–1930), from Heffron's monograph (61); in the early antibiotic era (Brooklyn, 1952–1962), from Austrian and Gold (11); and after well-established use (Chicago 1967–1970), from Mufson et al. (87). (Figure by Daniel Musher.)

mental and the practical in bringing vaccines into medical practice.

## THE EVOLVING PNEUMOCOCCUS

Even before penicillin was readily available, microbiologists were mindful of the rapid emergence of resistance to sulfapyridine and sought evidence that exposure to nonlethal doses of penicillin in vitro (81) or in mice could allow pneumococci to develop a measure of low-level resistance (110). It is remarkable that it took 25 years for resistance to emerge naturally in the clinical setting, first among immunocompromised individuals subjected to long-term treatment with penicillins (59, 111). Shortly thereafter, resistant strains appeared in Papua New Guinea during an investigation of penicillin prophylaxis against pneumonia (60). Within the decade, similar strains with intermediate resistance (now defined as those for which the MIC is 0.1 to 1 µg of penicillin/ml) were found in South Africa, Europe, and North America (70). Resistance proved not to be a matter of a few simple mutations in the penicillin binding proteins but involved several complex mechanisms. Multiple recombinant events among pneumococci resulted in an accumulation of gene variants, and penicillin binding protein gene elements were also borrowed from related species, such as *Streptococcus mitis* and *Streptococcus oralis*, and incorporated into the pneumococcal genome (121).

As the use of penicillin became worldwide, two important changes occurred. First, the most invasive epi-

demic types, 1, 2, 3, 5, and 7, became less frequent. These types were seldom carried but were often spread from sick individuals to healthy contacts. It appears that early treatment broke the chain of transmission and reduced both carriage and infection. Second, the less virulent "childhood" serotypes, 6, 9, 14, 19, and 23, were more successful colonizers and began to replace the epidemic types in serious infections (49). These types are commonly carried for prolonged periods and are the ones most frequently causing otitis media, the most common bacterial infection for which children receive antibiotics. Given widespread antibiotic use and the widely acknowledged misuse of antibiotics, it should come as no surprise that these were the first types to acquire resistance. The new pneumococcal conjugate vaccine (PCV7) has greatly reduced the frequency of invasive disease due to the seven vaccine serotypes (4, 6B, 9V, 14, 18C, 19F, and 23F). This result has also reduced the overall frequency of resistance, which occurs mainly among these serotypes (74). But nonvaccine types are beginning to reemerge, and the overall rate of pneumococcal infections in some populations has already returned to prevaccination levels (40, 112). There has also been a substantial increase in complicated pneumonia and empyema due to types 1, 3, and 19A (18). Few type 1 strains have ever become resistant to penicillin, but several new sensitive clones have been implicated in serious disease and may have mutated to be more successful colonizers (53, 63). Capsular type switching, originally observed in experiments with mice, has now been implicated in humans and may play an important role in the expansion of nonvaccine serotypes (102, 121).

Although these developments are unsettling for our vision of the future, it is important to remember how far we have come and how much we have learned from our struggle with "the enduring pneumococcus" (4).

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P. Helena Mäkelä  
Jay C. Butler

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## History of Pneumococcal Immunization

Those who cannot remember the past are condemned to repeat it.

—George Santayana

By the year 1800, 200 years ago, the smallpox vaccine had established the principle of preventing serious disease by active immunization. However, it took another hundred years before the principle was applied to other deadly diseases, such as cholera, typhoid fever, and lobar (pneumococcal) pneumonia. And still another hundred years later, there remains a need for research on improved pneumococcal vaccines.

The first clinical trial of a pneumococcal vaccine was conducted in 1911 among the native workers of gold and diamond mines in South Africa (61, 92), a target group with an extraordinarily high incidence of lobar pneumonia (up to 20 cases/1,000 persons/month) in the first months after arrival at the mine (54, 61). The vaccine in this first trial, and those performed over the following 30 years, consisted of killed bacteria.

In the 1940s, these whole-cell vaccines were supplanted by the next generation of pneumococcal vaccines, which consisted of the purified capsular poly-

saccharides (PS) of the bacteria. After a clear-cut demonstration of the efficacy of PS vaccine in a military population, Squibb and Sons developed and marketed two hexavalent pneumococcal PS vaccines in the United States in 1946 (64). Unfortunately, these vaccines were introduced at a time when new antimicrobial drugs (sulfonamides and penicillin, etc.) became available, and the apparent miraculous efficacy of these drugs for the treatment of pneumococcal pneumonia led to a widespread belief that pneumococcal infections were entirely curable. As a result, interest in the prevention of pneumococcal disease by vaccination waned, these vaccines were never widely used, and they were withdrawn from the market in 1954.

A renewed interest in vaccines for the prevention of pneumococcal infections during the 1960s and 1970s resulted in the current 23-valent PS vaccine. In spite of general recommendations for the immunization of large at-risk groups, including all people above retirement age, the adoption of this vaccine has been slow and largely restricted to the United States and Canada. The major shortcoming of the PS vaccine, lack of efficacy in infants, called for further improvement. This came when

the PS was chemically linked (conjugated) to a protein carrier.

The first pneumococcal conjugate vaccine was licensed in 2000. The experience accumulated during the first 5 years of its use is very promising for the prevention of pneumococcal disease worldwide. However, the capacity of the pneumococcus for evolution and adaptation to new circumstances warns against complacency and calls for continuing research and further vaccine development. The lessons learned during the past century of pneumococcal vaccine research will be instructive for the refinement of the current vaccines and for the development of further generations of vaccines.

## WHOLE-CELL PNEUMOCOCCAL VACCINES

As early as 1891, animal experiments showed that killed pneumococci elicited protective immunity to subsequent challenge with virulent bacteria (50). The first clinical trials were conducted among mine workers in South Africa starting in 1911 under the guidance of Sir Almroth Wright, called upon because of his pivotal experience in the development of a typhoid vaccine (61, 92). The vaccines consisted of broth-grown bacteria suspended in saline and killed by heating, given as two subcutaneous injections a week apart.

In order to understand the results of these trials, it is necessary to be aware of the special epidemiologic circumstances under which they were carried out. The mine workers were recruited by the thousands in their homelands and transported by foot, boat, and train to a central receiving compound in Johannesburg. After 3 to 4 weeks in this compound, those considered healthy enough for work underground were dispatched to the mines, where they would work for about 6 months. The situation, bringing together a large number of men in a new environment after a stressful journey, followed by hard physical labor, was ideal for the spread of respiratory infections, including pneumococcal infection. As a result, attack rates of lobar (pneumococcal) pneumonia were very high, varying by year, season, mine, and origin of the target population. With a case fatality rate of 20 to 40%, pneumococcal pneumonia was the main cause of death among the mine workers. A second consequence of the special epidemiology was that the pneumonia attack rates were highest in the first months after the arrival of the new recruits. Thus, if the vaccine was given upon arrival, as was logically the best way to prevent the largest number of cases, the vaccine-induced immunity was developing at the same time that the risk of disease was highest.

The very first trial enrolled approximately 12,000 recruits, who upon arrival were assigned to either a red line (for those to be vaccinated) or a blue line (for the controls). During the first month of follow-up, a total of 345 pneumonia cases were recorded in the hospital of the central compound, 147 among the vaccinated men and 198 among the controls. This translates into a vaccine efficacy (VE) of 29%, with the 95% confidence interval (95% CI) from 13 to 43% when recalculated by present methods. In subsequent months, the disease reporting from the mines was not perfect, and the reported data have been criticized as a result (61). The effect observed in the first month was considered encouraging, however, and further experiments were started. In the next trial, 20,000 men were vaccinated, but the experiment was terminated without reported results because of inappropriate selection of the controls. In further trials, designed to overcome the previous problems, the overall results indicated statistically significant protection from pneumonia, with a VE of 30 to 40% in the first 2 to 3 months after vaccination. In the subsequent months, no vaccine effect was apparent, but the numbers of cases were low. The vaccination had no effect on case fatality rates. In a separate trial, revaccination several months after the first injection was tested and found to be nonprotective (92). Attempts to identify the best dose of vaccine—a balance between immunizing power and tolerability—also gave little return.

During these early trials, the existence and importance of multiple pneumococcal serotypes was not yet understood. The vaccine used in these trials was prepared from locally isolated, uncharacterized pneumococcal strains, which fortunately were likely to be the predominant serotype causing disease among the miners. Understanding of the antigenic heterogeneity of the pneumococcus advanced greatly with the 1913 publication of articles that clearly showed that protective immunity to pneumococcal infection was serotype specific (23, 54, 55). Three serotypes (called I, II, and III, corresponding to types 1, 2, and 3 in the present nomenclature) were found to be the prevalent causes of lobar pneumonia in the United States (23). In South Africa, Lister had concurrently developed his own scheme of serotyping and also found three predominant types, called serogroups A, B, and C, which correspond to present types 5, 2, and 1, respectively (54, 55). Was the low VE seen in the first trials due to the lack of an important serotype in the vaccine, whereas a successful pneumococcal vaccine should be a mixture of the most prevalent types? A large controlled trial involving 55,000 men, in which every alternate recruit arriving in the central compound was immunized with a five-valent vac-

cine, was conducted in 1914 (57, 62). In the first 5 months of observation, the VE was 25%, with the 95% CI from 15 to 36%, while no efficacy was seen in subsequent months, an outcome supporting the results of the preceding trials.

Lister himself was, however, in favor of a different approach: to vaccinate all miners during pneumonia outbreaks as quickly as possible to bring about a general decrease of pneumonia incidence (54). Therefore, he did not include a nonvaccinated control group, arguing that a controlled trial would give a falsely low estimate of VE because the reduced transmission of infection from those vaccinated would decrease the risk of pneumococcal infection in the controls also (predicted herd immunity). Over 30,000 men at different mines were vaccinated with a trivalent vaccine consisting of serogroups A, B, and C. Without controls, it was not possible to estimate the VE, and without serotyping of isolates in the cases that occurred, it is very hard to draw definite conclusions as to the general effectiveness of the vaccinations. Lister, however, was pleased with the results and attributed a reduced incidence of lobar pneumonia among miners to the vaccine (54).

The influenza pandemic of 1918 to 1919, together with the First World War, created a situation conducive to a highly increased incidence of pneumococcal pneumonia. Because of travel and crowded living conditions, military recruits formed a special high-risk group for the epidemic spread of both infections, but also an ideal population for the testing of pneumococcal vaccines. Encouraged by the South African experience, researchers prepared and administered a trivalent (type I, II, and III) vaccine to 12,519 men in training at Camp Upton, NY (15). During observation for 10 weeks, 17 cases of pneumonia in the vaccinated men and 110 in the 14,160 controls were registered, giving a VE of 82% (95% CI from 70 to 89%). Pneumococci were found in 35% of the cases and subjected to serotyping. Among the isolates from the vaccinated men, only one was of a type included in the vaccine, compared to 18 isolates from the controls, indicating a serotype-specific VE of 94%. At the same time, there was a 47% reduction in the number of infections with types not in the vaccine (9 among the vaccinated men and 17 among the controls) and a 90% reduction in the number of nonpneumococcal cases of pneumonia (7 and 75, respectively). The data are consistent with the assumed serotype-specific protection, but the apparent effects of the vaccine on infections with other serotypes and especially on cases of pneumonia in which pneumococci were not isolated are disconcerting. The former suggests a less-than-ideal selection of the controls but may also be due to chance,

while the latter may be due to an overall low sensitivity in detecting pneumococci in the sputum (detection in most cases was based on culture alone, while the more efficient mouse inoculation method was used in one-fourth of the cases only). A second trial was soon conducted in Camp Wheeler, GA, with 13,460 vaccinated recruits, but the outcome was confounded by the coincident influenza epidemic (16).

The combined results of the trials with the killed whole-cell vaccines were considered very encouraging at the time. Even a 30% reduction in numbers of cases of lobar pneumonia was welcomed because there were no alternative prevention measures. Consequently, whole-cell vaccines were used extensively in the U.S. Armed Forces in 1918 and 1919 and taken into routine use in the South African mines starting in 1918. The experience from 6 years of use in South Africa was generally found "satisfactory" (57), although critical opinions were also recorded (65).

Very soon, however, accumulating evidence suggested changes in the patterns of clinical presentation of pneumonia: a relative decrease of lobar pneumonia, an increase of "atypical" and bronchopneumonia, and a diminishing role of the pneumococcus in general as well as of the three previously predominant serotypes in particular (56, 57), although these views were also criticized (65). As a concrete example, the prevalence of serogroups A, B, and C among isolates from pneumonia patients had dropped to 4.2% from the 69% observed in the prevaccination era (56). In the hope of covering a broader range of potential pneumonia etiologies, more serotypes of pneumococci and several other respiratory-tract bacteria were added to the pneumococcal vaccine. These mixed vaccines were used in controlled trials of various designs, mainly in the United States (46, 51, 73, 87). The results of most of these trials were considered positive at the time in terms of reduced pneumonia incidence, but the numbers of cases were small and the studies were not rigorously controlled. No firm evidence of efficacy for other respiratory infections was obtained. A similar mixed vaccine was put into use in South African mines in 1930 (57).

## WHAT WAS LEARNED DURING THE WHOLE-CELL VACCINE PERIOD?

What was learned during the whole-cell vaccine period? First, common methods for vaccine preparation, administration, and dosage were developed and applied on the basis of results from animal experiments complemented by those from the early trials. Second, methods

for measuring protective immunity to pneumococcal infection were developed. These advances resulted in the mouse protection assay, the most reliable method for the purpose, and the opsonophagocytic assay as an *in vitro* correlate of the mouse protection assay (72, 92), which is still an important tool of pneumococcal immunology. Third, methods for clinical trials matured through experience, which particularly highlighted the need for rigorous study designs to prevent bias between study groups.

Most importantly, the killed whole-cell vaccine was shown to work. Although several of the trials had problems with their selection of controls, the South African trials consistently showed a VE of about 30% for lobar pneumonia and also a reduction in mortality associated with pneumonia. Most of these trials were conducted in the preserotyping era, and the VE estimates refer to all-cause lobar pneumonia as a clinical entity and thus are both lower than the serotype-specific VE and strongly dependent on the relative frequencies of the vaccine serotypes during the study.

The duration of the vaccine-induced immunity could not be addressed in most of the controlled trials because of short observation times necessitated by the transient nature of the study populations. In the light of current understanding of the immune response and of later experience with other pneumococcal vaccines, it is surprising that the protective effect of the vaccine appeared to be very short lived, only 2 to 3 months, in several of the early trials. At the time the studies were reported, this issue was not commented on, and in hindsight, one can only guess about possible mechanisms, e.g., a rapid change of serotypes causing disease after the vaccine-induced immunity had decreased the spread of the vaccine serotypes.

## ENTRY OF PS VACCINES

The experimental research of Avery, Heidelberger, and Goebel in the 1920s formed the essential links between pneumococcal capsules, their serotype specificity, and the identity of the capsules with PS that could be isolated from the bacterial cultures by chemical means (4–6, 36, 39). These investigators could separate two major fractions, the PS (called the specific soluble substance) and a “nucleoprotein,” from the cultures. Sera from animals immunized with a pneumococcal culture contained antibodies to the PS that were specific to the serotype and antibodies to the nucleoprotein that were species specific; i.e., they recognized the pneumococcus but did not discriminate among different types. Only the type-specific antibodies were protective, while the species-

specific antibodies were not. These observations suggested that the PS might be used as a vaccine; however, in early experiments, the immunization of rabbits with the purified PS did not elicit type-specific antibodies (4, 5, 39). This observation raised questions: was the PS a “partial antigen,” or hapten? (75). Fortunately, this conundrum was resolved with the demonstration of type-specific immune responses to other preparations of pneumococcal PS in several experimental animals and, most importantly, in humans (30, 32, 75). Successful immunization with purified PS opened up the possibility of developing a second generation of pneumococcal vaccines. Obvious advantages expected of PS vaccines included better tolerability that would allow more serotypes to be combined in a truly polyvalent vaccine. The feasibility of this idea was supported by the demonstration of unimpaired immunogenicity when six different capsular PS were used as a mixed vaccine (40). A strict serotype specificity of the vaccine was expected because it consisted of the serotype-determining PS only.

After two inconclusive trials (26, 78), a definitive demonstration of the efficacy of a four-valent PS vaccine was provided in 1945 by MacLeod et al. (59). The vaccine contained the purified capsular PS of serotypes I, II, V, and VII in doses ranging from 0.03 to 0.06 mg each and was injected subcutaneously. Preliminary studies with each of the component PS had shown the production of protective antibodies in mice. The subjects of the trial were students at an army-air force technical school, and great care was devoted to the randomization of the men to receive either the vaccine or a saline placebo as well as to the even mixing of the study populations during daily activities. The incidence of pneumococcal pneumonia (diagnosed by the inoculation of mice with sputum and serotyping of the bacteria recovered from the mice) had been very high in the school in the two previous winters, and 60% of the pneumococci had been of vaccine types (34% of type II). During the follow-up period, four cases of pneumonia caused by any of the vaccine serotypes were observed among the 8,586 vaccinees and 26 were observed among the 8,449 controls, indicating a VE of 84%, with 95% CI from 54 to 96%. The difference in the incidence of type II cases alone (1 in vaccinees, 14 in controls) was also significant. Cases of pneumonia due to serotypes other than those in the vaccine were split evenly between vaccinees and controls (56 and 59 cases, respectively). The duration of immunity could not be assessed since most of the students had left the school in 6 months. The oropharyngeal carriage of pneumococci was also studied using mouse inoculation followed by serotyping. The results

indicated a vaccine effect on carriage: 1.79% of all pneumococci isolated from the vaccinated men were of vaccine serotypes, in contrast to 3.25% of those isolated from the controls, indicating a 45% (95% CI, 15 to 64%) reduction of carriage prevalence.

Thus, the results of this trial unequivocally showed the four-valent PS vaccine to be efficacious in preventing pneumococcal pneumonia due to vaccine serotypes and specifically to type II. It also showed a reduction of vaccine-type carriage in the vaccinees compared to the controls, a totally new finding that has not been repeated in subsequent studies with the PS vaccine in children (24, 58). This effect of the vaccine is of special interest in the light of present experience with the pneumococcal conjugate vaccine and its ability to reduce carriage and the transmission of vaccine serotypes from vaccinated children to others (35, 37). Was such an indirect effect observed in the study of MacLeod et al.? The authors indeed thought so and argued for a strong herd immunity effect, as the numbers of vaccine-type cases even in the control group were lower than expected on the basis of the previous years' experience. An alternative explanation is, however, also possible: the erratic behavior of pneumococcal epidemics. The school had experienced a pneumonia outbreak in two previous years, with serotype II dominating, and the study year may have coincided with the end of the outbreak, unrelated to the vaccination.

One would have expected a general interest in the new PS vaccines, and accordingly, two hexavalent vaccines were developed and marketed in 1946 in the United States by Squibb and Sons (64), one intended for adults, the other for children. At the same time, sulfonamides and penicillin had become readily available, and new antimicrobial drugs were on the way; the general expectation was that these new treatments had eliminated the threat of pneumonia. Thus, the vaccines were forgotten and were withdrawn from the market in 1954.

## RENEWED INTEREST IN PNEUMOCOCCAL VACCINES

It did not take long before it became clear that the antimicrobials had not eliminated pneumococcal disease and that better ways to reduce disease and death caused by the pneumococcus were needed. Austrian and Gold described the clinical course of over 2,000 cases of pneumococcal pneumonia treated at Kings County Hospital in Brooklyn, NY, between 1952 and 1962 (3). Even with antimicrobial drug treatment, nearly one in four patients admitted with pneumococcal bacteremia died. Mortality was greatest among the elderly and

among people with certain underlying medical conditions. Nearly half of pneumococcal bacteraemia cases in patients aged 60 years and older were fatal. Ten serotypes accounted for over 70% of pneumonia cases among the Brooklyn patients, indicating the potential for a polyvalent vaccine containing PS from a relatively limited proportion of the many known serotypes to prevent the majority of infections. Moreover, the emergence of strains of pneumococcus with reduced susceptibility to antimicrobial drugs in the 1960s suggested that the remarkable efficacy of the antimicrobial drugs might be short lived (38, 45, 74).

In 1967, the Vaccine Development Committee of the National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIH), recommended federal funding for research and development of new pneumococcal vaccines. Between 1968 and 1976, the NIH invested \$6.5 million to define the epidemiology of pneumococcal disease, to refine serologic assays for pneumococcal PS antibodies, and to determine the safety, immunogenicity, and clinical efficacy of candidate vaccine components (64). An important advance was the development of methods allowing the accurate measurement of the immune response to the vaccine PS—the radioimmunoassay, to be supplanted later by the enzyme immunoassay. The assays were believed to be highly specific because the antigen was the purified capsular PS, but soon it was shown that another pneumococcal cell wall component, the C PS, was copurified with the capsular PS (84) and that the antigen measured antibodies to both the type-specific capsular PS and the non-type-specific C polysaccharide (52). The problem could be overcome by removing the anti-C PS antibodies by preabsorption, now routinely included in the assay protocols.

In 1968, Eli Lilly began preparing pneumococcal vaccines under contract with the NIH. During the period from 1970 to 1978, Merck, Sharp, and Dohme spent an estimated \$6 million without direct federal funding to develop a pneumococcal vaccine and bring it to the market. Pasteur Mérieux Séums et Vaccins and Lederle Laboratories also began work on pneumococcal vaccines during this period. Documenting the efficacy of the newly developed pneumococcal vaccines was challenged by the diversity of clinical manifestations, the difficulty of making a definitive etiologic diagnosis, and the uncertain incidence of disease in the general population. Therefore, as was done for the evaluation of the whole-cell vaccines 60 years earlier, clinical efficacy trials were conducted with people at high risk for pneumococcal pneumonia. Austrian conducted trials among approximately 9,000 South African gold miners and

demonstrated a protective efficacy of 76 to 100% for pneumococcal pneumonia and radiographically confirmed pneumonia (1, 2, 10, 83). A placebo-controlled study of a 14-valent vaccine administered to 12,000 Papua New Guinea highlanders aged 10 years and older found no decline in the overall risk of pneumonia, but the risk of death from pneumonia was lower for those who were vaccinated (69).

## LICENSURE AND USE OF PS VACCINES

Based on data documenting the safety of vaccination with pneumococcal PS and the success of the clinical trials, the U.S. Food and Drug Administration and Health Canada's Health Products and Food Branch approved Merck's 14-valent pneumococcal PS vaccine (PNEUMOVAX) in 1977. Similar 14-valent vaccines were subsequently produced by Lederle (Pnu-Immune) and by Pasteur Mérieux. Fourteen-valent vaccines were licensed in other countries, including The Netherlands in 1978, the United Kingdom and Norway in 1979, France and Germany in 1980, Australia and Sweden in 1981, and Italy in 1982 (29). Each 0.5-ml dose of these vaccines contained 50 µg of purified PS of pneumococcal serotypes 1, 2, 3, 4, 6A, 7F, 8, 9N, 12F, 14, 18C, 19F, 23F, and 25F (18). These 14 serotypes were selected based on epidemiologic information from the United States, parts of Europe, and South Africa and on limited immunochemical information (70). The serotypes included in these vaccines accounted for 68% of pneumococcal isolates from normally sterile body sites identified through a national sentinel surveillance program in the United States in 1978 and 1979, and serologically related serotypes accounted for another 16% (11).

The 14-valent vaccine was reformulated after the emergence of additional data on the distribution of serotypes causing disease and on the biochemical and immunologic properties of the capsular PS (70). New 23-valent vaccines were produced by Merck (PNEUMOVAX-23) and by Lederle (Pnu-Immune 23) and were approved in the United States and Canada in 1983. The 23-valent PS vaccine remains the only pneumococcal vaccine for the immunization of adults in the early 21st century. It differs from the earlier 14-valent vaccine in several respects. The dose of each PS was reduced from 50 to 25 µg in the 23-valent vaccine on the basis of results from studies demonstrating comparable postimmunization antibody concentrations for most serotypes, as measured by radioimmunoassay, in volunteers vaccinated with the lower dose (41, 69). Serotype 25F was dropped and 10 additional serotypes, 5, 9V, 10A, 11A, 15B, 17F, 19A, 20, 22F, and 33, were included to give the 23-valent vaccine. Serotype 6A was

replaced by 6B PS, which is more chemically stable and is capable of stimulating antibodies cross-reactive to both serotypes (71, 93).

Recommendations for the use of PS vaccine have differed among developed countries and have evolved over time. The Advisory Committee on Immunization Practices of the U.S. Public Health Service issued its first recommendation for the use of the PS vaccine in 1981 and targeted persons aged ≥2 years with splenic dysfunction, anatomic asplenia, or chronic illnesses associated with an increased risk of pneumococcal disease or its complications (18). After the licensure of the 23-valent vaccine, Advisory Committee on Immunization Practices recommendations were expanded to include all persons aged 65 years and older and were revised to specify chronic conditions for which vaccination was recommended (21). These recommendations were further updated in 1989 and 1997 to include guidelines for the immunization of persons infected with human immunodeficiency virus (HIV) and for the improvement of vaccine uptake and delivery, respectively (19, 20). Canada, Australia, New Zealand, and most, but not all, European nations also issued public health recommendations for the use of the 23-valent vaccine, although conditions considered to be indications for pneumococcal vaccination varied considerably (29). In March 1988, a technical advisory group convened in Copenhagen, Denmark, by the World Health Organization Regional Office for Europe recommended pneumococcal PS vaccination for all elderly persons and for persons at high risk of acquiring pneumococcal infection regardless of age (28).

Despite recommendations for routine pneumococcal immunization of at-risk populations, the uptake of the vaccine was relatively slow. Nearly 10 years after the licensure of PS vaccine in the United States, only 10% of persons for whom the vaccine was recommended had ever been immunized (91). Reasons for the low uptake may have been related to uncertainty about which patients should be vaccinated and the need and timing of revaccination, skepticism concerning vaccine safety and efficacy in high-risk populations in developed countries, and a general lack of enthusiasm among the medical community for adult immunization. Pneumococcal PS vaccines have often been designated the "pneumonia vaccine." However, pneumococcal infections probably cause no more than one-third of all cases of pneumonia, and the occurrence of nonpneumococcal pneumonia without etiologic diagnosis has led many patients and clinicians to conclude that the vaccine is of no benefit. Nonetheless, vaccine use began to increase in the United States and in other developed countries by the mid-1990s. In 2000, the U.S. Department of Health and So-

cial Services set the goal of providing pneumococcal and influenza vaccination to at least 90% of people aged 65 years and older, 90% of residents of long-term care facilities, and 60% of high-risk adults aged 18 to 64 years by the year 2010 (86). By 2002, the proportion of Americans reporting ever having received a pneumococcal vaccine had increased to 56%; unfortunately, between 2003 and 2005, no additional increase in vaccine utilization was detected, and vaccination levels remain below the goals for 2010 (17). Outside the United States and Canada, the use of the 23-valent PS vaccine continues to be low or minimal (29).

Postlicensure epidemiologic studies have generally demonstrated moderate effectiveness of pneumococcal PS vaccine for the prevention of invasive pneumococcal infections, such as bacteremia and meningitis, among people at increased risk of disease because of advanced age or chronic underlying medical conditions (13, 22, 27, 63, 77). Economic analyses in the United States have shown that vaccination to prevent invasive infections among persons aged 65 years and older results in cost savings and that the vaccination of high-risk persons aged 50 to 64 years compares favorably with other prevention measures (80, 81).

Prospective studies and more than a quarter-century of clinical experience have confirmed the safety of PS vaccines. The most common side effect is injection site soreness within 48 h of vaccination. These reactions are more common and more pronounced with repeated doses of a vaccine but are relatively mild (43, 88). A meta-analysis of nine randomized, controlled trials of PS vaccine indicates that a mild local reaction (injection site discomfort, erythema, or swelling) occurs in less than one-third of vaccine recipients, and there were no reports of severe febrile reactions (31). Life-threatening reactions, such as anaphylactic reactions, to PS vaccine are extremely rare, and neurological disorders, such as Guillain-Barré syndrome, have not been linked to pneumococcal vaccination. Pneumococcal PS vaccine has been associated with a transient increase in the concentrations of HIV in the sera of infected persons (9), but this phenomenon is also observed after other immunizations and during episodes of acute bacterial pneumonia and is not of proven clinical significance (12, 85).

However, the data demonstrating the safety and effectiveness of the PS vaccine against invasive infection must be balanced with data that display the vaccine's shortcomings (34, 89). Protection against pneumococcal bacteremia provided by the 23-valent PS vaccine wanes over a period of 3 to 5 years, particularly in older people (77). Several large studies and meta-analyses of vaccine trials have not documented effectiveness against lower-respiratory-tract infection without bacteremia

among the elderly in developed countries, the population in which PS vaccines are most frequently used (42, 44, 60, 66, 79). Additionally, large epidemiologic studies have for the most part not documented any effectiveness of pneumococcal PS vaccine among people with immunocompromising medical conditions (14, 77). Studies among people infected with HIV have provided conflicting data (8, 33, 77). An epidemiologic study among Navajo Indians suggested that the effectiveness of PS vaccine among certain groups with high rates of invasive infection may be lower than what has been found for the general population (7).

## NEED FOR IMPROVED VACCINES

When the PS vaccines were introduced, the focus of attention was on pneumonia in adults, with little consideration of pneumococcal infections in young children. It has been estimated that each year, 2 million children less than 5 years old in developing countries die of acute respiratory infections, mostly pneumonia, with the pneumococcus as its main cause (90). In industrialized countries, pneumonia is less dangerous, and acute otitis media (AOM) is the most commonly identified pneumococcal infection in children under 2 years of age. About a third of AOM cases are caused by pneumococci (48, 49). Vaccines for the prevention of lower-respiratory-tract infections and otitis media in young children are badly needed.

With the licensure of the 14-valent PS vaccine, research to test its potential for the prevention of AOM was initiated. (47, 82). It soon became clear that the immune response to this vaccine (like other PS vaccines) was age dependent and weak in patients under 2 years of age (25, 52, 76). Controlled trials were conducted to evaluate the VE for AOM. In carefully designed trials, infants were enrolled at 2 to 3 months of age or after several previous attacks (to capture a possible effect on recurrent AOM). When AOM was identified clinically, tympanocentesis was performed to obtain middle-ear fluid for bacterial isolation and serotyping. The results were disappointing: while there was some indication of protection in the older children (those vaccinated when at least 3 years old) and of efficacy for serotypes giving the best antibody responses, no protection was seen after the immunization of infants (47, 82). On the other hand, the results suggested that the vaccine may well be protective if it could be made immunogenic in children in the critical age group of under 2 years.

At the same time that the trials of VE for AOM were conducted, Riley and colleagues carried out double-blind, controlled trials in Papua New Guinea, where acute lower-respiratory-tract infection (ALRI) was very

common in children (67). In Tari, 871 children 6 months to 5 years old were vaccinated with either the 14-valent vaccine or a saline placebo. The follow-up for subjects with clinically diagnosed end point cases of ALRI showed a VE of 37% if the child was at least 17 months old when vaccinated but no efficacy if the child was younger. The combined results of two subsequent studies indicated a statistically significant VE of 59% (95% CI from 20 to 79%) against death from ALRI as the sole cause (as determined by interviewing parents) in children immunized when younger than 5 years but not in those immunized when younger than 6 months of age (68). There was no effect on respiratory-tract-associated morbidity (53). Taken together, these results did not encourage the use of the PS vaccine in young children, among whom the incidence of pneumonia is the highest.

The clear message from this experience, supported by the numbers of cases potentially preventable by effective pneumococcal vaccines, was to encourage the development of vaccines that are efficacious when given in infancy to prevent pneumococcal disease during the first 2 years of life, when the risk of life-threatening infection and AOM is greatest. The 7-valent pneumococcal conjugate vaccine licensed in 2000 and plans for expanded-valency pneumococcal conjugate vaccines answer this need. Another group with a great need of pneumonia prevention is the elderly, for whom the PS vaccine has not fulfilled expectations, adding to the challenges for further vaccine development.

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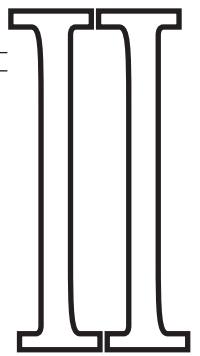
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# *Biological Basis*

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Janet Yother  
Stephen D. Bentley  
John P. Hennessey, Jr.

# 3

# Genetics, Biosynthesis, and Chemistry of Pneumococcal Capsular Polysaccharides

The *Streptococcus pneumoniae* capsules form a diverse group of polymers that are the most important and most recognized virulence factor of the organism. The capsular polysaccharides are essential for virulence and are the target for all current pneumococcal vaccines. To date, 91 distinct capsular serotypes have been recognized, and these differ with respect to the sugars and linkages that make up the repeating units. This chapter provides an overview of recent advances regarding the genetics, biosynthesis, and chemistry of pneumococcal capsules.

## CHEMISTRY AND SEROLOGY

### Serological Identity

For over 70 years, the fundamental empirical differentiator of pneumococcal isolates has been the unique serological identities of their capsular polysaccharides (87). Even before direct analysis could clearly reveal the chemical similarities and differences among the polysaccharides of pneumococcal isolates, antisera, typically raised in rabbits, could be used to distinguish between several dozen subspecies of *S. pneumoniae*. At present, a

total of 91 serologically distinct capsular polysaccharides have been described (47, 75). The Danish nomenclature for the naming of isolates has become the preferred system, as it reflects the cross-reactivity of the polysaccharide serotypes by placing them into 46 serogroups. This intricate system of defining the serogroup and serotype of a given isolate is based on a highly refined collection of factored antisera that permit the recognition of even subtle chemical differences between isolates. The use of such reagents has allowed the mapping of the antigenic epitopes of the polysaccharides to define both the unique and the cross-reactive sites within these 91 molecular entities at a level yet to be approached by chemical analyses (47, 75).

### Chemical Identity

In parallel with the increasing refinement of serological evaluations of pneumococcal polysaccharides, chemical analyses of increasing sophistication have been applied to unravel the chemistry of this family of biologically related molecules. Starting with the work of Avery and Heidelberger in the 1920s (7, 39), which established the carbohydrate nature of these “soluble specific

Janet Yother, Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL 35242. Stephen D. Bentley, Sanger Institute, Wellcome Trust Sanger Institute, Hinxton, Cambridge, United Kingdom. John P. Hennessey, Jr., Bioprocess Research & Development, Merck Research Laboratories, West Point, PA 19486.

substances” by chemical reactions, and continuing with modern analyses using high-field nuclear magnetic resonance, advances in the research have built an increasingly sophisticated understanding of the chemical compositions and structures. Details of the chemical structures of no fewer than 55 serotypes of pneumococcal polysaccharides, with compositional analyses of another 6 serotypes, are now available. The most recent compilation of chemical structures was provided by Kamerling (47), but since that time there have been five additional contributions to the literature on pneumococcal polysaccharide structures (44, 45, 53, 75, 84). All but one of these has been a refinement of previously published structures in which a better understanding of the O acetylation pattern of the carbohydrate ring structures has constituted one of the main refinements. However, in one contribution, Park et al. (75) established the chemical identity of the 91st serotype, type 6C, producing the first addition to the collection since 1995 (40).

## Physical Evaluation

Of the 55 serotypes that have been evaluated for composition and structure, only a subset have been evaluated for their physical properties. Not surprisingly, these evaluations have been focused mainly on the 23 serotypes used in the commercially available pneumococcal polysaccharide vaccines (PNEUMOVAX 23 [Merck & Co., Inc.] and PNEUMO 23 [Sanofi Pasteur]). One of the most important physical attributes of these molecules is their molecular size. Given the heterogeneous nature of polysaccharide preparations, molecular size can be fully reflected only in terms of average molecular size and polydispersity, which together reflect the size distribution of molecules in the preparation (9).

It has long been established that the molecular size distributions in polysaccharide (and other polymer) preparations can directly impact the biological activity of the polysaccharides as immunogens; i.e., the production of antibodies is reduced as the average molecular size of the immunogen is reduced below a threshold value, with the threshold value varying by polysaccharide (14, 41, 46, 60, 93). This phenomenon is likely also polysaccharide dependent. The fact that there is only limited evidence available to suggest that the functional activities of antibodies generated against very small polysaccharides can differ from those of antibodies generated against larger polysaccharides of the same composition (94) speaks to the complex nature of the chemistry and immunology of this class of compounds. Nonetheless, this complex relationship has been the driving force in establishing the historical importance of

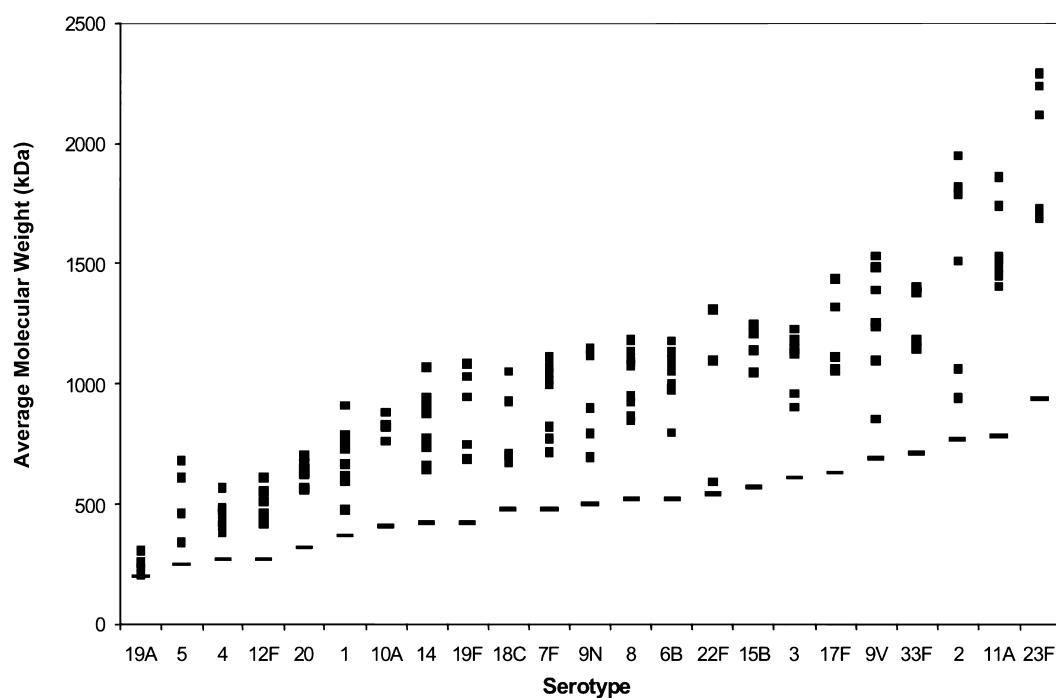
molecular size distribution as a physical parameter of biological significance.

Extensive studies have been conducted on the molecular size distributions in the polysaccharide preparations used in PNEUMOVAX 23 over the past 30 years (9, 58, 85). These studies have provided an extensive database for the assessment of appropriate manufacturing process capability-related product specifications for these polysaccharide preparations (57, 84) (Fig. 1). Historical use of the vaccine has shown no indication that these specifications, calling for average molecular sizes that exceed a minimum value, allow the use of material that may be of inferior immunogenicity.

One potential immunological impact of altered molecular size distribution is the creation or removal of conformational epitopes. Such epitopes have been shown to exist on pneumococcal serotype 14 polysaccharide (92) and the structurally related type III group B *Streptococcus* polysaccharide (101). Both of these polysaccharides have been shown to lose an immunogenic epitope upon molecular size reduction (93, 101), but there are limited data to support the immunological significance of losing this epitope (e.g., a change in the bactericidal activity of antibodies to the remaining epitopes).

## Impact of Genetics on Polysaccharide Structure and Function

Recently, a new era in differentiating pneumococcal isolates was initiated when, for the first time, preparations of monoclonal antibodies were used to describe the 91st serotype, a “subspecies” of serotype 6A now dubbed 6C (75). Though this new serotype was immunologically hidden from detection with factored polyclonal reagents, the polysaccharide was shown to be an antigenically and chemically distinct species and has been confirmed to be present in serum isolates collected as long ago as the late 1970s. Park et al. (75) provide an interesting glimpse into the dynamics of pneumococcal genetics in showing that 6B and 6C polysaccharides differ in one carbohydrate component (galactose versus glucose [Glc]) that results from a gene insertion (74a), whereas serotype 6A and 6B polysaccharides show only a linkage difference (rhamnose [Rha]-1,3-ribitol versus Rha-1,4-ribitol) that is the result of a nucleotide change in a single gene (61). In this example, the antigenic epitopes on each molecule are clearly influenced by each of these chemical differences in the resultant polysaccharides. These polysaccharides stand in contrast to polysaccharides of other serotypes in which repeating-unit structures containing as many as eight chemical components



**Figure 1** Range of average molecular size of purified pneumococcal polysaccharides from preparations made on a commercial scale and the acceptance criteria for each serotype. Data were generated as described in reference 58.

(e.g., the serotype 45 polysaccharide) have but a single known antigenic epitope (47). Moreover, polysaccharides of various serotypes that otherwise differ only by their extent of O acetylation can show differences in their epitope structures (e.g., serotype 9A and 9V polysaccharides) but little difference in their cross-reactive abilities (47).

This intriguing observation establishes two new considerations in our collective understanding of the immunology and chemistry of pneumococcal polysaccharides: (i) with more-selective immunological reagents, more hidden serotypes and unique epitopes are almost certain to be discovered, and (ii) with more-refined attention to the isolation of clonal organisms and more-detailed evaluation of factors that impact polysaccharide biosynthetic pathways, previously hidden chemical structures may start to emerge. Alternatively, serotypes with closely related chemical structures may be shown to be phenotypic variants of the same genotype.

As to the first point, there is practical relevance to be gained in better dissection of the immunological structures of these polysaccharide entities. The intricate cross-reactivities of serotypes 6A, 6B, and 6C provide an interesting reflection on a historical conflict in opinions on the cross-protection afforded by 6B and 6A in

polysaccharide and polysaccharide-protein conjugate vaccines. This observation also poses a new question to consider in evaluating breakthrough cases of pneumococcal infection with vaccine serotypes (e.g., 19F in conjugate vaccine clinical trials) among vaccinees.

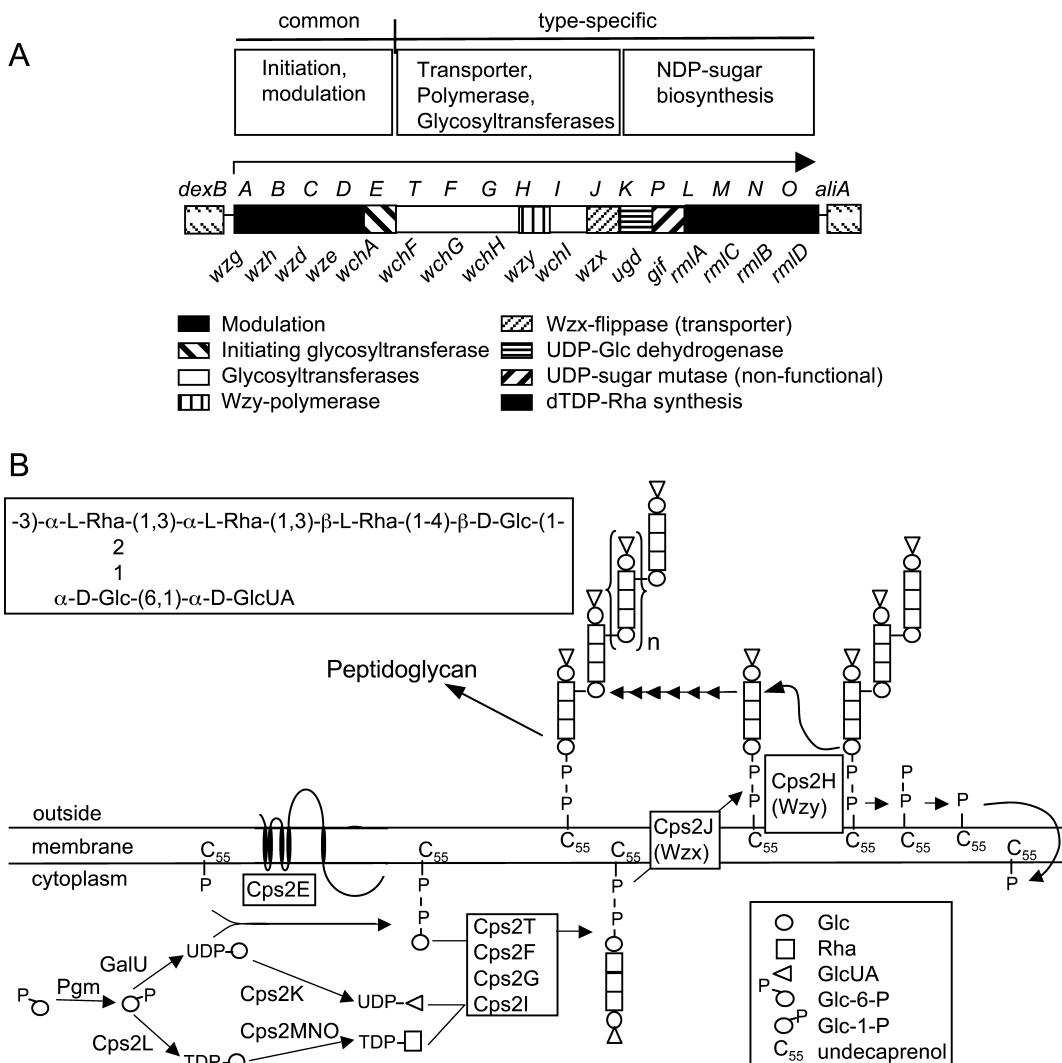
As to the second point, there is reason to consider that many serotypes may in fact contain multiple chemotypes that cannot be distinguished based on (present) serological criteria. In addition to the serogroup 6 example given above, multiple chemical structures have been reported for serotype 19A (52) and differences in the O acetylation patterns of several serotypes are evident from the literature (e.g., references 44, 52, and 83). Given that many of these differences are at the level of minor substitutions on a given ring structure, it is possible that some of these structures are anomalies of the clonal isolate, the conditions used to grow the bacterial cultures, or even the means of polysaccharide purification.

## GENETIC BASIS FOR CAPSULE EXPRESSION

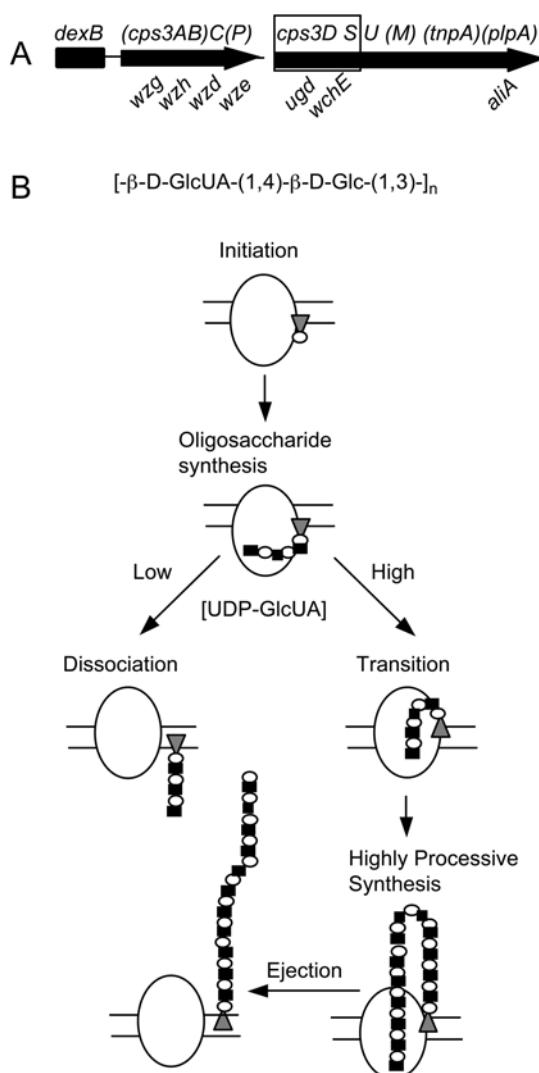
Early work led to the conclusions that genes required for capsule synthesis in *S. pneumoniae* were closely linked on the chromosome but that genes outside this region were also important (references 6, 30, 31, 57,

and 78; reviewed in reference 98). Modern molecular genetic studies have confirmed and expanded on these observations. The first DNA sequences of the capsule loci were obtained for serotypes 3, 19F, 14, 1, and 19B. From the results of these studies and hybridization analyses performed therein, it was evident that the capsule genetic loci exhibit a conserved organization wherein sequences unique to a given capsular serotype are flanked by sequences apparently common to all types (3, 4, 28, 29, 35, 36, 49, 51, 67, 69, 72). In each

capsule type, the flanking sequences are *dexB* and *aliA*, indicating a common location in the chromosome. It was also evident from the sequences that at least two different mechanisms are utilized in the synthesis of pneumococcal capsules—one, observed in types 19F and 14, is similar to that for capsules from group B streptococci (*Streptococcus agalactiae*); the other, observed in type 3, is similar to that for the hyaluronic acid capsule of group A streptococci (*Streptococcus pyogenes*). The details for both mechanisms of synthesis, referred



**Figure 2** Wzy-dependent capsules. (A) The top panel shows the typical organization of a Wzy genetic locus. The map shows the genes for the serotype 2 locus. Gene designations above the line are from reference 42; gene designations below the line are from reference 12. *cpsE* (*wchA*) homologues are common to 65 of the 88 *S. pneumoniae* Wzy loci. The arrow indicates the direction of transcription and putative transcript length. (B) Pathway for serotype 2 synthesis. The structure of the type 2 repeat unit is shown in the box. GalU and Pgm are encoded outside the capsule locus. NDP, nucleotide diphosphate; n, any number.



**Figure 3** Synthase-dependent type 3 capsule. (A) Genetic locus. Gene designations above the line are from references 16, 28, 29, and 100; gene designations below the line are from reference 12. Arrows indicate the direction of transcription of *cps3DSUM-tnpA-plpA* and the direction of genes *cps3ABC*, which are not transcribed. Sequences in parentheses are mutated. Only *cps3D* and *cps3S* (boxed) are required for type 3 synthesis. (B) Structure of type 3 polymer and model for type 3 synthesis. The model assumes high UDP-Glc concentrations, as in the cell. Triangles, phosphatidylglycerol; circles, Glc; squares, GlcUA; n, any number.

to as synthase dependent and Wzy dependent, respectively, according to the polymerase involved, have been expanded in the ensuing years and are described in depth below. Figures 2 and 3 show the typical genetic loci and biosynthetic pathways.

The conclusions derived from the first identified sequences have been largely borne out by sequence analy-

ses of additional serotypes (42, 43, 55, 64, 68, 71, 88) and most recently by the results of a study determining the sequences of the capsule loci from strains representing the 90 serotypes known at the time (1, 12, 60a). Sequencing of the loci in the latter study took advantage of the common chromosomal location of the capsule genes, allowing for the amplification and cloning of the loci using PCR primers specific for the flanking genes, *dexB* and *aliA*, whose functions are apparently unrelated to capsule synthesis. The strategy proved successful for all 90 serotypes, generating sequences ranging from 10,337 bp (type 3) to 30,298 bp (type 38), with an average size of 20,714 bp (12). The data obtained provide a detailed overall view of pneumococcal capsule genetics and will also enable the generation of comprehensive molecular capsule-typing schemes allowing robust, high-throughput screening of large numbers of pneumococcal isolates.

Eighty-eight of the 90 serotypes exhibit sequences consistent with the Wzy-dependent synthesis mechanism, whereas only two types, 3 and 37, utilize the synthase-dependent mechanism (12, 54, 56). As further described below, the type 3 locus displays differences from the other loci that are consistent with the different biosynthetic mechanism. More striking, however, is the observation that the synthase-encoding gene (*tts*) of serotype 37 is not located within the capsule locus and that an apparently silenced type 33F locus is present between *dexB* and *aliA*; thus, type 37 pneumococci are described as natural, genetically binary strains (56). The assumption that the serotype is determined by the genes present between *dexB* and *aliA* is therefore not always correct, and caution is required when the genes at the capsule locus appear to be inconsistent with those expected from the known repeat unit structure or the serological reactions.

The locus containing the genes required for capsule production is generally referred to as *cps* (capsular polysaccharide synthesis) based on nomenclature described in 1994 (28, 29, 36, 63). Here, the locus name is followed by a number designating the serotype and a letter designating a specific gene (e.g., *cps19fA* for gene A in the type 19F locus and *cps3D* for the type 3 UDP-Glc dehydrogenase gene). Earlier designations included *cap* (13), and this nomenclature has been retained in some instances (1a, 4, 55, 56, 71, 72). The genes located at the 5' ends of the Wzy-dependent capsule loci, *cpsABCDE*, are conserved among most of the 88 *S. pneumoniae* serotypes, as well as the capsule loci of many other gram-positive cocci, and these designations have also been adopted for those systems (23, 80, 83). Beyond *cpsE*, however, the genes become specific for serotype,

and for example, *cpsF* in one serotype may not correspond to *cpsF* in another serotype. This complexity in nomenclature led to the use of a system devised in 1996 (79) for naming the genes identified as part of the project to sequence the 90 serotypes (12, 43). While this system lacks the ability to easily identify the serotypic origin of a gene, the general functions and orthologies of other gene clusters can be readily recognized.

### Organization of Wzy-Dependent Loci

The Wzy-encoding loci are conserved with respect to their location between *dexB* and *aliA*, the presence of common sequences in the 5' ends of the loci, and the presence of genes specific for serotype between the common genes and *aliA* (Fig. 2A). In most loci, the order of the first four genes is *cpsABCD* (*wzg*, *wzh*, *wzd*, and *wze*). In types 25F, 25A, and 38, a rearrangement changes the order to *cpsCD-tnp-cpsAB*, where *tnp* is a transposase gene. As described below (see Biosynthesis and Regulation), *cpsABCD* encode proteins involved in the modulation of capsule expression. The fifth gene encodes the initiating glycosyltransferase, which in most cases (65 of 88 serotypes) is a homologue of CpsE (WchA), which adds Glc-1-P to undecaprenyl-P (see Biosynthesis and Regulation below). When Glc is not present in the capsule structure, the fifth gene encodes an initiating glycosyltransferase of a different homology group (WciI, WcjG, or WcjH).

The initiating glycosyltransferase gene is followed by genes encoding proteins for the synthesis and modification of the oligosaccharide repeat units (glycosyltransferases), the transport of the repeat units across the cytoplasmic membrane (Wzx flippase), and the linkage of the repeat units to form the mature polysaccharide (Wzy polymerase). When nonhousekeeping sugars are included in the capsule, the genes for their synthesis are always present within the *cps* locus and generally form the 3' portion of the gene cluster (Fig. 2A).

Between the *cps* genes proper and the flanking *dexB* and *aliA* genes, there are additional sequences with homology to pneumococcal insertion sequence (IS) element transposase genes. It is tempting to suggest that such sequences may coordinate the mobilization of the *cps* loci, but in most cases the genes are incomplete or are interrupted by other IS elements. Regions occupied by these IS elements appear to be tolerant of such insertion events, though it is not clear why they have not been purged from the genome. It is plausible that such conserved sequence elements at equivalent flanking locations may facilitate recombination, allowing for the exchange or acquisition of nonhomologous *cps* genes or the deletion of the locus.

Transcription of the *cps* genes is unidirectional and is expected to proceed from a single promoter located upstream of *cpsA* (51, 67, 72). In many instances, the ribosome-binding site is located either within the prior gene or closely after its termination. Both cotranscriptional and cotranslational regulation may thus be important in capsule expression.

The overall G+C content of *cps* DNA sequences is below the average for pneumococcal genomes. Such deviations are generally due to functional constraints on sequence divergence that disallow the alteration of the genomics background (e.g., RNA and protein genes) or are a possible indicator of mobile DNA and/or recent lateral acquisition. There is often a distinct G+C profile across the *cps* locus, where a low-G+C-content trough is associated with the central region encoding the glycosyltransferases, Wzx flippase, and Wzy polymerase. The genes in this region are often referred to as the serotype-specific genes, and it may be that their low G+C content reflects a propensity for lateral transfer. The mixing of sugar linkage genes may theoretically lead to the generation of novel capsule types, though the substrate specificity of the Wzx flippase and Wzy polymerase proteins would present a significant hurdle.

### Analysis of Sequences from Wzy-Dependent Loci

The identification and assignment of *cpsABCD* (*wzg*, *wzh*, *wzd*, and *wze*) and any nonhousekeeping sugar biosynthesis genes are fairly straightforward, as their sequences are highly conserved. Conversely, the sugar linkage and modification proteins, the Wzx flippase, and the Wzy polymerase have highly divergent sequences reflecting an ability to generate and process a broad range of oligosaccharide repeat units. The Wzx flippase and Wzy polymerase are most easily recognized by the presence and number of membrane-spanning regions, as seen on a standard hydrophobicity plot: Wzx flippases normally have 12 membrane-spanning regions, while Wzy polymerases normally have 11 to 13 (77).

The enzymes that link individual sugars to the repeat unit are broadly classed as glycosyltransferases. Comparing their sequences against those in the public protein databases using BLASTP or FASTA identifies matches to other glycosyltransferases, but the sequence diversity, poor annotation, and lack of characterization usually result in few clues to the specificity of the enzyme. Assignment to broad glycosyltransferase families can be done using the Pfam database (<http://www.sanger.ac.uk/Software/Pfam/>) (8, 32) or the CAZy database (<http://afmb.cnrs-mrs.fr/CAZY/>) (25), but these will give information about only the type of linkage per-

formed. On the basis of sequence similarity, Bentley et al. clustered all the proteins from the 88 *cps* loci into homology groups (HGs), which could then be correlated with known capsule structures and serological profiles (12). The cooccurrence of HGs corresponding to *cps* genes and particular sugar linkages in capsule structures provided good evidence for the assignment of specific substrates to HGs, and these data could then be extrapolated to predict currently unknown capsular polysaccharide structures with a degree of confidence. Interestingly, there is a strong correlation between the order of glycosyltransferase-encoding genes in the *cps* loci and the order of sugar linkages in the known capsular polysaccharide repeat unit structures, suggesting a physically integrated protein complex for the sequential linking of sugars possibly generated through the coupled translation of a long *cps* transcript.

The assignment of all genes to HGs effectively digitizes the *cps* loci with a profile that corresponds to the HGs present. The loci can then be clustered according to the proportion of shared HGs and weighted for the sequence similarities among the shared *cps* genes to identify groups of similar loci. It should be noted, however, that these loci are likely to have been generated through a long and complex evolutionary path such that standard phylogenetic interpretation is inappropriate for this data set. However, the clustering analysis has been useful in describing coarse groupings (clusters) and highlighting closely related groups of loci (subclusters).

Generally, the clustering analysis lends support to the serological groupings but also identifies some interesting relationships. Serotypes fall into 21 subclusters. In six cases (serogroups 7, 16, 17, 33, 35, and 47), serotypes within the same serogroup are in different subclusters, and conversely, nine subclusters include completely different serotypes. Thus, the sequences and gene orders of the *cps* loci of types 44 and 46 show extensive identity to those of the locus of serogroup 12, as do those of the loci of serotypes 35A, 35C, and 42 to those of the loci of serotypes 7B, 7C, and 40. The similarities among loci of different serotypes are sometimes greater than those among serotypes within the same serogroup. For example, serotype 40 is more similar in sequence to type 7B than 7B is to 7C and is much more similar to the latter two serotypes than these are to serotypes 7F and 7A.

Extending the clustering analysis to include Wzy-dependent gene clusters from other streptococci shows that the loci encoding *Streptococcus oralis* and *Streptococcus mitis* polysaccharide biosynthesis proteins are likely to share a recent ancestor with a subcluster that also includes *S. pneumoniae* serotype 21, whereas the

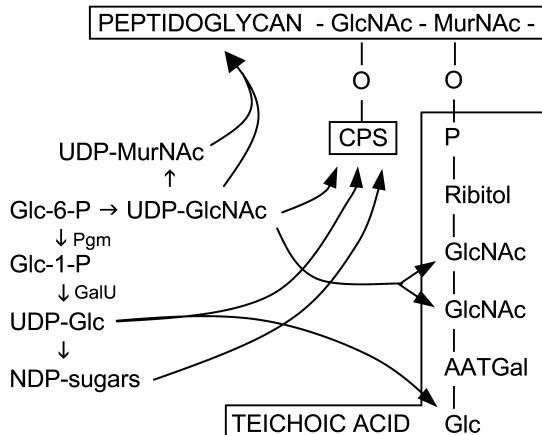
*S. agalactiae* *cps* loci form a divergent cluster that is not closely related to any of the pneumococcal *cps* loci.

### Synthase-Dependent Loci

As noted above, the type 37 *cps* locus is occupied by a cryptic type 33F locus, and the type 37 synthase-encoding gene (*tts*) is located elsewhere on the chromosome (56). It is the only capsule-specific gene necessary for the synthesis of the type 37 Glc polymer. In the serotype 3 locus (Fig. 3A), *cpsABCD* homologues are present but they are not transcribed and all but *cpsC* are mutated (3, 16, 100). Similarly, *aliA* (originally designated *plpA*) is truncated at the 5' end (16, 29). The type 3 *cps* genes are transcribed as an operon from a promoter located upstream of the first type-specific gene (3, 16). The transcript corresponds to *cps3DSUM-tnpA-plpA*, where *tnpA* is a truncated or defective IS1167. *cps3DSUM* (also referred to as *capABCD*) encode a UDP-Glc dehydrogenase (a homologue of the product of *ugd*) required for the synthesis of the precursor UDP-glucuronic acid (GlcUA), the type 3 synthase (WchE family), a Glc-1-P uridylyltransferase, and a C-terminally truncated (and defective) phosphoglucomutase (2–5, 16, 28, 29). Although the Glc-1-P uridylyltransferase encoded by *cps3U* is functional, neither it nor the defective phosphoglucomutase (Cps3M) are required or sufficient for type 3 synthesis, as these functions are provided by the cellular enzymes encoded by *galU* and *pgm*, located elsewhere on the chromosome (16, 28, 37, 62) (see Biosynthesis and Regulation below).

## BIOSYNTHESIS AND REGULATION

Capsule synthesis requires the production of nucleotide sugar precursors, the synthesis of which relies on enzymes encoded both within and outside the capsule genetic loci. For sugars such as GlcUA and Rha, which do not occur in other cellular structures, the synthesis of the precursors (UDP-GlcUA and TDP-Rha, respectively) is catalyzed by enzymes encoded within the capsule loci. In contrast, precursors such as UDP-Glc, UDP-N-acetylglucosamine (UDP-GlcNAc), UDP-N-acetylgalactosamine (UDP-GalNAc), and UDP-2-acetamido-4-amino-2,4,6-trideoxy- $\alpha$ -D-galactose (UDP-AAT-Gal), which are utilized in other cellular pathways, are derived from cellular pools and require enzymes encoded by genes located outside the capsule loci. These nucleotide sugars are also used in the synthesis of peptidoglycan, teichoic acid, and lipoteichoic acid (Fig. 4). Most capsules contain at least some sugars that are derived from cellular nucleotide sugar pools, and some, such as the *S. pneumoniae* type 14 capsule (containing



**Figure 4** Common pathways involved in the synthesis of surface polymers. Teichoic acid and a Wzy-dependent capsular polysaccharide (CPS) are shown linked to peptidoglycan. NDP, nucleotide diphosphate.

GlcNAc, Gal, and Glc), rely totally on these pools. Of central importance is the synthesis of UDP-Glc, which requires the cellular enzymes phosphoglucomutase (Pgm; Glc-6-P → Glc-1-P) and Glc-1-P uridylyltransferase (GalU; Glc-1-P → UDP-Glc). Mutations affecting these enzymes not only result in reduced capsule synthesis but also can be lethal due to their effects on other pathways (Fig. 4) (37, 38, 62). In addition, the synthesis of most *S. pneumoniae* capsules involves initiation on the same lipid acceptor that is used in peptidoglycan and teichoic acid synthesis (see below). Teichoic acid and Wzy-dependent capsules are ultimately linked to the peptidoglycan (Fig. 4), and as discussed below, certain mutations that affect capsule-specific genes are lethal due to effects on this part of the pathway.

Mechanisms for synthesizing pneumococcal capsules are closely related to those used in the synthesis of lipopolysaccharide O-antigens, and their classification is based on that defined for O-antigen synthesis (96). Three mechanisms of synthesis are known, but only the Wzy- and synthase-dependent mechanisms occur in *S. pneumoniae*. Not represented is the ATP-binding cassette (ABC) transporter-dependent mechanism, in which a completed polysaccharide is assembled on the cytoplasmic face of the membrane and then transported to the external face of the membrane by an ABC transporter.

### Wzy-Dependent Capsule Synthesis

Wzy-dependent, or block type, synthesis is similar to the synthesis of peptidoglycan. In addition to being the most common mechanism for synthesizing pneumococcal capsules, it is used in most streptococci and related

organisms, including *S. agalactiae*, *Streptococcus thermophilus*, and *Lactococcus lactis*, as well as many other gram-positive and gram-negative bacteria (95, 99). The basic steps involve the assembly of a repeat unit on a lipid acceptor located on the cytoplasmic face of the membrane, the transport of the lipid-linked repeat unit to the extracellular face of the membrane, the polymerization of repeat units into a long-chain polymer, and the transfer of the polymer to the cell wall (Fig. 2B).

Polymer synthesis likely initiates on undecaprenyl-P ( $C_{55}\text{-P}$  or the bactoprenyl-P), the same lipid acceptor used to initiate the synthesis of peptidoglycan and teichoic acids. In *S. pneumoniae*, the acceptor is a polyprenyl-P whose size and properties are consistent with undecaprenyl-P (18). In Glc-containing polymers, synthesis initiates by the addition of Glc-1-P to undecaprenyl-P and is catalyzed by a homologue of CpsE (WchA family) (18, 49, 50, 76, 88). These proteins consist of four N-terminal transmembrane domains, a large extracellular loop, and a large cytoplasmic domain that contains the glycosyltransferase activity (Fig. 2B). The role of the extracellular domain is unknown, but this sequence is absent in *S. pneumoniae* glycosyltransferases that mediate the initiation of capsules lacking Glc. It is present, however, in homologues found in gram-negative bacteria that lack Glc in their polymers, and thus its significance in relation to the initiating sugar is unclear (97).

Following initiation, the complete repeat unit is assembled on the cytoplasmic face of the membrane by the action of multiple glycosyltransferases. The glycosyltransferases are generally unique to a given locus, and their activity determines both the sugar added and its linkage. By analogy to O-antigen assembly, the transport of the repeat unit to the extracellular face of the membrane is expected to be mediated by the Wzx flipase, which is encoded in all Wzy-dependent polysaccharide loci. On the extracellular face of the membrane, the Wzy polymerase mediates polysaccharide assembly by linking the growing polymer at its reducing end to a single lipid-linked repeat unit. A large percentage of the polymer is covalently linked to the peptidoglycan (10, 81), and by analogy to the *S. agalactiae* type III capsule (27), the linkage is likely to be via the GlcNAc residue of the peptidoglycan (Fig. 4). The enzymes involved in transfer to the cell wall are not known, but transfer appears to be independent of polymer size, as the same range of short to long polymers occurs in both membrane and cell wall fractions (10).

The repeating units of many capsules are branched (Fig. 2B), and it is evident that the lack of the complete repeat unit (which ultimately forms the side chain) can

be detrimental, at least in the case of serotype 2 (97). Here, mutants lacking UDP-Glc dehydrogenase (encoded by *cps2K*, a *ugd* homologue) are unable to synthesize UDP-GlcUA, the precursor of the terminal sugar, GlcUA. These mutants make significantly reduced levels of capsular polysaccharide, which is not transferred to the cell wall. Additionally, they contain secondary mutations that occur primarily in the initiating glycosyltransferase, Cps2E. The presence of these secondary, suppressor mutations is necessary to sustain viability. Reductions in repeat unit initiation are apparently essential due to the retention of the polymer on undecaprenyl-P and either the destabilization of the membrane or the lack of available undecaprenyl-P for other pathways. The deletion of *wzx* or *wzy* is likewise detrimental to the cell and leads to selection for isolates containing secondary mutations, again in Cps2E (97). Wzx mutant proteins are expected to accumulate single undecaprenyl-P-linked repeat units on the cytoplasmic face of the membrane and thus to be lethal for the reasons discussed above. The lethality of the Wzy mutations suggests that either single lipid-linked repeat units are not transferred from undecaprenyl-P or the Wzy polymerase itself is important in the transfer. Parental *S. pneumoniae* strains contain high levels of membrane-bound polymers, but it is not known whether the polymer is linked to undecaprenyl-P or is transferred to another acceptor. In the UDP-Glc dehydrogenase mutants described above, only low levels of membrane-bound polymers are observed, despite the ability of the mutants to polymerize long chains. Thus, an alternate or intermediate acceptor (en route to the cell wall) may be involved, and the lethality of the *cps2K* and *wzy* mutations may result, in part, from failure to transfer repeat units from undecaprenyl-P to either the alternate acceptor or the cell wall.

The modulation of Wzy-dependent capsule synthesis occurs, at least in part, through the action of a tyrosine-phosphoregulatory system. In gram-positive bacteria, the components of this system are an autophosphorylating tyrosine kinase (CpsD and homologues), its cognate membrane component (CpsC and homologues), and a protein-tyrosine phosphatase that is also a kinase inhibitor (CpsB and homologues) (10, 11, 65, 70, 82). In gram-negative bacteria, the CpsC and CpsD components are contained in a single protein (Wzc), and the phosphatase component lacks homology to its gram-positive counterpart (95). The fourth common protein encoded at the upstream end of the *cps* locus, CpsA, may be involved in transcriptional regulation based on its homology to LytR, a transcriptional regulator in *Bacillus subtilis* (36). The deletion of *cps2A* in serotype

2 *S. pneumoniae*, or that of its homologous sequence in the *S. agalactiae* capsule locus, results in decreased transcription of the capsule locus (unpublished data and reference 24), but the mechanisms involved have not been defined. The precise mechanism by which the tyrosine-phosphoregulatory system is related to capsule synthesis remains under study and may vary in different systems. In *S. pneumoniae*, the level of CpsD phosphorylation has been reported to correlate both positively (10, 91) and negatively (70) with the level of capsule production. The differences observed may relate to the use of wild-type, clinical isolates in the former studies and, in the latter, a laboratory-passaged strain that has lost components of the system (10). In studies with clinical isolates, increased levels of capsular polysaccharide and tyrosine-phosphorylated CpsD were observed during growth in reduced oxygen environments (91). In studies using the recombinant proteins, the phosphorylation of CpsD required CpsC but, once phosphorylated, CpsD could transphosphorylate CpsD and other proteins in vitro (11). Thus far, however, no other tyrosine-phosphorylated proteins that may be targets of this transphosphorylation have been identified. CpsC has been reported to be important in the transfer of polymers to the cell wall (66); however, it is not required for this function, as both *cpsC* and *cpsD* deletion mutants exhibit parental ratios of membrane- and cell wall-associated polymers (10). These mutants produce only short polymers, consistent with observations of the *Sinorhizobium meliloti* succinoglycan system, demonstrating a role for the homologous phosphoregulatory system in chain length determination (74).

### Synthase-Dependent Synthesis

The synthase-dependent mechanism is utilized in the production of only the type 37 and type 3 capsules, and the details have been explored for the latter. Because the type 37 capsule is a Glc homopolymer, only the synthase is required for its assembly, as the precursor sugar is derived from cellular pools. The synthesis of the Glc-GlcUA type 3 polysaccharide requires two enzymes encoded within the capsule locus, the UDP-Glc dehydrogenase that catalyzes the synthesis of UDP-GlcUA from UDP-Glc (which is derived from cellular pools) and the membrane-bound synthase (2, 4, 5, 28, 29). The synthase is a processive  $\beta$ -glycosyltransferase that is a member of glycosyltransferase family 2, which also includes synthases for cellulose, chitin, the Nod factor from *Sinorhizobium*, and hyaluronan in both eukaryotes and prokaryotes (including *S. pyogenes*) (17, 28, 48). These enzymes are integral membrane proteins that exhibit common topological characteristics, generally including

four transmembrane domains and a large cytoplasmic loop that contains the glycosyltransferase activity. The type 3 synthase is responsible for the initiation, elongation, and export of the type 3 polysaccharide and must therefore contain functional domains involved in multiple stages of synthesis. Based on hydrophobic cluster analysis, sequence homology, and X-ray crystallographic data, a model for the action of polymerases that utilize two distinct nucleotide sugars has emerged (22, 26, 86). It is proposed that a single nucleotide sugar-binding site exists and that the binding of one nucleotide sugar fine-tunes the affinity of the synthase for the next nucleotide sugar. Consistent with this model, during *in vitro* reactions the affinities of the type 3 synthase for the nucleotide sugars differ when both nucleotide sugars are present and when the nucleotide sugars are present individually (33).

The analysis of type 3 synthesis during *in vitro* reactions has shed much light on the fundamentals of this process (Fig. 3B). The synthesis of the polymer initiates by the transfer of Glc from UDP-Glc to phosphatidylglycerol and proceeds by the alternate addition of GlcUA and Glc to the nonreducing end of the growing polymer (19–21). During synthesis, the polymer remains linked to phosphatidylglycerol at its reducing end (21). The generation of an oligosaccharide-lipid that contains eight sugar residues appears to be a critical point in synthesis, as it is at this time that the growing chain is postulated to reorient from the inner to the outer face of the cytoplasmic membrane, leading to a highly processive mode of synthesis and the transport of the polymer across the membrane (34). The octasaccharide allows for tight binding between the growing polymer and the carbohydrate-binding site of the synthase. The ability to attain an octasaccharide length is dependent on the substrate concentrations, in particular that of UDP-GlcUA. This capsule-specific precursor is present at a low concentration (low micromolar range) in the cell compared to UDP-Glc (present at a concentration in the millimolar range), which is used in many other pathways. If UDP-GlcUA is lacking or is present at a reduced concentration, the high concentration of UDP-Glc causes an abortive translocation of the growing polymer, resulting in the ejection of the chain from the synthase and the termination of synthesis for that chain (33, 34). As the concentration of UDP-GlcUA increases, the frequency of chain termination decreases, allowing for the synthesis of the octasaccharide-lipid and the transition to the highly processive mode with the rapid synthesis of high-molecular-weight polymers (20, 33, 34). Both *in vitro* and *in vivo*, the concentration of UDP-GlcUA determines the frequency of chain termination

and the lengths of the polymers. Mutants that produce defective UDP-Glc dehydrogenase enzymes, and hence reduced levels of UDP-GlcUA, exhibit reduced amounts of total capsular polysaccharides, with corresponding reductions in the lengths of the chains (89). The synthase appears to be preset to make high-molecular-weight polymers, with the main controlling factor being the substrate concentrations.

Unlike Wzy-dependent capsules, the type 3 polymer is not covalently linked to the cell wall (81), and commercial preparations are therefore generally free of teichoic acid (C. Abeygunawardana, Merck, personal communication). Cell association of the type 3 polymer occurs via linkage to the phosphatidylglycerol primer or interactions with the synthase (21). However, approximately half of the total polymer synthesized during laboratory culture can be detected in the culture medium (37, 89). This released polymer appears to result from either multiple breaks in the polysaccharide chain or both a break in the chain and the ejection of the polymer from the synthase. Mutants that produce only short-chain polymers do not release capsular polysaccharide into the culture medium, and the polymer remains linked to phosphatidylglycerol (89). In contrast, about half the polymer in the parent type 3 strain is not linked to the lipid but can be ejected from the synthase *in vitro*, as likely occurs at some frequency *in vivo* to yield released polymers. Breaks in the polymer are postulated to be due to an undefined enzymatic activity, as mechanical forces other than sonication have not been found to disrupt the polymer (89). Released polymers may have biological significance, as they are frequently detected in the blood and urine of patients with pneumococcal pneumonia (15).

## CONCLUSIONS AND FUTURE DIRECTIONS

The *S. pneumoniae* capsular polysaccharides have long been the object of intense study. Studies over the past decade have brought a unity to descriptions of the chemistry, genetics, and biosynthetic mechanisms that had been lacking in the past. It is now difficult to discuss one of these aspects without discussing the others, as our understanding of them has become more detailed and intertwined. The ability to distinguish capsular serotypes with monoclonal antibodies and to obtain the complete sequences of capsule loci may bring to light previously unrecognized serotypes and may provide information concerning the genetic mechanisms for generating new serotypes. A better understanding of the regulation of capsule expression and chain length is critical not only in the isolation of polymers for vaccine prepa-

rations but in the development of a full picture of pneumococcal virulence, as reduced capsule levels occur during the colonization of the nasopharynx while elevated levels are essential for systemic disease (10, 38, 59, 73, 90, 92). Finally, the ability to block capsule synthesis by targeting functionally equivalent enzymes that occur in many bacteria, and simultaneously crippling the cell due to effects on essential pathways, may provide a novel therapeutic approach to the treatment of pneumococcal infections.

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David E. Briles  
Susan K. Hollingshead  
Ingileif Jonsdottir

# 4

## Animal Models of Invasive Pneumococcal Disease

Researchers use animal models of invasive pneumococcal disease to attempt to provide surrogates for invasive pneumococcal disease in humans (see chapters 8 and 9). Although this chapter focuses on the application of animal infection models to evaluate the potential efficacy of candidate vaccine antigens, the models are equally applicable to studies of (i) alternative vaccination strategies (mucosal, DNA, and live vaccines, etc.), (ii) new adjuvants, (iii) the efficacy of antibiotics, (iv) mechanisms of innate and adaptive immunity, (v) screens for pneumococcal virulence factors, and (vi) the *in vivo* relevance of suspected pneumococcal virulence factors.

From the very beginning, the development of capsule-based vaccines utilized animal models extensively as surrogates for protection in humans (1, 29). Even though the opsonophagocytosis and killing assay has been found to be a very useful *in vitro* surrogate to study the protective effects of antibody to capsular polysaccharide (50), animal models continue to be useful in the development of polysaccharide-protein conjugates to demonstrate that new polysaccharides, protein car-

riers, or formulations will elicit protective immunity *in vivo* (33, 34, 53, 68).

Animal models are especially critical in studies both to identify and to compare the protective efficacies of candidate noncapsular vaccine antigens, most of which are proteins (see chapter 28). Since the functions of many of these antigens and the protective mechanisms of immunity to them are largely undefined, validated *in vitro* surrogates for them do not yet exist.

Pneumococcal disease results when colonizing pneumococci in the nasopharynx (see chapters 5 and 19) successfully invade sterile sites. The first half of this chapter focuses on individual disease models and provides general descriptions of the commonly used models. It also discusses considerations important to the application of these models to studies of vaccine antigens and some of the pros and cons of each model. The latter half of the chapter presents experimental considerations that relate to animal models in general: the need for multiple models, inclusion criteria for experiments, and statistics, etc.

## MODELS OF INVASIVE DISEASE

### Bacteremia

Bacteremia is a potential outcome in virtually all models of pneumococcal infection and is easily evaluated. When virulent pneumococci are administered intraperitoneally (i.p.) or into the lungs of mice or other animals, they can appear in the blood within a few hours. The effects of active or passive immunization on the numbers of CFU of pneumococci in the blood are measured by quantifying the CFU in sequential blood samples postinfection. By monitoring the numbers of CFU in the blood, it is possible to monitor the disease state without having to sacrifice mice at each time point (8, 26, 53). Although the study of bacteremia would appear to have application for measuring transit from nasal surfaces into the blood, pneumococci are normally cleared efficiently from the blood and the clearance is accelerated by immunity.

When pneumococci are inoculated into the lung or peritoneal cavity, they replicate in these sites and continually enter the blood. As a result, measuring the density of CFU in the blood does not distinguish among immune effects that may be operative in the infected cavities, effects that may control transit into the blood, and immune effects on survival in the blood. The changes in the numbers of viable bacteria in the peritoneal cavity or in the lungs are best studied quantitatively by sacrificing groups of mice at different times postinfection and plating the contents of these tissues (10, 30). The infections at these sites can also be monitored semi-quantitatively without killing the mice if the infections are with luminescent pneumococci (23, 47, 53) (see below).

### Blood Clearance

To study the effects of active or passive immunity on the clearance of pneumococci from the blood (reduction in bacteremia), a dose of  $10^5$  to  $10^6$  CFU is injected intravenously (i.v.) and the number of CFU in the blood over time is monitored by plating sequential blood samples onto blood agar (3, 39). No more than  $10^6$  CFU/mouse should be injected since doses of more than  $10^6$  CFU/ml of some strains of pneumococci elicit significant host immune responses that accelerate clearance from the blood (3, 4), which could complicate the interpretation of the results.

After i.v. injection, the mice are bled at 2 min and at 4, 8, and 24 h and the blood is plated onto blood agar to determine the number of CFU per milliliter. The bleed at 2 min postinjection provides confirmation that the inoculation was in fact i.v. The number of CFU per milliliter

at the 2-min time point is more variable than those at later points, probably due to incomplete mixing of the bacteria in each mouse. By 5 to 10 min postinoculation, significant clearance may already have occurred. Thus, it is best to use the number of CFU injected rather than the number of CFU per milliliter at 2 min as the baseline for calculations of percent clearance. Depending on the state of the pneumococci injected, the numbers of virulent pneumococci can decrease for the first 4 h even in nonimmune mice (39). Whether this decrease in CFU occurs may depend on the percentage of the inoculated pneumococci in the opaque phase. From 4 h on, the number of CFU per milliliter in a nonimmune mouse generally increases until the death of the mouse, at which time the number of CFU per milliliter can be  $\geq 10^9$ .

To be used in a blood clearance assay, the challenge strain must be relatively resistant to clearance in the absence of immunity. Strains of capsular types 2, 3, 4, and 5 usually resist clearance in the absence of immunity. Capsular type 2, 3, and 5 strains show relatively unhampered growth from 4 h postinjection until the death of the mice, whereas the growth of capsular type 4 strains can level off at about  $10^6$  CFU from 24 to 72 h, with most mice dying by 96 h postinoculation (3). In contrast, strains of types 14, 19, and 23 are generally cleared almost immediately after inoculation. Strains of capsular types 6A and 6B are frequently cleared to a level of around  $10^4$  to  $10^5$  per ml and then remain at these levels for as long as a week (3). In this model, it is now known that the 6A and 6B bacteria can create a focal infection in the kidney, from which location they maintain their low numbers in the blood (D. E. Briles, unpublished data). Thus, while bacteremia can be used to monitor the course of infection following i.v. inoculation of capsular type 6A or 6B pneumococci, these strains are not useful for blood clearance studies.

The number of times a mouse can be bled within 24 h is determined by the local animal welfare guidelines. It is recommended that no more than 100  $\mu$ l of blood be removed from a mouse per week (21). Thus, tail bleeds of very small volumes, rather than eye bleeds, would be the best way to monitor clearance from the blood over time. A second advantage of tail bleeds is that anesthesia is not required.

For blood clearance studies the pneumococci are injected i.v. into the tail. In principle, any vein can receive the injection, but the lateral vein near the tip of the mouse's tail is preferred. The distal end of the mouse tail has one dorsal and two lateral veins. A few centimeters from the tip of the tail, the dorsal vein splits and joins the two lateral tail veins. At this bifurcation, one branch

of the dorsal vein is larger than the other, resulting in a larger lateral tail vein on the corresponding side. The i.v. injection should be in the largest of the lateral veins right after it is joined by the branch of the dorsal vein. To administer the injection, restrain the mouse's body using a 50-ml plastic conical tube with a slit cut down one side to allow the tail to exit the point in the tube and hold the tip of the tail to keep it taut. With good direct illumination, insert the needle into the tail toward the mouse's body. Injections closer to the mouse's body must go through thicker tissue to reach the vein and are more difficult to carry out even though the vein is larger. If the first injection is not successful, make the next attempt a little further up the vein toward the mouse's body. In this way, the injected material will not run out of the hole left by the first attempt. Prior to an i.v. injection, squirt 70% ethanol onto the tail. This both sterilizes the tail surface and makes it optically smooth, which greatly facilitates visualization of the vein through the skin. Do not use 100% ethanol as it removes protective oils from the mouse's skin.

Pneumococci should be injected i.v. in 0.2 ml of sterile Ringer's injection solution or saline through a sharp 27-gauge, 1/2-in. BD needle. If the 0.2-ml volume is injected without significant back pressure, swelling of the tail, or an increase in the opacity of the skin, the i.v. injection is successful; these signs are more difficult to judge if a smaller volume is used. Another mark of a good injection is that the injected fluid will rapidly replace the blood for the entire length of the vein as soon as the injection begins. A final mark of a successful i.v. injection is that the tail will always bleed after the inoculum is delivered.

It is common to put the cage of mice under a heat lamp for a few minutes to warm up the animals prior to injection. The heat causes the dilation of the tail veins, making injections easier. It is important, however, not to leave the mice under the lamp for too long and to monitor them carefully for overheating. One sign that a mouse is under heat stress is that it spreads saliva over its face to reduce its body temperature.

Blood clearance studies can be conducted with almost any strain of mice (or other animals) in which the challenge strain of pneumococcus resists clearance from the blood in the absence of immunity. CBA mice that have the *Btk(Xid)* phenotype are particularly well suited for blood clearance studies. Mice homozygous for the *btk(xid)* allele fail to make normal antibody responses to polysaccharides (11, 36, 67). The *Btk(Xid)* strains used most commonly for such studies are CBA/N and CBA/CAHN-XID/J. Other CBA strains lack the *btk* (*xid*) allele and are not as susceptible to

pneumococcal infection. *Btk(Xid)* mice lack natural antibodies in their sera that react with the phosphocholine epitopes of pneumococcal lipoteichoic and teichoic acids. These antibodies to phosphocholine accelerate the clearance of pneumococci from the blood and can protect mice against pneumococcal infection (7, 8, 12, 39, 41). Thus, mice with the *Btk(Xid)* phenotype provide an especially sensitive background on which to measure the effects on blood clearance induced by active immunity or passive antibodies to pneumococcal antigens.

Although blood clearance studies provide a means of studying an important protective mechanism, we have observed that they are a less sensitive means of detecting protection against i.v. challenge than is the model of protection against the moribund state (61; Briles, unpublished).

To quantify pneumococci in studies of bacteremia, blood from each draw is serially diluted (1/3) in Ringer's injection solution and plated onto blood agar plates in spots containing 50 µl of diluted blood. The use of blood agar is recommended because contaminating bacteria can be easily distinguished by their lack of alpha-hemolysis. After overnight incubation, colonies are counted. Each colony is assumed to be the product of a short chain of pneumococci. Commonly, CFU are counted in the most concentrated countable spot and the number of CFU per milliliter of blood is calculated by multiplying by the dilution factor.

### Sepsis and Moribund Sepsis

The classic mouse model for assessing protective immunity has been the use of otherwise fatal infections following i.p. challenge to detect the effects of active immunization or passive antibodies. Similar models have also been developed using i.v. or lung challenge. With the changes in animal welfare policies over the last 20 years, the use of fatal end points is no longer acceptable in most jurisdictions, and the moribund state is now used as an end point. Prior to death, mice undergo a loss of the righting response, a loss of response to stimuli such as a tail pinch, and a significant reduction in body temperature (2). Once mice reach this state, they are considered to be moribund since they will invariably die within a few hours. To use this end point, mice are checked every 6 h postinfection until the termination of the experiment, generally between 10 and 21 days postinfection. Mice observed to be moribund are scored as such, and the time of observation is recorded. Moribund mice are then euthanized, and their carcasses are disposed of as biohazard material.

In studies of invasive disease, monitoring body weight, surface temperature, and numbers of CFU per milliliter of blood can provide additional information about the protective effects of active immunity or passive antibodies (31, 35, 52). Changes in body weight can be an especially sensitive measure of disease because when mice feel bad they stop eating and drinking. Surface temperature can also be monitored; a fall in temperature indicates the moribund state (2), and elevation in temperature can provide evidence of inflammation.

**Protection against Sepsis following i.p. Challenge**  
 The model of protection against sepsis following i.p. challenge has been used in the 60-plus years of development of the capsular vaccines and is considered to predict protection by antipolysaccharide antibodies against agents of human disease (29). The model is also frequently used in studies of protection-eliciting proteins (15, 38, 43–46). The high level of virulence of pneumococci in this model means that a broad range of challenge strains can be used. The very low challenge doses required minimize the chance of having the inoculum absorb a significant proportion of the circulating antibody. Since pneumococci can grow in the peritoneal cavity without being subjected to filtration in the spleen and liver, this route may tend to work better for antibodies to toxins (such as pneumolysin) than for antibodies that facilitate clearance by phagocytes in the reticuloendothelial system. However, the model mimics neither a natural route of infection nor an important aspect of the natural history of human pneumococcal disease.

In this model, it is important that the inoculum be delivered i.p. directly through both the skin and the peritoneal wall. It is also important that care be taken not to deliver the inoculum into the pleural cavity or too low within the peritoneal cavity, where unintended injection of the bladder may occur. To reduce the chance of injecting or nicking the intestines, liver, and spleen, it is best to use a 3/8-in. tuberculin needle. This needle is sharp enough and long enough to enter the peritoneal cavity, but it is too blunt and too short to easily penetrate the internal organs.

**Protection against Sepsis following i.v. Challenge**  
 Pneumococcal pneumonia is lobar and generally does not cause respiratory failure. Instead, human fatalities result primarily from bacteremia and sepsis, which occur when the invasion of pneumococci into the blood is no longer controlled by complement, antibodies, and phagocytes. i.v. challenge has been used for over 25

years and allows a very reproducible evaluation of the ability of elicited immunity to noncapsular antigens to protect against bacteremia and sepsis (7, 9, 12, 14, 30, 40). i.p. and intranasal (i.n.) challenges with pneumococci also lead to bacteremia and sepsis, but the kinetics of the transit of bacteria from these cavity infections into the blood in individual mice cannot be controlled experimentally. Moreover, in sepsis following i.p. or i.n. challenge, pneumococci continually enter the blood in significant numbers from a depot of pneumococci growing in the peritoneal cavity or lungs, thus complicating studies of protection of the blood from infection. The protection seen in this model was positively correlated with the level of antibody to PspA (pneumococcal surface protein A) elicited in the sera of immunized volunteers (9).

The inoculation protocol for the i.v. challenge sepsis model is the same as that for blood clearance studies (see above). Many different mouse strains can be used in this model (8, 39). However, CBA mice with the *btk(xid)* defect have proved particularly useful. The high susceptibility of these mice to pneumococcal infection means that with most strains of capsular types 2, 3, 4, 5, and 6, a moribund state can be achieved with 300 or fewer bacteria (7). This in turn means that the infection can be initiated with a small inoculum, making it possible to study protection against a developing disease rather than protection against the almost instant sepsis that results when doses of  $\geq 10^6$  CFU must be given to other strains of mice. Because of the low doses generally used for inoculation, one also avoids protective effects of host inflammation, which occurs when high challenge doses are used.

**Protection against Sepsis following i.n. Challenge**  
 Challenge i.n. while mice are lightly anesthetized allows much of the inoculum to be aspirated into the lungs, from which the pneumococci invade the blood, causing bacteremia and sepsis (10, 28, 53). Lung infection with sepsis-causing pneumococci can also be accomplished by intubation (68), but this is a more time-consuming procedure and runs the risk of unintended trauma or delivery into the stomach. The model of infection through i.n. aspiration allows both the protection that occurs in the lungs and the protection against bacteremia and sepsis to have an impact on the final outcome, which is usually protection against a moribund condition or the numbers of CFU per lung or per milliliter of blood at specific time points.

To induce aspiration, mice are anesthetized immediately prior to i.n. inoculation with pneumococci in 50  $\mu$ l of saline diluent (53). The mice are anesthetized with sodium phenobarbital (50 mg/kg of body weight i.p.) or

fentanyl-fluanisone and midazolam (Hypnorm and Dormicum; 5 mg/ml each, diluted with equal volumes of sterile water to produce 0.05 to 0.075 ml of working solution per 10 g body weight, for subcutaneous inoculation), hung on a wire by their upper incisors so that their noses are uppermost, and then inoculated slowly with bacteria in 50  $\mu$ l of diluent. It should be noted that the anesthesia used induces sleep just deep enough to cause aspiration of the fluid delivered into the nose. These mice are unconscious for only a few minutes. The induced aspiration is probably more like the aspiration of upper airway fluids that occurs every night when humans sleep, rather than the aspiration of stomach contents that can occur when humans are severely intoxicated.

This model has been used extensively to measure protection elicited by active immunization with polysaccharide-protein conjugate vaccines. In this model, protection is detected by determining the numbers of CFU in the lungs and blood at 24 h postinoculation. The numbers of CFU at these sites is inversely correlated with the levels of polysaccharide-specific antibodies in serum (33, 34). Protection elicited by passive i.p. immunization with sera from infants vaccinated with conjugate vaccines was also shown to be proportional to polysaccharide-specific antibody levels and to the opsonic activities of the infant sera (35, 52). The level of polysaccharide-specific antibodies needed to prevent bacteremia is about 1/10 of that needed to clear the lungs (33).

The aspiration and lung inoculation models have also been used successfully to study the immunogenicity and protective efficacy of several pneumococcal protein vaccine candidates (68; I. Jonsdottir et al., unpublished results). This i.n. challenge sepsis model has several advantages: (i) it mimics fatal pneumonia in humans in which a lung infection leads to fatal sepsis; (ii) up to 80 mice can be reproducibly infected at one time; (iii) the inoculum is a little less virulent when given by aspiration into the lungs than when given i.p., thus making it easier to detect the protective effects of immunity; (iv) it is possible to study infection and inflammation of the lungs, invasion into the blood, and clearance from the blood (33); and (v) for highly invasive strains, a moribund state can be used as an end point (68).

This model also has limitations: (i) the ability to induce the moribund state (if that is the end point) at a high enough frequency for successful statistical analysis requires the use of especially virulent strains of pneumococci; (ii) for a moribund-state end point, high inoculation doses ( $1 \times 10^6$  to  $5 \times 10^6$  CFU) are needed for conventional mice, and only highly invasive strains cause bacteremia and sepsis; and (iii) most mice have concur-

rent pneumonia and sepsis, thus precluding unambiguous evaluation of protection against pneumonia per se.

A variant of this model uses nasal inoculation of high doses ( $\geq 10^7$  CFU) of highly virulent pneumococci without anesthesia. This approach avoids variability induced by variations in anesthesia delivery, but it results in potentially increased variability because only a small and variable fraction of the inoculated CFU ever reach the lungs, with a net result that the aspiration model can reduce the overall variability in the development of pneumonia among the mice (10).

#### Neonatal and Infant Mouse Model of Lung Infection with or without Bacteremia

As neonates and infants are a major target for pneumococcal vaccination, the murine model of i.n. challenge with aspiration has been adapted to 1-week-old mice (31), whose immune system corresponds to that of the human neonate, and 3-week-old mice, whose immune system corresponds to that of the human infant (58). This early-life murine infection model has been used with pneumococcal vaccines to assess protective efficacy in relation to antibody levels, isotypes, and avidity (31); the generation and persistence of B memory cells (5); and T-cell responses (32), all of which are highly age dependent. Maternal immunization protects the young offspring, and the protection is dependent on the level of maternal antibodies (48). Protective levels of maternal polysaccharide-specific antibodies appear to enhance the immune responses of neonatal and infant mice to conjugate vaccines, while only the highest levels of maternal antibodies interfere with immune responses to conjugate vaccines (49).

#### Infant Rat Lung Model with Injection of Pneumococci in Agar Particles through the Chest Wall

In an infant rat lung model, pneumococci in agar particles are injected through the chest wall (54). After liquid agar is cooled, bacteria are added just before the agar sets. The agar with the entrapped live pneumococci is then passed through progressively smaller needles until it will pass through a 27-gauge needle. This procedure allows the use of a low inoculum that can result in pneumonia, bacteremia, meningitis, and death in infant rats. The inclusion of the pneumococci in agar particles protects them from rapid clearance from the lungs and allows even low-virulence strains of diverse capsular types to be used. This model provides a highly sensitive method of evaluating passive protection studies using immune serum (60).

A histologic end point is precluded since the injection of agar alone without pneumococci results in inflammation, which has the histological characteristics of pneumonia. Another complication of the model is that for any particular infant rat, it is difficult to know exactly where the agar is deposited.

### Focal Pneumonia (In the Absence of Sepsis)

In the pneumococcal models described above, there are generally concomitant sepsis and pneumonia, which makes it difficult to know how much of the protection against the moribund state or even against the numbers of CFU in the lungs is the result of protection against sepsis and how much is due to protection against lung infection per se. Perfusion of the lungs prior to homogenization can prevent the need to count the CFU in the blood in the vessels of the lungs but cannot compensate for the fact that the lungs are in a septic mouse in which polymorphonuclear leukocytes, complement, and antibody have been consumed. Moreover, in humans most cases of pneumococcal pneumonia do not lead to sepsis. To study the protection of the lungs against pneumonia, i.n. inoculation and aspiration are carried out with strains of pneumococci that are not able to survive in the blood and thus cause focal pneumonia without causing bacteremia or sepsis (10, 62).

The bacterial strains useful for this approach may include most of the 91 different capsular types, since most are poorly virulent in mice (6, 42). To date, however, this model has been used only with pneumococci of capsular types 14, 19, and 23, all of which are important in otitis media and pneumonia in children and adults. The ability of pneumococci of these capsular types to survive in the blood of mice mixed with cobra venom factor as an anticomplement (George Carbone [CDC], personal communication) may indicate that interactions between the capsular polysaccharides of these strains and mouse complement may explain the avirulence of these strains in mice.

In this model, lightly anesthetized mice are inoculated i.n. with about  $5 \times 10^6$  CFU in 40 to 50  $\mu\text{l}$  of diluent. Agents used for anesthesia have included methoxyfurane (Metofane; Schering-Plough) (10) and isoflurane (Attane; Minrad Inc.). With the use of capsular type 19F strain EF3030, this inoculation results in progressive focal pneumonia in one or two of the five lung lobes and the infection peaks (as determined by numbers of CFU) around 5 to 7 days after inoculation and is largely resolved in the mice by 3 to 4 weeks. The readout for these studies is most commonly a determination of the total number of CFU in the pooled lung lobes at 5 to 7 days postinoculation, but it can also be the results from

histological examination of the lungs. This model has been used to demonstrate synergy between the antigens PspA and pneumolysin in eliciting protection against pneumococcal pneumonia in mice (10).

### Otitis Media

Otitis media models in several animal species have been developed to evaluate vaccine effects (19, 27, 51, 66). The classic model of otitis media was developed using the chinchilla and has been well reviewed by Giebink (27). This model allows bacteria to be deposited in the middle ear or administered i.n. In this model, otitis media can be monitored histologically, by examining the tympanic membrane and determining numbers of bacteria in the middle ear exudates, or radiologically (24, 27). This model provides a means of evaluating the ability of vaccines, passive antibodies, or antimicrobials to protect against otitis media infection. The inoculum usually contains a few hundred to a few thousand viable bacteria. A higher dose of bacteria is likely to cause fatal disease. A similar model was developed using the rat, in which otitis is also monitored by examination of the tympanic membrane, histological analysis, and enumeration of viable bacteria in the middle ear (66). Because adult rats are smaller than chinchillas, the manipulations are more difficult to carry out, but problems of animal availability and costs are reduced. An additional advantage is that the level of susceptibility of the rat to pneumococcal sepsis is much lower than that of the chinchilla and is thus more similar to the susceptibility of humans to sepsis, where otitis media is normally not likely to lead to death.

### Meningitis

A classic rabbit meningitis model in which meningitis is induced by intracisternal inoculation of  $10^4$  to  $10^5$  pneumococci was developed decades ago (20). Male New Zealand White rabbits are anesthetized intramuscularly with ketamine (50 mg/kg) and acepromazine (4 mg/kg) before every procedure. Flunixin meglumine (1.1 mg/kg) is administered intramuscularly every 12 h for analgesia. Animals are immobilized in stereotactic frames, and a spinal needle is introduced into the cisterna magna to withdraw 250  $\mu\text{l}$  of cerebrospinal fluid and inject an equal volume of inoculum.

Murine meningitis models have also been developed, where meningitis is induced by the transcutaneous injection of  $10^4$  to  $10^6$  CFU of pneumococci into the right forebrain or cisterna magna under short-term anesthesia with halothane. The clinical score is monitored from 6 to 120 h postinfection, and the numbers of CFU per milliliter in the cerebrospinal fluid and cerebellum ho-

mogenates are determined (25). A simple method to induce pneumococcal meningitis in mice is by the intracranial subarachnoidal route of infection. This model is easy to carry out, the results are reproducible irrespective of the serotype of pneumococci used, and the infection histologically closely resembles the disease in humans (18).

## TECHNICAL CONSIDERATIONS FOR ALL MODELS

### Importance of Diverse Invasive Models and Multiple Challenge Strains

The original mouse model of pneumococcal infection was septic death following i.p. infection (29, 42, 44). Human pneumococcal disease is both diverse and complex and cannot be adequately modeled with any one animal model. Invasive models that are highly relevant to specific aspects of pneumococcal disease are those of sepsis following i.v. challenge, sepsis following i.n. challenge, bacteremia, focal pneumonia, otitis media, and meningitis (8–10, 27, 53, 57, 63).

Pneumococci are extremely diverse; identical strains are rare even when they are members of the same clonally related group (22). There are several reasons to test each noncapsular vaccine antigen against several challenge strains to obtain a good estimate of its potential efficacy: (i) different strains can cause very different courses of disease even in the same model and thus may be more or less affected by immunity to a particular antigen; (ii) strains will differ in their levels of virulence in the model used and thus may differ in their sensitivities to immunity; (iii) antigens are not expressed equivalently by all strains; and (iv) the sequences of alleles of the target antigen in different strains may differ, and thus the cross-reactivities of the antigen will differ. Based on all of the above-listed considerations, it is likely that (i) no single animal model is appropriate for all test antigens, (ii) tests of the efficacy of immunity to any particular antigen should be conducted with more than one challenge strain, and (iii) since it is not possible to predict what the best models are, results from more than one model are likely to provide the best indication of an antigen's potential protective efficacy in humans.

### Choice and Handling of Pneumococcal Challenge Strains

Careful attention should be given to the choice of pneumococcal strains for assessing the protection elicited by test antigens. The challenge strains selected should be

virulent in the model used and representative of strains in the geographic region where the vaccine is intended for use. Pneumococci for use in a particular series of experiments should be frozen in aliquots in 8 to 10% glycerol so that multiple experiments can each be conducted with separate vials of exactly the same stock. Ideally, the challenge strains would be epidemiologically significant strains recently isolated from humans. To minimize genetic changes, isolates should be exposed to minimal laboratory and animal passage between isolation from humans and the generation of infection stocks.

It is helpful to use well-characterized strains known to cause reproducible infection in the animal model. Such strains make it easy to do well-controlled experiments and to adapt the model to different laboratories. The importance of using recent isolates, however, is in conflict with the use of well-characterized strains because most commonly used challenge strains were isolated from humans between 10 and 80 years ago. New challenge strains should be derived from freshly collected isolates from known anatomical sites of patients with a known diagnosis. Strains should come from those parts of the world for which the vaccine is intended.

The general consensus is that strains chosen for use in animal models should, in most situations, be those originally isolated from humans with invasive disease, even though there is conflicting evidence as to whether strains recovered from colonized humans are any less able to cause serious disease than strains of the same capsular types isolated from humans with invasive disease (17, 56). Recent data indicate that, although some serotypes are more invasive than others, the differences in invasiveness in humans can also be related to clonal type (16, 59).

Most cultures of pneumococci are mixtures of transparent-phase and opaque-phase pneumococci (13, 64). Careful examination of these cultures may also reveal a series of less well defined intermediate phases of pneumococci between the most transparent and most opaque forms (64, 65). The transition between phases can be spontaneous but may also be induced. Pneumococci with a transparent phenotype are best suited for colonization, and those of an opaque phenotype are best suited for invasive disease (37, 64). It is possible that some of the intermediate forms are appropriate for different niches in the host and are important for specific aspects of disease. Since the first half of the 20th century, it has been known that a single mouse passage of a low-virulence strain can sometimes cause a large increase in virulence (12, 29). It is likely that most of this increase in virulence results from the enrichment during the passage of opaque-phase pneumococci in infectious

stocks that had come to comprise largely transparent-phase pneumococci during multiple laboratory passages.

Almost all challenge strains used in animal models are undoubtedly mixtures of the transparent and opaque phenotypes, and differences in the virulence of different stocks of the same strain are related to differences in their relative opacities. Rather than being purified for particular phase variants, stocks can be examined for opacity, and if pneumococci of each phase constitute between 30 and 70% of the stock, necessary adjustments in the challenge dose can easily be made. Freezing down infectious stocks directly from murine passage or from growth in media containing human serum has also been used to reduce variability in phase variation and virulence.

#### Bioluminescent Pneumococci for Studies of Tropism, Virulence, Invasiveness, and Disease Potential

The quantitation of bioluminescent pneumococci can be used as an alternative readout in infection models. Stable bioluminescent derivatives of pneumococcal isolates have been created using chromosomal DNA from the highly bioluminescent *Streptococcus pneumoniae* strain D39 Xen 7, which carries the Tn4001 luxABCDE Km<sup>r</sup> cassette (23). The injection of such derivatives results in the production of luciferase and its substrate and in the emission of light, some of which passes through the skin, bones, and hair of the inoculated animals. The light emitted from the infected mice can be monitored with a charge-coupled device camera (Xenogen). Although the recovery of bacteria from the mice has shown that the bioluminescent signal is roughly proportional to the number of CFU (23), the studies are semi-quantitative and most studies rely on CFU enumeration in the final data (47). By using luminescent pneumococci for challenge by the i.n. route, it is possible, without killing the mice, to monitor the time course of pneumococci infection and colonization and to document the spread of pneumococci to different organs in otitis media, lung infection, bacteremia and sepsis, or meningitis (47, 55). Bioluminescence readout in mouse challenge experiments should be useful to study the roles and protective capacities of novel pneumococcal proteins.

#### Control Groups and Inclusion Criteria for Successful Experiments

Some individual experiments cannot be used for decision making because of experimental error, unforeseen differences in animal husbandry, and differences in the conditions of the infectious stocks. To avoid any bias in decisions about which experiments should be excluded,

it is necessary to have preestablished inclusion criteria for the use of experimental data in decision making. These criteria should be based on the results obtained with the positive and negative control groups.

In studies measuring the protective efficacy of vaccines, animals that receive adjuvant but no antigen constitute the negative control groups. In studies of passive protection by human sera in mice, the control mice should be given preimmune sera from the immunized individual as a negative control. In studies of passive protection by mouse immune sera, the control mice should receive sera from mice immunized with adjuvant and diluent but no antigen. Animals receiving antigen known to elicit strong protection in the model are used as the positive control group. It is also critical that antibody levels be monitored in each study so that a failure to protect will not be confused with a failure to immunize. In addition to the positive and negative control groups, it is also important to have a group of naïve animals. This control group allows a test of whether the infection stock is working as expected and controls for the possibility that the adjuvant or diluent may elicit protection if it causes enough inflammation or contains an antigen that cross-reacts with an antigen of the challenge bacteria.

If the experimental end point is protection from becoming moribund, then the inclusion criteria may require, for example, that  $\geq 90\%$  of the mice in the negative control group and  $\leq 10\%$  of the mice in the positive control group become moribund. If these criteria are satisfied, there is a good chance that the results with a test antigen will be informative.

#### Statistical Comparisons

In studies to determine whether a particular immunization leads to immunity, the results from the experimental group should be compared with those from the appropriate negative control group in a two-tailed statistical test. The appropriate statistical test will depend in part on the end point used. In general, nonparametric statistics are appropriate. The Mann-Whitney two-sample rank test (nonparametric) is always appropriate for comparisons of the test group and the negative control using data on the time to a moribund state, the number of CFU in the blood or lungs, body weight, or body temperature. The advantage of the Mann-Whitney test in animal model studies is that the validity of the calculated *P* values is not affected by the lack of a Gaussian (normal) distribution in one or more of the data sets or significant differences in the standard deviations of the test and control data sets. Parametric statistical tests require Gaussian data distributions and similar standard

deviations in each group. However, when they can be used, parametric statistical tests such as Student's *t* test are preferable and can provide somewhat higher *P* values, especially if the group size is <20.

If pneumococcal density is used as an indicator of disease and most of the mice in each group have countable CFU, it is often possible to obtain Gaussian distributions for the data by log<sub>10</sub> transformation of the numbers of CFU in the blood. However, if the data set is bimodal, it cannot be transformed into a Gaussian distribution. Bimodal data sets are common in infection studies because some mice in a group may survive with little infection while others will die. Most computer programs for Student's *t* test will indicate when the *t* test is not valid. With some programs (for example, InStat by GraphPad Software Inc.), the Mann-Whitney test will not calculate a result if all values in one group are identical. This problem can be circumvented without adversely affecting the *P* value by making one of the values in the group 1% larger than the observed value and another 1% smaller.

If comparisons are intended to be made between several groups in the same experiment, as would be the case if several immunogens were being compared, then the Kruskal-Wallis test (nonparametric analysis of variance) and Dunn's multiple-comparison test (69) should be employed. To look for correlations between antibody levels and protection, the Spearman rank correlation is appropriate.

In some cases, a qualitative comparison is desired, as in comparisons of moribund versus nonmoribund or bacteremic versus nonbacteremic. For qualitative comparisons in which the data fall into two discrete groups, the Fisher exact test for data in a two-by-two contingency table is preferred. In general, however, comparisons will be statistically more informative if the time to a moribund state or actual CFU numbers are analyzed with a Mann-Whitney test or Student's *t* test.

### Biohazard Considerations

*S. pneumoniae* is a biosafety level 2 agent for work on the lab bench and for use in animal models. The normal host for pneumococci is humans, and to our knowledge, there have been no reports of the transmission of pneumococci from experimental animals to humans or even between adult mice in different cages. Even so, every precaution should be taken to protect animal care workers, technicians, and laboratory animals from the possibility of acquiring *S. pneumoniae* while working with infected animals. Extra care is warranted if an antibiotic-resistant strain is used. Infected animals must be kept in an appropriately labeled negative-pressure

module, separated from any uninfected animals. Mice infected with *S. pneumoniae* should be kept in microisolation cages with filter tops and should always be handled using disposable gloves. All euthanized mice, used cages, used gloves, and leftover food, water, and bedding must be autoclaved prior to disposal. Employees with sickle-cell disease and those with abnormally low levels of immunoglobulin, complement, or splenic filtration should not work with pneumococci.

### CONCLUDING REMARKS

Standardized and validated animal models of invasive pneumococcal diseases are useful to study the virulence, invasiveness, and disease potential of different pneumococcal clones, the pathogenic process, and the role of different pneumococcal components in various steps of the pathogenesis. Novel treatments, the protective capacities of pneumococcal vaccines and vaccine candidates, and the mechanisms of protection from disease can also be studied in such models. To obtain reliable, consistent, and meaningful results, the study design, the selection of mouse strains, the selection and handling of pneumococcal challenge strains, the experimental readout, and other technical aspects are critical. Advantages and limitations of the various animal models of invasive pneumococcal diseases discussed above emphasize the importance of a careful selection of animal models to address different scientific questions. Multiple models will be needed to increase our understanding of the complex interaction between the pneumococcus and the human host and how to intervene for the benefit of the host.

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Richard Malley  
Jeffrey N. Weiser

5

# Animal Models of Pneumococcal Colonization

With the exception of horses, which are hosts to a capsular type 3 variant, humans represent the main ecological niche for *Streptococcus pneumoniae* (55). The survival and transmission of pneumococci are thus highly dependent on the ability of these organisms to colonize the human nasopharynx, in the face of competition from other organisms and innate as well as acquired immune effectors. Much of what has been learned in this regard has been derived from various animal models of pneumococcal colonization. The purpose of this chapter is to describe existing animal models of colonization and review what these various models have taught us about pneumococcal carriage.

## EXISTING ANIMAL MODELS OF PNEUMOCOCCAL NASOPHARYNGEAL COLONIZATION

The most widely studied model of pneumococcal colonization is the mouse model. There are many reasons for this choice. First, it should be noted that mice are readily colonized by pneumococci of multiple types but

are highly susceptible to invasive infection with only certain pneumococcal types, those designated by lower numbers, which were the first to be isolated using mouse passage (57). The availability of reagents and targeted gene knockout strains of mice also make this animal model more attractive. Furthermore, the intranasal inoculation of anesthetized mice with selected strains can subsequently lead to invasive disease, in a pattern that may mimic natural infection following aspiration in humans (4, 46). Several variations of the model have been used, but in most cases, a bacterial inoculum is dropped atraumatically onto the nares of unanesthetized mice. The presence and density of pneumococcal colonization can then be assessed over the ensuing days, generally by obtaining retrograde tracheal wash fluids from recently euthanized animals (57). These models have been studied by using mice of various genetic backgrounds (such as CD-1, C57BL/6, BALB/c, and CBA/n). The persistence of colonization or carriage has also been studied; in one particular strain of mice, colonization was shown to last several weeks, which again may be viewed as representative of what is believed to occur in humans (10, 15, 34).

Infant rat models of pneumococcal colonization have also been described in several studies (29, 37, 52, 54). A direct inoculation model with infant rats was employed to demonstrate significant differences in the abilities of transparent and opaque pneumococcal strains to colonize (54). In this model, infant Sprague-Dawley rats are inoculated nasally with pneumococci; colonization is assessed by obtaining nasal wash fluids. While the persistence of carriage in each individual rat can be readily assessed, the density of colonization is more difficult to establish, and the results can be, at best, described as semiquantitative. A variation of this model relies on intralitter spread among infant rats (29). In this model, 10 to 12 Sprague-Dawley infant rats 3 to 4 days of age are housed in a cage with a postpartum dam. Two of the infant rats (those representing the index cases) are inoculated with live pneumococci, while the remaining rats in the cage are left untouched. This model offers the possibility of studying the ability of pneumococci to persist in one host as well as spread to other naïve infant rats in the cage. A buccal mucosa model was also developed to evaluate the impact of ambient temperatures on pneumococcal colonization (37).

The chinchilla model, which was developed initially to understand the pathophysiology of pneumococcal otitis media, was also adapted to study pneumococcal colonization (7, 8). This modification of the model is useful, as the initial approach (direct inoculation of pneumococci into the middle ear) is likely to be an extremely aggressive test of any therapeutic intervention, since it bypasses the natural defenses against pneumococcal pathogenesis. The direct nasal inoculation of chinchillas with pneumococci is associated with nasopharyngeal colonization of several weeks' duration. This model has also been adapted for the study of interactions between pneumococci and respiratory viruses (1, 8, 9, 48–50). As an example, influenza A virus was shown to synergize with pneumococci with respect to the development of acute otitis media (8). Peak pneumococcal susceptibility occurred 4 days after influenza virus inoculation. These findings have allowed for the study of vaccine efficacy against mucosal infection following colonization and in the context of influenza infection. The major limitation of this model is the relative lack of chinchilla reagents, as well as the expense. Affinity-purified chinchilla immunoglobulin reagents have been developed and used in this model but are not routinely available.

It has long been known that certain nonhuman primate infants are susceptible to pneumococcal infections (16, 40, 44). One of the first reported cases dates from 1936, when an infant chimpanzee named Panacea born

at the Johns Hopkins Department of Anatomy died of pneumococcal pneumonia due to a type 3 strain (45). More recently, a pneumococcal outbreak was suspected in the Tai National Park in 1999 when, in the process of investigating an epidemic of acute respiratory disease in wild chimpanzees, investigators isolated a type 3 pneumococcal strain from the lung of a deceased chimpanzee. It was concluded that the outbreak was likely the result of the infection of animals with pneumococci transmitted from either visitors or people working in the park (20).

Nonhuman primate models of pneumococcal infection have been used in the past but have generally focused on pneumonia or sepsis, not colonization. Because studies with nonhuman primates that involve morbidity and/or mortality are both expensive and extremely unlikely to be approved by animal use committees, models that use asymptomatic colonization as an end point may be attractive. To date, there are no published reports of nonhuman primate colonization models, although there has been some experience with rhesus macaque (*Macaca mulatta*) colonization studies (H. Keyserling, personal communication). A recently published report of *S. pneumoniae* pneumonia in rhesus macaques concluded that the clinical course of pneumonia in these animals mimics aspects of human disease (39). This experience suggests that a colonization model, perhaps in infant macaques, may well be feasible and may provide insight into mechanisms of pneumococcal colonization. Although such a model would clearly be very expensive, it may be worth pursuing because nonhuman primates appear to have natural susceptibility to the pathogen at an early age. A reproducible nonhuman primate model would thus potentially be of relevance to human disease and could be thoroughly studied, given the general availability of immunological reagents. It is worthy of note that the rhesus monkeys used in the previous study had preexisting pneumococcal antibodies, suggesting the possibility that they may have been colonized with pneumococci in the past (39). It is thus tempting to speculate that these rhesus macaques developed this immune response following pneumococcal exposure through their handlers.

Experimental models of colonization have also been applied to the human host. Limited use of this model demonstrated the nasal inoculum required to establish colonization in healthy adults (a 50% colonizing dose is estimated to be  $10^3$  to  $10^4$  CFU), defined a duration of colonization (range, 0 to 122 days), and permitted an analysis of the mucosal and systemic humoral response to a single carriage event (32, 33). Susceptibility to the establishment of the carrier state was shown to be asso-

ciated with lower levels of preexisting antibody recognizing the pneumococcal surface protein A (PspA) of the challenge strain. The characterization of experimental nasal colonization in humans also provided a basis for assessing the applicability of animal models of carriage. For example, the same isolate used in human studies was found to colonize adult mice in association with a similar inoculum dose, duration, and immune response (34). In addition, recent experience with experimental human colonization offers a paradigm for carrying out closely monitored studies in which *S. pneumoniae* can be safely studied in its natural host. Such studies may be particularly useful in the future in the early testing of vaccines that target carriage.

## BACTERIAL FACTORS IN COLONIZATION

Animal models have provided information about the contribution of specific bacterial components to colonization, the first step in the pathogenesis of all pneumococcal disease. Before we describe studies using these models, several limitations of the models should be mentioned. As discussed above, much of the work has been performed with mice as the most convenient model. Different laboratories have used different mouse strains, which often vary in susceptibility to pneumococcal colonization and disease. A further consideration is that *S. pneumoniae* is a genetically heterogeneous and unstable species, causing different strains or even the same strain maintained in separate laboratories to yield different results when tested in the same model system (38). Much of the work in this field has relied on a single isolate, D39, which is useful for studying invasive disease but colonizes mice relatively inefficiently compared to most other isolates. Finally, colonization in many of these studies has often been assessed by the presence of bacteria in the lower rather than the upper airway following nasal inoculation under anesthesia, a procedure commonly complicated by aspiration.

Since the environmental niche of the pneumococcus is the mucosal surface of the upper airway, it is likely that much of the pneumococcal genome contributes to survival or persistence at this site. Clearly, we are in the early stages of understanding the biology of pneumococcal colonization. Some important colonization factors cannot be fully appreciated using standard animal models. The secreted immunoglobulin A1 protease expressed by pneumococci, for instance, has specificity for its human target (18). Many of the bacterial factors whose roles have been assessed using animal models of colonization have also been the targets of protein- or non-protein-based-component vaccines. In most cases,

these bacterial factors appear to have an even greater role in events beyond the mucosal surface, although several studies have defined tissue-specific determinants that affect survival within the respiratory tract (13, 36). For example, a whole-genome approach to identify such genes revealed a pathogenicity island present in some strains that encodes a surface adhesin now known to be a pilus-like structure (5, 14, 21).

A major feature of *S. pneumoniae* and a target of all currently licensed vaccines is its polysaccharide capsule. Although the capsule allows for the evasion of opsonization and subsequent phagocytosis during invasive infection, its role during mucosal colonization, the organism's commensal state, has remained largely unexplored. In the mouse model, there is a minimum amount of capsular polysaccharide required for efficient colonization (24). Unencapsulated mutants remain capable of nasal colonization but at a reduced density and duration compared to their encapsulated parent strains (35). Unlike that in invasive infection, the capsule-related deficit in colonization is not due to increased susceptibility to opsonophagocytic clearance involving complement, antibody, or the influx of neutrophils seen during carriage. Rather, unencapsulated mutants remain agglutinated within luminal mucus and, thus, are less likely to transit to the epithelial surface, where stable colonization occurs. Results from in vitro binding studies with immobilized human airway mucus confirm the inhibitory effect of encapsulation. Likewise, pneumococcal variants expressing larger amounts of negatively charged capsule per cell than their wild-type counterparts are less likely to adhere to surfaces coated with human mucus and more likely to evade initial clearance in vivo. The removal of negatively charged sialic acid residues by the pretreatment of mucus with neuraminidase diminishes the antiadhesive effect of encapsulation. This finding suggests that the inhibitory effect of encapsulation on mucus binding may be mediated by electrostatic repulsion and offers an explanation for the predominance of anionic polysaccharides among the diverse array of unique capsule types. Thus, the capsule may confer an advantage on *S. pneumoniae* distinct from its role in the inhibition of opsonophagocytosis and escape from entrapment in luminal mucus.

Among the protein targets of experimental vaccines, only a few have been evaluated for their role in colonization. The protein that seems to have the greatest impact in murine colonization models is choline-binding protein A (CbpA), also referred to as PspC or SpsA (4, 42). Functions attributed to this surface protein include adhering to host cells, binding to the human secretory component, and inhibiting complement activation through

the binding of factor H (6, 11). In contrast, the importance of another choline-binding surface protein, PspA, seems to be more limited in these models, although the mucosal immune response to this protein may be protective (4). Pneumolysin, a cholesterol-dependent pore-forming toxin, promotes an acute inflammatory response in the nasal mucosa that facilitates the clearance of the organism, although there are also reports of findings from studies using different strains suggesting that pneumolysin enhances or has no role in murine colonization (17, 43, 53). Likewise, the contribution of pneumococcal neuraminidase, NanA, which removes terminal sialic acid, exposing glycoconjugate receptors, has varied in different models in different laboratories (19, 31, 47).

### INNATE IMMUNITY IN COLONIZATION

The availability of genetically modified mice has allowed for the characterization of host factors that contribute to colonization. These studies generally examine a single host component and, thus, are informative only in identifying nonredundant pathways. Another consideration in these studies is the need for thorough back-crossing of mice derived from mixed genetic backgrounds since, as noted above, different lines often vary markedly in their susceptibility. These reports have focused on host factors that have an impact on the clearance of colonization, particularly aspects of innate or adaptive immunity. Host determinants of the initial establishment of the carrier state or host-to-host transmission have proven more difficult to model using animals (29). Putative host receptors identified in cell culture models, including the receptor for platelet-activating factor and the secretory component of the polymeric immunoglobulin receptor, have not yet been analyzed for their role in the upper airway (41). In the latter case, this analysis may require the use of transgenic animals expressing the human secretory component (12).

A number of components of innate immunity in mice have been examined for their contribution to the clearance of pneumococci from the mucosal surface of the upper airway following the establishment of carriage. A limitation in the use of immunodeficient mice to study colonization is that these mice may harbor altered flora affecting pneumococcal carriage. In mice, the initial colonization of the nasal mucosa leads to the acute production of chemokines, such as macrophage inflammatory protein 2, and subsequent influx of neutrophils into the nasal spaces (53). Between 1 and 3 days post-inoculation, colonizing pneumococci are seen to be associated predominately with luminal neutrophils. The

neutrophil influx, however, is ineffective at reducing the numbers of viable pneumococci unless there are additional stimuli, such as cocolonization with another species providing different inflammatory mediators (23). The depletion of complement also has little effect on initial colonization in this model (53). Pneumococci are eventually cleared over a period of weeks from the upper airways of colonized immunocompetent mice. As described below, this effect requires CD4<sup>+</sup> T cells rather than antibody. The specific effectors of the immune response associated with this delayed killing remain unknown. The signaling events required for clearance are dependent on members of a family of Toll-like receptors, which recognize pathogen-associated molecular patterns. Mice deficient in Toll-like receptor 2 (TLR2), which is involved in the response to lipoteichoic acid, show delayed clearance (53). The expression of cholesterol-dependent cytotoxins, including pneumolysin, has been shown to affect TLR4-mediated signaling (25). In vivo studies in separate laboratories examining the effect of TLR4 on pneumococcal colonization have yielded different results (25, 53). A role for TLR9, an intracellular sensor of bacterial DNA, in the immune response to pneumococci in the lung was recently described but has not been examined with regard to the upper airway (2). Additional evidence for the importance of signaling mediated by Toll-like receptors is the increased susceptibility of mice lacking myeloid differentiation factor 88, an adaptor protein required for signaling for many Toll-like receptors (3).

### ACQUIRED IMMUNITY TO COLONIZATION

Murine models of pneumococcal colonization have helped enhance our understanding of acquired immunity to pneumococcal colonization. The infant rat model of intralitter spread described above was used to evaluate the impact of systemic anticapsular antibodies in the prevention of pneumococcal colonization (29). The administration of bacterial polysaccharide immune globulin (from hyperimmune sera obtained from adults immunized with pneumococcal, *Haemophilus influenzae* type b, and meningococcal pure-polysaccharide vaccines) to infant rats reduced the likelihood of pneumococcal colonization by about 50%. The concentrations of anticapsular antibody achieved in the infant rats were similar to that which could be reached by conjugate vaccine immunization. Therefore, this study showed that parenterally administered anticapsular immunoglobulin G was protective against the acquisition of pneumococci. Of some interest, the same dose of bacte-

rial polysaccharide immune globulin was ineffective in preventing colonization following the direct inoculation of pneumococci into infant rats, presumably because this challenge, which may be less representative of the mechanisms of natural spread, may be too stringent.

More recently, two groups have demonstrated that acquired immunity to colonization can occur independently of antibodies. Focusing on naturally acquired mechanisms of clearance, McCool and Weiser demonstrated that the eradication of pneumococcal colonization occurs in an antibody-independent fashion: mice that lacked virtually any immunoglobulin G, A, or M ( $\mu$ MT<sup>-/-</sup> mice with a C57BL/6 background) cleared colonization just as efficiently as wild-type mice (34). Using a different approach, Malley et al. evaluated mechanisms of immunity to pneumococcal colonization following immunization with a vaccine consisting of killed, unencapsulated whole-cell bacteria and an adjuvant. In this mouse model, this whole-cell vaccine (WCV) conferred protection against colonization by a variety of encapsulated pneumococci (26, 27, 30). Whereas wild-type,  $\mu$ MT<sup>-/-</sup>, and major histocompatibility complex class I-deficient mice were all significantly protected by the WCV, nude mice (which lack T cells) and major histocompatibility complex class II-deficient mice were not, implying a role for CD4<sup>+</sup> T cells in WCV-induced protection against colonization (30). Subsequent studies showed that these CD4<sup>+</sup> T cells are effectors of protection, as CD4<sup>+</sup> T-cell depletion at the time of challenge abolished WCV-induced immunity whereas CD8<sup>+</sup>-T-cell depletion had no effect. The nature of the T cells has been suggested by the findings of a similar study in which mucosal immunization with the conserved pneumococcal cell wall polysaccharide (CWPS) was shown also to confer significant protection against nasopharyngeal colonization in an antibody-independent, CD4<sup>+</sup>-T-cell dependent fashion (28). In this study, the neutralization of the cytokine interleukin-17A at the time of challenge abolished protection, suggesting that TH17 cells may be responsible for protection by CWPS. Studies using interleukin-17A receptor knockout mice have subsequently confirmed this finding (R. Malley et al., unpublished data), and thus, it is likely that immunization with WCV or CWPS elicits protection via TH17 CD4<sup>+</sup> T cells. Exposure to live pneumococci has also been shown to protect against pneumococcal colonization. In one approach, repeated intranasal exposure to live pneumococci provided significant protection against subsequent colonization with heterologous strains, again in an antibody-independent, CD4<sup>+</sup>-T-cell dependent manner (30, 51). A single intranasal exposure to an attenuated strain (with combi-

nations of deletions in genes encoding the capsular polysaccharide, pneumolysin, and PspA) conferred both systemic and mucosal protection against the parent wild-type strain and a distantly related isolate (Roche, S. J. King, and J. N. Weiser, unpublished data). In this model, there appeared to be a significant contribution of antibody to full protection, as  $\mu$ MT-deficient mice were not protected.

## CONCLUSIONS

The development of strategies to prevent or reduce pneumococcal colonization in a species-specific (as opposed to serotype-specific) manner has been the subject of intense research over the last several years. Paradoxically, the impressive success of the pneumococcal conjugate vaccine in reducing invasive disease provides further rationale for developing alternative strategies aimed at reducing colonization. Indeed, as discussed elsewhere, the conjugate vaccine is now estimated to prevent more invasive disease by its indirect effect than by its direct effect on immunized hosts (22, 56). At the same time, the prospect of global implementation of this strategy faces the challenges of worldwide serotype diversity, cost, and availability, as well as serotype replacement. The development of purified-protein vaccines or whole-cell vaccines, however, faces several challenges, including the inherent difficulty of studying colonization in animals that normally do not carry *S. pneumoniae* in their respiratory trees. In this respect, the development of a highly reproducible model of colonization in the nonhuman primate and further experiments using the human colonization model (clearly the most biologically relevant) would be of great importance.

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David Goldblatt  
Tracy Assari  
Clifford Snapper

# 6

## The Immunobiology of Polysaccharide and Conjugate Vaccines

Infections with polysaccharide (PS)-encapsulated extracellular bacteria are a major source of global morbidity and mortality among infants, as well as the elderly and immunosuppressed individuals. The PS capsule is a major virulence factor, largely through its ability to diminish the phagocytic uptake of bacteria by neutrophils and macrophages (17) via resistance to and degradation of complement components (42). Anti-PS antibodies can afford protection against these pathogens by binding to the capsule, acting as a receptor for complement components, and thereby promoting complement- and Fc-dependent opsonophagocytosis (4, 147). Pneumococci that lack a capsule are normally avirulent because of their inability to resist innate immunity (151). Purified capsular PSs have therefore formed the basis of vaccines designed to prevent infection with encapsulated bacteria. The limitations of the use of purified PSs in the very young and the very old and in those most vulnerable to infection have become apparent. A better understanding of the interaction between PS antigens and the immune system has contributed to the development of alternative strategies to induce immune responses to PS antigens, particularly in the very young, through the use

of glycoconjugates. An understanding of the immunological basis of glycoconjugate interactions is still emerging but is already helping shape strategies to improve conjugate responses.

### IMMUNOLOGICAL BASIS OF RESPONSE TO PS ANTIGENS

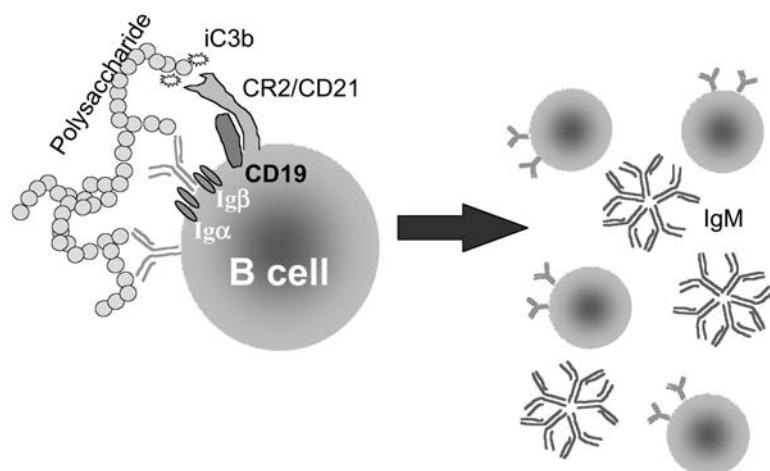
Purified PS antigens are poorly immunogenic in infants, although they can elicit protective antibody responses in adults (103). With the exception of zwitterionic PS (30, 141), purified PS antigens fail to associate with major histocompatibility complex class II (MHC-II) molecules (despite being taken up by antigen-presenting cells [APCs] such as dendritic cells [102]) and thus do not recruit classical cognate CD4<sup>+</sup> T-cell help (55, 62). Although PS antigens are reported to elicit nonclassical forms of T-cell help and suppression (8), as well as CD40 ligand-dependent help (70, 71), their ability, in contrast to that of protein antigens, to elicit significant immunoglobulin (Ig) responses in T-cell-deficient mice continues to warrant their description as T-cell-independent (TI) antigens. In this regard, Ig responses to purified PS

antigens are largely of the IgM isotype and are generally limited in their elicitation of germinal center reactions, Ig class switching, somatic hypermutation and affinity maturation, and immunologic memory (Fig. 1) (37, 85, 103, 145). PS antigens are also known to induce variable-region genes in humans in a restricted or biased fashion (2, 12). A recent study demonstrated TI induction of long-lived PS-specific memory B cells exhibiting a unique phenotype relative to T-cell-dependent memory B cells in mice in response to a purified TI antigen (110). In this study, the presence of circulating antigen-specific IgG markedly inhibited the subsequent elicitation of the secondary response. A PS-specific IgM<sup>+</sup> memory-B-cell population responsive to the 23-valent pneumococcal PS (PPSV23) vaccine in humans has also been described and is thought to have a role in natural immunity to the pneumococcus. These cells originate in the marginal zone of the spleen (75), and thus, their absence in splenectomized individuals (24) may contribute to the susceptibility of such individuals to pneumococcal infection. Levels of PS-specific IgM<sup>+</sup> memory B cells are also thought to be reduced in elderly individuals (134), perhaps explaining in part the susceptibility of the elderly to pneumococcal disease.

Genetic factors in humans that are known to influence susceptibility to infection with the pneumococcus may also provide a clue to the key factors associated with the immune response to PS antigens. IgG2 plays a pivotal role in defense against pneumococcal infection, and its receptor, human Fc $\gamma$ RIIa, has two codominantly

expressed allotypes, which differ greatly in their ability to ligate IgG2 (99). Whereas Fc $\gamma$ RIIa-R131 binds only weakly to IgG2, Fc $\gamma$ RIIa-H131 binds with high affinity. Two studies have demonstrated an association between the Fc $\gamma$ RIIa-H131 allotype and protection against invasive pneumococcal disease (159, 161). C-reactive protein is an acute-phase protein known to bind to the C polysaccharide of pneumococci (144), and C-reactive protein polymorphisms have been shown to be associated with susceptibility to invasive pneumococcal disease (122). Mutations in three codons of the gene for mannose-binding lectin, a key mediator of innate host immunity that activates the complement pathway and directly opsonizes some infectious pathogens, have also been shown to be associated with susceptibility to invasive pneumococcal disease (123). Deficient Toll-like receptor (TLR)-mediated cytokine production has also been described in association with susceptibility to pneumococcal disease (33), and cytokine receptor polymorphisms may also influence the height of the antibody response to pneumococcal vaccines (154). Abnormal interleukin receptor-associated kinase 4 expression and NF- $\kappa$ B translocation, with the consequences of abnormal TLR signaling and cytokine expression, are also associated with susceptibility to pneumococcal disease (76).

In contrast to PS antigens, some, although not all, proteins elicit Ig responses in infants roughly comparable to those observed in adults. Unlike most PS antigens, proteins are degraded into peptides within the lyso-



**Figure 1** Interaction between PS antigen and B cells. B cells recognize the antigen via the B-cell receptor, and this interaction is sufficient to trigger clonal expansion of the B cells and antibody production. Antibody responses are dominated by the production of IgM, and no affinity maturation of antibodies or memory-B-cell production occurs. CR2, complement receptor 2.

somes of APCs and are presented on the APC surface in association with MHC-II molecules. This presentation results in the recruitment of CD4<sup>+</sup> T-cell help and the subsequent induction of the germinal center reaction, class switching to IgG, and somatic hypermutation and affinity maturation, as well as the generation of memory (101).

## IMMUNOLOGICAL BASIS OF RESPONSE TO GLYCOCONJUGATES

The covalent linkage of PS antigens to immunogenic proteins capable of recruiting CD4<sup>+</sup> T-cell help to produce conjugate vaccine, first described in the 1930s (7), results in the elicitation of protective, high-titer-IgG anti-PS responses and the generation of immunologic memory, as well as immunogenicity in the infant host (3, 14, 127). In the absence of a Th1-inducing adjuvant (see below), conjugate vaccines, relative to PS alone, induce a shift in Ig isotypes from IgM and IgG3 (mouse) or IgG2 (human) to primarily IgG1, associated with a large increase in the bactericidal activity of the serum (124). In addition to conjugate vaccines in current clinical use (*Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Neisseria meningitidis* vaccines), numerous animal studies have tested the potential of various conjugates to protect against a variety of other bacterial infections (Table 1).

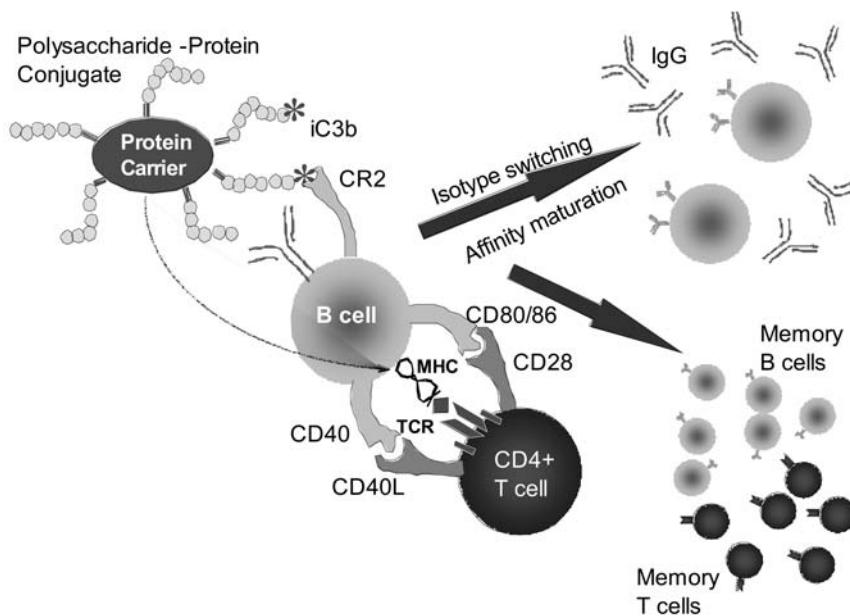
A limited number of studies have investigated the immune mechanisms underlying the Ig response to conjugate vaccines. Adoptive transfer studies utilizing cells from mice immunized with the type 3 capsular PS from group B streptococci conjugated to the protein tetanus

toxoid (TT) demonstrated the ability of the conjugate to elicit PS-specific memory (51). This memory was characterized by an anamnestic response exhibiting more-rapid kinetics, greater dominance of IgG over IgM, and 10-fold-higher IgG anti-type 3 PS titers than the primary response (51). The anti-PS response to the type 3 PS-TT conjugate requires MHC-II-T-cell receptor, B7-CD28, and CD40-CD40L interactions (50) shown previously to be critical for Ig responses to purified protein antigens (29). A similar role for specific CD4<sup>+</sup>-T-cell help was shown for murine IgG anti-pneumococcal PS type 14 (PPS14) responses to both intact *S. pneumoniae* cells of capsular type 14 (156, 157) and a conjugate of PPS14 and pneumococcal surface protein A (PspA) (74). PS-specific memory induced by a conjugate vaccine can be elicited by boosting with either the conjugate itself or the purified PS antigen that was used in the conjugate (104, 124), the latter dependent upon the dose of the conjugate used for priming (114). However, the presence of relatively high doses of unconjugated PS in the conjugate preparation can inhibit the PS-specific Ig response to the conjugate itself (114, 121). The ability of conjugate-primed mice to elicit a boosted IgG anti-PS response to the PS alone can be transferred to adoptive recipients solely by B cells derived from mice primed with the conjugate but not from mice primed with PS alone (see Fig. 2) (124). Similar results were reported in a more recent study in which recipients of adoptively transferred splenocytes from conjugate-primed, rather than PS-primed, mice exhibited an anamnestic response characterized by more rapid kinetics, isotype switching from IgM to IgG, and higher anti-PS Ig titers in response to boosting with PS alone (48).

CD4<sup>+</sup> T-cell clones generated from mice immunized with a meningococcal serotype C capsular PS vaccine (MCV-C) conjugated to TT (MCV-C-T) were largely TT specific and MHC restricted, as expected (107). Surprisingly, however, non-MHC-restricted CD4<sup>+</sup> T-cell clones specific for meningococcal C PS alone and MHC-restricted CD4<sup>+</sup> T cells specific for MCV-C-T were also isolated. All T-cell clones, regardless of MHC restriction, required contact with APCs for maximal stimulation. Of interest, PS antigens may also influence the ability of APCs to present associated protein antigens to CD4<sup>+</sup> T cells (86). Thus, the ability of spleen cells from HLA-DR1 transgenic mice, pulsed with individual pneumococcal PS-cross-reactive material (CRM) conjugates found within a heptavalent conjugate vaccine, to stimulate a CRM-specific, DR1-restricted CD4<sup>+</sup> T-cell hybridoma was dependent on the particular capsular PS serotype in the conjugate. A significant correlation between IgG anti-PS and IgG anti-carrier protein re-

**Table 1** New bacterial conjugate vaccines tested in animals

Bacterial species	Reference(s)
<i>Bacillus anthracis</i>	77, 128
<i>Escherichia coli</i>	41
<i>Francisella tularensis</i>	31
<i>Klebsiella pneumoniae</i>	26
<i>Moraxella catarrhalis</i>	61, 160
<i>Mycobacterium tuberculosis</i>	54
Nontypeable <i>Haemophilus influenzae</i>	57, 58, 60, 155 (NTHi)
<i>Pseudomonas aeruginosa</i>	142, 162
<i>Salmonella enterica</i> serovar Typhimurium	53, 69
<i>Shigella flexneri</i> 2a	116
<i>Staphylococcus aureus</i>	41, 91
Group A <i>Streptococcus (pyogenes)</i>	125, 129
Group B <i>Streptococcus</i>	50, 51, 66, 111
<i>Vibrio cholerae</i>	21, 25, 112



**Figure 2** Interaction between PS-protein conjugate and B cells. B cells recognize the PS antigen via the B-cell receptor while also taking up the carrier protein, processing it, and presenting it to T cells in the context of MHC-II molecules. The CD4<sup>+</sup> T cell provides the necessary costimulation for the switching of antibody class, affinity maturation, and the generation of memory B cells. CR2, complement receptor 2; TCR, T-cell receptor. Reprinted from *Science* (135) with permission of the publisher.

sponses is observed in response to immunization with a conjugate vaccine (64), but conjugates that induce relatively low titers of anti-PS antibody may still elicit a strong T-cell response (100).

## GLYCOCONJUGATE VACCINES IN THE ELDERLY

Whereas conjugate vaccines have clearly proven their usefulness in the infant population, they appear to work only a little better than purified PS vaccines in the elderly (1, 117). Thus, in contrast to infants, who receive the pneumococcal conjugate vaccine (e.g., Prevnar), the elderly continue to receive the older PPSV23 vaccine for the prevention of serious pneumococcal infections. However, some controversy exists regarding the relative effectiveness of the PPSV23 vaccine in the elderly, indicating a need for better pneumococcal vaccines for this group (6, 59, 63, 89, 109, 150). In light of the key role of carrier protein-dependent CD4<sup>+</sup> T-cell help in the induction of the Ig response to conjugate vaccines, it is of interest that the age-related decline of the adaptive immune response is largely due to defective CD4<sup>+</sup> T-cell help (56). Of note, when CD4<sup>+</sup> T cells from young adult mice were transferred into aged mice, they in-

duced B-cell proliferation and IgG production comparable to those seen upon similar transfer into young adult recipients (39). Exposure to inflammatory cytokines in vivo could eliminate the defect in CD4<sup>+</sup> T-cell function in aged mice (39). These observations suggest the possibility that the use of a suitable adjuvant with a conjugate vaccine may enhance the anti-PS response in the elderly by restoring, at least in part, adequate CD4<sup>+</sup> T-cell function. In this regard, a recent study demonstrated the ability of CpG-ODN (a TLR9 ligand) to improve defective anti-PS responses to a pneumococcal conjugate vaccine in aged mice such that the responses reached levels comparable to those seen in young adult mice (110). This improvement was associated with a partial amelioration of the defective carrier-specific CD4<sup>+</sup> T-cell priming. The anti-PS response to conjugate-CpG-ODN in the aged mice was dramatically higher than that observed using unconjugated PS as the immunogen. Of interest, anti-PS titers in the elderly do not necessarily correlate with opsonizing activity, and indeed, sera from elderly vaccinated individuals have been found to have lower pneumococcal opsonizing activity than sera from their younger counterparts, despite similar titers (106). Thus, optimizing conjugate vaccine efficacy in the elderly may involve issues in addition to simply raising anti-PS titers.

## CARRIER PROTEIN INTERACTIONS WITH GLYCONJUGATE VACCINES

Conjugate vaccines used clinically are produced by employing a limited number of carrier proteins (e.g., TT, diphtheria toxin or its genetic mutant form CRM<sub>197</sub>, the outer membrane protein complex [OMPC] of *N. meningitidis*, and more recently, protein D derived from *H. influenzae*). This has led to the potential problem of diminished anti-PS responses to conjugate vaccines when the same carrier protein is used for several different vaccine types or combined with multiple serotypes within the same vaccine. This diminution occurs when the vaccines containing the carrier protein are injected at the same or different times. This effect would appear in part to be due to epitopic overload and carrier-induced suppression. An early study demonstrated that the preimmunization of mice with TT suppressed a subsequent antibody response to synthetic peptides conjugated to TT (131), that this result was due to clonal dominance (130), and that the response required CD4<sup>+</sup> T cells for induction (81).

Preexisting immunity of mice to the cholera toxin B subunit (CTB) inhibited both the pulmonary mucosal and serum antibody responses to dextran upon intranasal (i.n.) immunization with a dextran-CTB conjugate (13). This effect, however, could be overcome by using higher conjugate doses and delaying immunization until the CTB antibody titers had declined. The addition of free recombinant *Pseudomonas aeruginosa* exotoxin A (rEPA), but not that of diphtheria toxin, to a conjugate of rEPA and the type 5 capsular PS of *Staphylococcus aureus* resulted in a 64% reduction in anti-type 5 PS antibodies (41). Likewise, immunization with a mixture of rEPA-conjugated type 5 and type 8 PS vaccines resulted in a 30% reduction in anti-type 8 PS Ig relative to immunization with the single type 8 PS conjugate alone. In addition, 30 to 90% reductions in serotype-specific anti-PS responses were observed using a 12-valent *Escherichia coli* lipopolysaccharide (LPS) conjugate vaccine relative to the responses observed using each monovalent vaccine separately (41). Combining an *H. influenzae* type b (Hib) PS, polyribosyl ribitol phosphate (PRP), conjugated to TT (PRP-T) with a vaccine consisting of diphtheria toxin, TT, and acellular pertussis proteins (DTaP) resulted in a reduced anti-PRP response in mice relative to immunization with PRP-T alone (97). A follow-up study with rats demonstrated that the Ig response to the various components within the DTaP vaccine were unaffected by coimmunization with PRP-T, although the anti-PRP response was again reduced relative to the response to immunization with

PRP-T alone (96). The rat model set up by Mawas and colleagues (96) (and the guinea pig model of Gupta and colleagues [49]) was designed to model the phenomenon first noticed in humans when Hib conjugate vaccines were given in combination with acellular pertussis protein-containing combination vaccines. In the original studies describing this phenomenon, Hib PS responses were attenuated when PRP-T was given together with DTaP (40). Initially, the mechanism was thought to be due to the effect of aluminum hydroxide on the integrity of the PRP-T conjugate, but subsequent studies revealed that the reduction in anti-PRP responses to PRP-T were directly correlated with the total amount of TT administered (34). Whole-cell pertussis pathogen-containing combinations in general overcome the TT-induced suppression of responses (through adjuvant-like activity), but the potential for suppression is unmasked when acellular pertussis protein combinations are used. In a study of a pneumococcal-bicarrier conjugate vaccine using both diphtheria toxin and TT carriers for different PSs, increasing doses of TT were associated with the attenuation of primary responses to the pneumococcal PSs conjugated to TT (35). Higher doses of diphtheria toxin carrier were, however, associated with only small reductions in secondary but not primary responses. CRM has proved to be the carrier which appears least likely to induce carrier-associated hyporesponsiveness, although large amounts of CRM may paradoxically reduce responses to a concomitantly administered diphtheria vaccine (20).

In contrast to the observations cited above, a number of murine studies have demonstrated no inhibitory effects of the same carrier protein used in combination vaccines. Thus, the preimmunity of mice to bovine serum albumin (BSA) had no effect on the antibody response to the O-specific PS of *Vibrio cholerae* O1, serotype Ogawa conjugated to BSA, although the preimmunity did enhance the protective capacity of the antisera (25). Recombinant staphylococcal enterotoxin C1 (rSEC) was conjugated to group A meningococcal PS or W135 capsular PS. A bivalent formulation of group A meningococcal PS-rSEC and W135 PS-rSEC elicited anti-C polysaccharide IgG at levels comparable to those induced by the conjugates administered separately (72). Combining a PRP-T conjugate with a vaccine consisting of DTaP and inactivated poliovirus did not reduce the anti-PRP response (97). The tumor antigens ganglioside GD3 and neutral glycolipid Lewis<sup>y</sup> (Le<sup>y</sup>) and mucins MUC1 and MUC2 were conjugated to keyhole limpet hemocyanin for testing as components of a possible antibody-based antitumor conjugate vaccine (119). The immunogenicities of the four individual antigens

conjugated to keyhole limpet hemocyanin following the immunization of mice in the presence of the adjuvant, QS-21, were not affected by mixing the four together, as opposed to injecting them individually, or by administering them subcutaneously at a single site. The co-administration of MCV-C-CRM and PRP-CRM i.n. resulted in anti-PS responses in mice similar to those observed following single-conjugate immunization (146).

The effect of carrier prepriming on the anti-PS response to subsequent immunization with a PS-carrier conjugate may depend on the dose of carrier used for priming and the nature of the conjugate itself. Thus, an increase in the anti-PS Ig response to PPS4-TT and MCV-C-T, but not MCV-C-CRM, was observed with low-dose carrier priming (0.025 to 0.25 µg/mouse) (113). However, the suppression of the anti-PS response to the PPS4-TT and MCV-C-T, but not MCV-C-CRM, was observed with high-dose (25-µg/mouse) carrier priming. Similarly, reducing the amount of PRP-T and/or free TT when the two were used in combination was shown to reduce the suppression of the anti-PRP Ig response in guinea pigs relative to the use of PRP-T alone (49). In humans, carrier priming at birth with diphtheria toxin and TT had little effect on subsequent responses to Hib conjugated to CRM or TT (88), although priming at 6 weeks with diphtheria-tetanus-pertussis vaccine significantly increased the response to a dose of PRP-T at 10 weeks compared to the response to a first dose at 6 weeks without carrier priming in children in Niger (22). In contrast, in older children (aged either between 3.5 and 6 years or 13 and 18 years) resident in the United Kingdom, carrier priming with diphtheria toxin reduced subsequent responses to MCV-C-T but not MCV-C-CRM (19), and similarly, the response to PRP-T was reduced in pregnant women who had received prior TT during the same pregnancy (105).

Responses to conjugate vaccines may also be affected by the nature of the PSs to which the carriers are conjugated. McCool et al. (100) showed that the conjugation of different pneumococcal PSs to the same carrier protein altered the peptide specificity of T-cell responses, suggesting that variations in carrier-induced T-cell help may contribute to differences in the PS-specific immunogenicities of conjugate vaccines. Collectively, these data indicate a need to consider carrier-mediated suppressive effects as a possible, though not invariable, outcome of using combinations of vaccines containing the same protein. In this regard, clinical testing of additional carrier proteins suitable for conjugate vaccines is warranted and exemplified by a new 10-valent pneumococcal vaccine using an outer membrane protein of *H. influenzae*, protein D, which is close to licensure (118). A summary of animal studies testing the use of new car-

**Table 2** Potential new carrier proteins tested in animals

New carrier(s)	Reference(s)
rSEC .....	72
CTB .....	13
P64k .....	23, 48
rEPA .....	41, 77, 128
Recombinant PorB (meningococcal porin) .....	44
CD, UspA ( <i>Moraxella catarrhalis</i> outer membrane proteins)	61
Recombinant <i>Bacillus anthracis</i> protective antigen .....	77, 128
Recombinant pneumolysin Ply .....	79, 82, 83
Autolysin (Aly) .....	83
OmpA ( <i>Klebsiella pneumoniae</i> ) .....	87
Flagella .....	148
Nontypeable <i>Haemophilus influenzae</i> outer membrane protein P6 .....	155
Recombinant P40 ( <i>Klebsiella pneumoniae</i> outer membrane 40-kDa protein) .....	53

rier proteins relevant to potential clinical use is given in Table 2.

## ADJUVANTS AND THE USE OF GLYCOCOCONJUGATE VACCINES

Currently, only aluminum salts (alum) are used clinically as adjuvants for anti-PS responses to conjugate vaccines. Alum acts to promote immunity, likely by serving as a depot for antigen, thus enhancing uptake by APCs for presentation of the protein component to CD4<sup>+</sup> T cells. In this regard, alum fails as an adjuvant in TI anti-PS responses to purified PS antigens. Of interest, PNEUMOVAX 23, the PPSV23 vaccine, is suspended only in saline, whereas Prevnar, the seven-valent pneumococcal conjugate vaccine, is suspended in aluminum phosphate. Alum elicits minimal, if any, inflammatory sequelae and is thus found to be safe for clinical use. However, this very property also limits its adjuvant capacity. In comparative studies of an experimental 11-valent bicarrier pneumococcal conjugate vaccine in Finnish and Israeli infants, there was no significantly increased immunogenicity of the aluminum hydroxide adjuvant formulation, although it did increase the avidity of responses to one of the 11 serotypes (serotype 5) (158).

The upregulation of innate immunity is largely mediated by signaling via TLRs, present within the cytoplasm or on the cell membrane, of various cell populations (e.g., dendritic cells, macrophages, and neutrophils); hence, ligands for TLRs are attracting increasing attention as potential adjuvants. Numerous animal studies

have demonstrated the ability of certain TLR (e.g., TLR2, TLR4, and TLR9) as well as non-TLR ligands (in addition to alum) to enhance anti-PS responses to conjugate vaccines either individually or in combination (Table 3). Mice immunized intradermally or subcutaneously with PRP-T or PRP-CRM exhibited a predominantly IgG1 anti-PRP response (149). The addition of CpG-ODN resulted in an increased total IgG anti-PRP response resulting from the selective induction of IgG3 and IgG2a, with no further enhancement of IgG1 anti-PRP antibody. This result correlated with CpG-ODN-mediated enhancement in serum anti-TT and anti-CRM IgG titers and the induction of interleukin-12 (IL-12) and gamma interferon (IfN- $\gamma$ ), cytokines important in eliciting Th1 responses, which are dominated by the IgG3 and IgG2a isotypes (137, 138). Thus, CpG-ODN likely enhances CD4 $^{+}$  T-cell help for anti-PRP responses and Th1 differentiation via the activation of APCs and their release of IL-12, respectively. Consistent with this notion was the failure of CpG-ODN to enhance the TI anti-PRP response to PRP alone. In a separate study, CpG-ODN was found to actually suppress the antibody

response to intraperitoneal (i.p.) immunization with the purified high-molecular-weight PS component of the *P. aeruginosa* LPS O-specific side chain (143).

In a similar study, mice immunized i.p. with PPS19F and PPS6B conjugated to CRM elicited predominantly IgM and IgG1 anti-PPS19F and IgM anti-PPS6B responses, respectively (27). Coimmunization with CpG-ODN had no effect on the IgG1 anti-PPS19F response but selectively induced IgG2a and IgG3. Likewise, CpG-ODN induced IgG2a and IgG3 anti-PPS6B responses, as well as inducing IgG1. IgG2a and IgG3 anti-CRM responses were also selectively induced by CpG-ODN. CpG-ODN also enhanced protein (i.e., hen egg lysozyme)-specific IgG2a responses, via the induction of Th1 immunity, to the protein antigen alone (28). Thus, CpG-ODN is a potent Th1 adjuvant for anti-PS and anti-carrier responses to conjugate vaccines injected systemically.

CpG-ODN has also been shown to be a strong adjuvant when delivered with conjugate vaccine i.n., enhancing both systemic and local, mucosal Ig responses. Thus, serum PPS9V-specific IgG and IgA titers were enhanced in mice immunized i.n. with PPS9V conjugated to inactivated pneumolysin (Ply) when the vaccine was delivered with CpG-ODN (16). Of interest, the mucosal delivery of CpG-ODN with unconjugated PPS9V also enhanced serum IgG and IgA anti-PPS9V titers. Further, CpG-ODN enhanced serum IgG and IgA anti-PRP responses following i.n. immunization with Hib-CRM, with preferential enhancement of the Th1 isotypes IgG3 and IgG2a (94). Mucosal anti-PRP IgA responses measured in bronchoalveolar lavage fluid and saliva were also induced in the presence, but not the absence, of CpG-ODN, in contrast to responses to a comparable systemic immunization. Systemic and mucosal anti-CRM responses were similarly enhanced by mucosal codelivery of CpG-ODN.

In addition to CpG-ODN, other TLR ligands have proven effective in augmenting anti-PS responses to conjugate vaccines. For example, porin proteins, expressed in the outer membranes of *N. meningitidis* and *Neisseria gonorrhoeae*, are polyclonal B-cell activators that can enhance antigen presentation, proliferation, and Ig secretion by B cells (95, 140, 152, 153) through a TLR2- and MyD88-dependent pathway (95). OMPC, although not diphtheria toxin, also activates macrophages in vivo in a TI manner (5). A conjugate of Hib PRP and OMPC, which contains porin protein, was shown to induce protective anti-PS antibody titers after only one dose following the i.p. injection of mice (80). However, PRP-OMPC was much less immunogenic in TLR2 $^{-/-}$  mice, indicating that OMPC, in addition to recruiting CD4 $^{+}$  T-cell help, acts as an adjuvant via

Table 3 Adjuvants tested in animals

Adjuvant	Reference(s)
TLR ligands	
TLR2	
Porin .....	44
OMPC .....	5, 47, 80, 115
Pam <sub>3</sub> Cys .....	78
MALP-2 .....	129
LT-II .....	52
LT-K63 .....	10, 11, 15, 64, 65, 67, 98, 146
LT-R72 .....	64, 68, 98, 146
TLR4	
Monophosphoryl lipid A .....	148
Ribi adjuvant system .....	155
dLPS .....	9
TLR9	
CpG-ODN delivered .....	27, 84, 92, 93, 132, 149 systemically
CpG-ODN delivered .....	82, 94, 162 mucosally
Non-TLR ligands	
Cholera toxin (Th2; GM1 .....	13, 57, 73, 98, 129, 162 ganglioside)
Aluminum salts (Th2) .....	77, 93, 96, 136
MF59 .....	46
Saponin type (Th1): QuilA .....	36, 84, 148
QS-21 .....	78, 119
IL-12 (Th1) .....	18, 90, 126
RhinoVax .....	68

TLR2 signaling. Splenocytes from OMPC-immunized TLR2<sup>-/-</sup> mice also produced significantly less IL-6 and tumor necrosis factor alpha than those from wild-type mice. This finding is of interest in light of the observation that Hib conjugated to other non-TLR ligand carrier proteins, in contrast to PRP-OMPC, requires multiple doses to induce protective Ig (38, 45). The enhanced anti-PRP response to PRP-OMPC relative to that to PRP-T was largely due to an increase in IgG2a and IgG3 anti-PRP (115), indicating that, like CpG-ODN (TLR9 ligand), OMPC (TLR2 ligand) promotes Th1 immunity in response to conjugate vaccines. In a recent study, OMPC induced CD86, CD80, and CD40 costimulatory molecules on human neonatal and murine B cells and Th1 cytokines (115). Porins do not fully account for the effects of OMPC observed in the latter study. Similarly, PRP conjugated to a recombinant class 3 porin of *N. meningitidis*, serogroup B elicited anti-PRP responses in rats that were 1 to 2 orders of magnitude greater than those induced by PRP conjugated to a number of other non-TLR ligand carrier proteins (44). Additional TLR2 ligands, such as Pam<sub>3</sub>Cys (78) and MALP-2 (129), have also been used experimentally as adjuvants in conjugate vaccines.

Similar to CpG-ODN and OMPC or porin, monophosphoryl lipid A, acting as an adjuvant through TLR4, also enhanced anti-PS responses to conjugate via a Th1 pathway. Thus, PPS14 conjugated to BSA in the presence of the block copolymer adjuvant L121 elicits a predominantly IgG1 (Th2) anti-PPS14 response (148). The addition of monophosphoryl lipid A to PPS14-BSA-L121 resulted in a reduced IgG1 response but enhanced IgG3, IgG2b, and IgG2a responses.

Collectively, these studies suggest that TLR (TLR2, TLR4, and TLR9) ligands used as adjuvants for conjugate vaccines favor class switching to the Th1 IgG isotypes (IgG3, IgG2b, and/or IgG2a). Of interest in this regard, a recent report demonstrated the presence of a contaminating TLR2 ligand(s) in the pneumococcal conjugate vaccine Prevnar (133). TLR2<sup>-/-</sup> mice immunized with Prevnar had a significant reduction in the PS-specific IgG response relative to that of wild-type mice, especially in the case of the Th1 isotypes, IgG3 and IgG2a.

## TH1 VERSUS TH2 ANTIBODY ISOTYPES AND GLYCOCOCONJUGATE VACCINE RESPONSES

In addition to TLR2, TLR4, and TLR9 ligands (Th1) and cholera toxin and L121 (Th2), other predominantly Th1- and Th2-inducing adjuvants have been used in

combination with conjugate vaccines to enhance serum anti-PS IgG titers relative to those observed using conjugate alone (Table 3). This variety raises the interesting question of whether PS-specific Th1 and Th2 isotypes elicited in response to conjugate vaccines differ in their protective capacity. The immunization of mice subcutaneously with a trisaccharide derived from the capsular PS of *S. pneumoniae* serotype 3 conjugated to CRM in the presence of a number of different adjuvants elicited predominantly either Th1 (IgG3, IgG2a, and IgG2b, in the case of QuilA and CpG-ODN) or Th2 (IgG1, in the case of monophosphoryl lipid A and alum) anti-PPS3 isotypes (84). The phagocytic capacity of the elicited antibody, a predictor of protection, was correlated most strongly with Th1-associated IgG2a and IgG2b levels and, to a lesser extent, with Th2-associated IgG1. Similar data were obtained for mice immunized subcutaneously with detoxified LPS (dLPS) from *V. cholerae* linked to a glucan matrix and conjugated to human serum albumin as a carrier protein (112). Anti-dLPS-dependent in vitro vibriocidal activity more strongly correlated with the Th1 isotype IgG2b than the Th2 isotype, IgG1. Of interest, IgA anti-dLPS showed the strongest correlation. Finally, mice immunized i.n. with PPS3-CRM in the presence of the Th1-inducing cytokine IL-12 exhibited higher systemic IgM, IgG2a, and IgG3, but not IgG1, anti-PPS3 responses than mice immunized with PPS3-CRM alone (90). Although total IgG anti-PPS titers were similar between the two groups, sera from the IL-12-treated mice exhibited more efficient in vitro opsonophagocytosis of homologous *S. pneumoniae* bacteria, likely due to the induction of IgG2a. IL-12-treated mice also had higher rates of survival of systemic infection following i.p. challenge with *S. pneumoniae*. The findings of these limited studies suggest that the induction of PS-specific IgG by a conjugate in a Th1 cytokine milieu may result in the production of a qualitatively more protective antibody at any given titer. This idea is consistent with in vitro data demonstrating that IgG2a and IgG2b bind relatively better to Fc<sub>y</sub> receptors (120) and, together with IgG3, fix complement better than IgG1 (108), both processes important in opsonophagocytosis. Nevertheless, IgG1 can also confer protection, albeit less efficiently.

## MUCOSAL DELIVERY OF GLYCOCOCONJUGATE VACCINES

The delivery of a conjugate in the presence of a suitable mucosal adjuvant has the potential advantage of inducing protective mucosal immunity, in addition to systemic immunity. Thus, in addition to cholera toxin and

CpG-ODN, other mucosal adjuvants have been shown to be effective for conjugate vaccines in mice, including the Th1-inducing cytokine IL-12, variants of the heat-labile enterotoxin (LT) of *E. coli* (LT-K63 and LT-R72), and RhinoVax (caprylic-capric glycerides dissolved in polysorbate 20-water) (67). TT-conjugated PPS1 delivered to mice i.n. induced strong systemic IgG and IgA and mucosal IgA anti-PPS1 responses in the presence, but not the absence, of the mucosal adjuvant RhinoVax (67) or a nontoxic mutant of *E. coli* LT (LT-K63 or LT-R72) (67). Immunized mice were protected against both bacteremia and pneumonia after i.n. challenge with a lethal dose of *S. pneumoniae* capsular type 1. The survival of mice correlated with serum IgG anti-PPS1 titers. Mice inoculated i.n. with PPS3-CRM in the presence, but not the absence, of IL-12 not only elicited better systemic immunity against i.p. challenge with *S. pneumoniae* but also induced mucosal IgA in bronchoalveolar lavage fluid (90). As demonstrated using IgA<sup>-/-</sup> mice, this IL-12-induced IgA afforded protection against pneumococcal colonization of the upper respiratory tract. In contrast, intramuscular immunization in the presence of IL-12 had only minimal effects on pneumococcal carriage. In an analogous study, the immunization of neonatal mice i.n. with PPS14-CRM in the presence, but not the absence, of IL-12 resulted in the induction of mucosal (middle ear) and enhanced serum anti-PPS14 IgA, IgG1, IgG2a, IgG3, and IgM titers (126). The Ig-inducing effect of IL-12 was IFN-γ-dependent, as demonstrated using IFN-γ<sup>-/-</sup> mice, consistent with previous in vitro data showing a differential effect of IFN-γ directly on activated B cells (139). The induction of Ig in the middle ear following i.n. immunization with PPS14-CRM in the presence, but not the absence, of IL-12 resulted in enhanced clearance of homologous *S. pneumoniae* bacteria from the middle ear, a reduction in the incidence of experimental otitis media, and increased survival after otitis media-induced invasive pneumococcal infection (126).

Collectively, these and other (32, 58, 94, 125, 129, 146, 162) data illustrate that mucosal immunization via the i.n. route can elicit strong systemic and mucosal immunity. Effective i.n. immunization appears to require a strong mucosal adjuvant, of which there are several candidates potentially suitable for human use (e.g., CpG-ODN, LT, and RhinoVax). The induction of mucosal immunity can decrease colonization in the upper respiratory tract and the middle ear and, in so doing, prevent both local infection and invasive disease. Mucosal IgA may play a critical role in this protective response. Of interest, a single study suggests that, through the use of microparticles, it may also be possible to induce sys-

temic PS-specific IgG in response to conjugate vaccine delivered orally, although the induction of mucosal IgA was not observed in this study (43).

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Margaret K. Hostetter

7

# Interactions of *Streptococcus pneumoniae* with Complement Proteins

At the present time, the complement system includes approximately 20 soluble serum proteins and 10 cellular receptors (Table 1) that form the first line of innate immune defense against the pneumococcus. Complement proteins interact both with other components of innate immunity, such as Toll-like receptors 1, 2, 4, 6, and 9 (10, 46, 53, 64, 68, 80), and with components of the adaptive arm of the immune system (64). Thus, the complement proteins—and, most particularly, the third component of complement (C3)—serve as the linchpin that unites broad surveillance, acute microbicidal activities, and long-term protection against invasion or reinfection.

More than a century ago, Wright and Douglas were the first to prove that serum components are essential for the phagocytosis of virulent pneumococci (78). Ward and Enders distinguished the roles of heat-labile (complement) and heat-stable (antibody) serum proteins by demonstrating that the addition of fresh human serum to anticapsular antibodies accelerated the phago-

cytosis of a serotype 3 organism more than sevenfold (74). These experiments sparked continuing investigations into the role of the complement proteins in the opsonization, phagocytosis, and killing of *Streptococcus pneumoniae*. Since these processes are critical to innate immune defense against invasive pneumococcal infection, it is perhaps not surprising that *S. pneumoniae* has developed a number of mechanisms to subvert them.

Broadly, these activities involve at least four distinct processes: (i) the degradation of soluble complement proteins, (ii) interference with complement activation, (iii) inhibition of the deposition of opsonic C3 ligands, and (iv) perturbation of ligand-receptor interactions involved in phagocytosis. This chapter will briefly review some salient features of complement-mediated opsono-phagocytosis, discuss the inhibitory mechanisms attributed to specific pneumococcal proteins and polysaccharides, and review those human immunodeficiencies of particular relevance to complement-mediated killing of *S. pneumoniae*.

**Table 1** Role of CRs in pneumococcal pathogenesis

CR	CD protein(s)	Cell type in which CR is expressed	Ligand(s)	Function(s)
CR1	CD35	Phagocytes	C3b	Attachment
CR2	CD21	B lymphocytes	C3d, C3dg	B-cell activation and augmentation of antibody response
CR3	CD11b/CD18	Phagocytes	iC3b	Phagocytosis and killing
CR4	CD11c/CD18	Phagocytes	iC3b	Phagocytosis and killing
CR Ig		Tissue macrophages	C3b, iC3b	Phagocytosis

## COMPLEMENT PATHWAYS

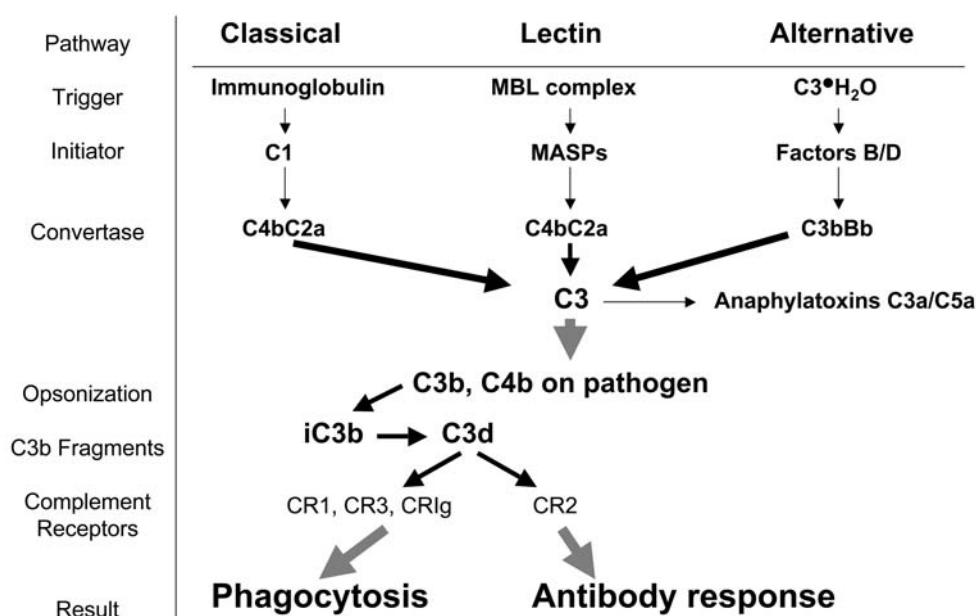
As shown in Fig. 1, three complement pathways are currently recognized:

- The classical complement pathway, activated by immunoglobulin complexes and by antibody-independent mechanisms such as SIGN-R1,
- The lectin complement pathway, initiated by the binding of mannose-binding lectin (MBL), in conjunction with MBL-associated serum proteinase1 (MASP-1), MASP-2, or MASP-3, to specific carbohydrates on the bacterial surface, and
- The alternative complement pathway, triggered by the binding of hydrolyzed C3 ( $C3 \cdot H_2O$ ) to factor B.

Complement activation by each pathway generates a specific C3 convertase (Fig. 1) that cleaves soluble C3

into two fragments: the anaphylatoxin C3a ( $\sim 10$  kDa) and the opsonic fragment C3b ( $\sim 175$  kDa). As a result of this cleavage event, C3b undergoes a conformational change that exposes the components of a thioester bond: a sulphydryl residue ( $Cys_{688}$ ) and a reactive carbonyl group on a glutamyl residue just 3 amino acids distant ( $Glu_{691}$ ) (70, 71). The conservation of the thioester heptapeptide in invertebrates and humans indicates its central importance in innate immunity (48).

The conformational change initiated by the cleavage of the C3a moiety is quite dramatic. The crystal structure at 4-Å resolution shows marked structural rearrangements that shift atomic positions up to 95 Å. In particular, conformational change in an amino-terminal segment of the C3  $\alpha$ -chain (residues 727 to 745) exposes four acidic residues that are important for the



**Figure 1** Diagram of the three pathways of complement activation. Each convertase cleaves circulating C3 and results in the deposition of C3b onto the pathogen. Interaction with factors H and I cleaves C3b to iC3b, C3c, and C3d. Serving as ligands for complement receptors on leukocytes, iC3b and C3d mediate phagocytic killing and antibody response, respectively. Adapted from *Cell* (64) with permission of the publisher.

binding of factor B. Binding sites for properdin, factor H, and complement receptor 1 (CR1), CR2, and CR3 are also exposed on C3b but not on native C3 (36). Interestingly, the binding site for factor B overlaps with those for factor H and CR1, sterically hindering the ability of the latter two proteins to dissociate the C3bBb complex and serve as cofactors for the proteolytic degradation of C3b into iC3b and thence into C3d (36).

In the 60 to 100  $\mu$ s of its half-life, the reactive glutamyl residue binds covalently to free hydroxyl or amino groups in an ester or amide linkage, respectively (21, 33, 50, 69). Amino groups of pneumococcal proteins and free hydroxyl or amino groups on pneumococcal polysaccharides can be *trans*-acylated by the reactive glutamyl (32). In the absence of surface hydroxyl or amino groups, the thioester is hydrolyzed, generating  $C_3 \cdot H_2O$  and rendering the protein incapable of further opsonic activity.

### Classical Pathway

Traditionally, the binding of complement protein C1q to the Fc portion of either pentavalent immunoglobulin M (IgM) or IgG initiates the classical complement pathway (Fig. 1). The triggering antibodies typically recognize either capsular polysaccharides or surface proteins of *S. pneumoniae*; IgG molecules recognizing surface polysaccharides are thought to be somewhat more efficient in mediating phagocytosis (13). The binding of C1q initiates a series of proteolytic events that culminate in the generation of the classical pathway C3 convertase, C4bC2a.

The direct binding of C1q by SIGN-R1, a cell surface C-type lectin receptor expressed on marginal-zone macrophages in the murine spleen (25, 42–44, 49), represents a novel, antibody-independent mechanism to activate the classical pathway (42). SIGN-R1 mediates splenic uptake of purified capsular polysaccharides from serotypes 3 and 14 (43). SIGN-R1<sup>−/−</sup> mice have a two-fold increase in mortality after intraperitoneal injection with serotype 2 or 14 (49) and show corresponding deficits in the proteolysis of C3 and the opsonic deposition of C3b onto the pneumococcal surface compared to their wild-type counterparts (42). If this pathway exists in humans, the activity of SIGN-R1 in splenic clearance of *S. pneumoniae* may explain the association between asplenia and fulminant pneumococcal bacteremia.

### Lectin Pathway

The MBL complex, composed of three to six subunits of MBL, preferentially recognizes mannose, mannans, and N-acetylglucosamine (GlcNAc). The binding of the MBL complex to these surface components activates MASP-1,

MASP-2, and MASP-3, which cleave C4 to C4b, thereby generating the lectin pathway C3 convertase, C4bC2a (27, 59). The covalent deposition of C3b and the subsequent degradation of C3b into iC3b, C3dg, or C3d are thought to recapitulate the effects of the classical complement pathway.

### Alternative Complement Pathway

Since it does not require the presence of antibodies and is operative even in invertebrates, the alternative complement pathway is thought to be the most primitive of opsonic mechanisms, although some advocate for the lectin pathway (20, 82). The integrity of the alternative complement pathway is thought to be critical for those hosts lacking type-specific anticapsular antibodies or proteins required for classical pathway activation (e.g., C2). The binding of factor B to hydrolyzed C3 ( $C_3 \cdot H_2O$ ), which is continually produced in serum at very low levels by the hydrolysis of the C3 thioester (58, 77), forms the alternative pathway C3 convertase, which is stabilized by properdin. Stabilization of the alternative pathway convertase generates an “amplification loop,” which partially compensates for the slower kinetics of this pathway (58).

When <sup>3</sup>H-labeled purified human C3 was employed to reconstitute the alternative complement pathway in agammaglobulinemic serum in which C3 and C4 had been inactivated by a nucleophile (32), the covalent deposition of C3b onto both the capsules and the cell walls of encapsulated serotypes occurred. Reconstitution with biochemically inactivated C3 failed to opsonize the identical pneumococcal serotypes, and no phagocytosis occurred. These experiments show that the covalent deposition of C3b is essential for pneumococcal opsonization and phagocytosis in the absence of antibody.

### COMPARATIVE EFFICACY

Comparative studies of pneumococcal bacteremia in C57BL/6 mice genetically deficient in C1q, factor B (encoded by *Bf*), C3, C4, or natural IgM pointed to the importance of the classical complement pathway in the absence of IgG (14). The proportion of *S. pneumoniae* strains found to be positive for C3 by flow cytometry was significantly reduced in C1qa<sup>−/−</sup> and C4<sup>−/−</sup> mice but not in *Bf*<sup>−/−</sup> mice. However, the amount of C3 deposition per bacterium in *Bf*<sup>−/−</sup> mice was significantly reduced compared to that in their wild-type counterparts, suggesting that the classical complement pathway initiated complement activation even in the absence of IgG but that the alternative complement pathway was

primarily responsible for augmenting C3 deposition, presumably through the amplification loop. The use of C1qa<sup>-/-</sup> Bf<sup>-/-</sup> mice showed that only a very small proportion of C3 binding was dependent on the lectin complement pathway.

### COMPLEMENT-MEDIATED PHAGOCYTOSIS

Since the cell wall of *S. pneumoniae* makes the organism resistant to complement-mediated lysis, phagocytosis via leukocyte CRs is a major mechanism of pneumococcal killing. The deposition of C3b onto the capsules of virulent pneumococci (serotype 25) was initially shown by antibody localization (26, 34). Although these early antibodies could not distinguish among the species of C3 deposited (C3b, iC3b, or C3d) and covalent versus noncovalent binding, studies with <sup>125</sup>I-labeled guinea pig C3 demonstrated the binding of C3b to the cell walls of both encapsulated and nonencapsulated pneumococcal strains (34). Subsequent experiments showed that pneumococcal polysaccharides or proteins with exposed hydroxyl or amino groups could also serve as acceptors for C3b (32).

The cleavage of deposited C3b by factor I, a serum protease, generates iC3b, C3dg, and C3d. Each of these fragments remains covalently attached to the organism and serves as a ligand for CRs on leukocytes (Table 1). On phagocytic cells, CRs mediate the attachment of opsonized organisms, engulfment, and killing (66); on B lymphocytes, CRs upregulate cellular activity more than 10,000-fold, in cooperation with costimulatory molecules and B-cell receptor complexes (15, 19).

CR1 (CD35) recognizes C3b and C4b as preferential ligands but is much less effective than CR3 (CD11b/CD18) in promoting pneumococcal phagocytosis (26), presumably because of the absence of a transmembrane domain in CR1. CR2 (CD21) is expressed primarily on B cells and follicular dendritic cells but can also be found on subsets of CD4<sup>+</sup> and CD8<sup>+</sup> peripheral T cells, on activated T cells, and on basophils, mast cells, keratinocytes, and epithelial cells. C3d binding to CR2 results in a 3-log reduction of the B-cell activation threshold (19).

CR3 (CD11b/CD18) and CR4 (CD11c/CD18), expressed on neutrophils, monocytes, and macrophages, recognize iC3b, C3c, and C3dg, as well as a number of other ligands, including fibronectin, intracellular adhesion molecules 1 and 2,  $\beta$ -glucan, and lipopolysaccharide. The degradation of covalently bound C3b into iC3b and C3dg is a result of competition between soluble factors B, H, and I. A sialic acid-rich environment, such as human tissues or erythrocytes, favors the binding of factor H over factor B; the C3-C5 convertase

does not form, and the amplification loop of the alternative complement pathway is halted (54). This reaction is thought to prevent complement activation in the absence of pathogens. Because *S. pneumoniae* strains do not produce sialic acid, factor H serves as the cofactor for the binding of factor I, and the degradation of C3b into iC3b is favored. The ligation of CR3 onto activated neutrophils or monocytes triggers an inflammatory response that encompasses both oxidative and nonoxidative mechanisms of killing in response to pneumococcal phagocytosis (26).

CR Ig, the newest complement receptor, is found on macrophages residing in tissues, including hepatic Kupffer cells, and participates with CR3 in the removal of particles such as sheep erythrocytes opsonized with C3b or iC3b (29). Hepatic clearance of bacteremia due to *Listeria monocytogenes* and *Staphylococcus aureus* is impaired in CR Ig<sup>-/-</sup> mice, even in the presence of CR3. The binding of C3b to CR Ig selectively interferes with the generation of the alternative pathway convertase but not with that of the classical pathway (76).

### PNEUMOCOCCAL PROTEINS INTERFERING WITH COMPLEMENT FUNCTIONS

As might be expected, the pneumococcus has evolved a number of mechanisms to avert complement-mediated opsonization and phagocytosis: (i) the degradation of soluble complement proteins, (ii) interference with complement activation, (iii) inhibition of the deposition of opsonic C3 ligands, and (iv) perturbation of ligand-receptor interactions involved in phagocytosis. For the proteins discussed below, interfering mechanisms are summarized in Table 2.

#### Degradation of Soluble Complement Protein PhpA

Exponentially growing wild-type *S. pneumoniae* strains of serotypes 3, 4, and 14 are able to degrade both  $\alpha$ - and  $\beta$ -chains of purified human C3 in the absence of other serum proteins (6). PhpA, a histidine-rich protein with a molecular mass of 79 kDa, is responsible for cleaving the C3  $\alpha$ -chain (83). The depletion of soluble C3 or the degradation of the  $\alpha$ -chain, the site of the thioester bond, would clearly interfere with the formation of the C3 convertase and the generation of opsonic C3b.

#### Interference with Complement Activation

##### Hic

A surface protein called factor H inhibitor of complement (Hic) impedes the formation of the C3 convertase and accelerates the degradation of C3b into iC3b by

**Table 2** Mechanisms of pneumococcal interference with complement pathways

Mechanism of interference	Proteins interfering with the following process:			
	Classical pathway	Lectin pathway	Alternative pathway	Phagocytosis
Degradation of C3	PhpA	PhpA	PhpA	
Activation/generation of convertase	Ply		Hic, PspC	
Opsonic deposition	PspA, Ply			
Ligands for complement receptors				Hic, PspA/C, Ply, CppA

binding factor H (28, 37, 39, 65). Short consensus repeats 8 to 11 and 12 to 14 on factor H constitute the binding sites for Hic (23, 39); these sites differ from those recognizing heparin or C3b, which do not inhibit the binding of Hic (23). Interestingly, heparin inhibits the binding of factor H to  $\beta$ , a group B streptococcal protein, even though the two microbial proteins have significant identity (38). The phagocytosis of an unencapsulated Hic $^+$  strain is inhibited compared to that of an unencapsulated Hic $^-$  strain (37), although virulence studies with isogenic Hic mutants of encapsulated strains have not been reported.

### PspC

Although Hic and PspC are encoded by the *pspC* locus, PspC (also known as CbpA, SpsA, and PbcA) binds factor H through short consensus repeats 6 to 10, a site separate from that utilized for the binding of secretory IgA by PspC (18). The first 89 amino acids of PspC are required for the binding of factor H (51). PspC also binds C3, C3b, and secretory IgA (16, 28, 65) and elicits interleukin-8 from pulmonary epithelial cells (52). PspC variants differ in both sequence and virulence. In a murine model of pneumonia, the deletion of *pspC* in serotypes 2, 3, and 19F had no effect on host survival while the *pspC* mutant with the TIGR4 background was significantly reduced in virulence (45). Approximately 35% of sequenced PspC variants from clinical strains and laboratory isolates display an LPXTG motif at the C terminus, instead of the choline-binding domain observed in CbpA (35).

### Pneumolysin

The release of pneumolysin (Ply) during autolysis leads to the activation of the classical complement pathway, possibly through the binding of the Fc portion of IgG (55). Soluble pneumolysin may thereby serve as a decoy for C3b deposition, diverting opsonic C3 fragments away from the surface of the organism and ultimately depleting C3. The mutation of Asp<sub>385</sub> to Asn abolishes complement-activating activity but retains the hemoly-

tic effects of a pore-forming toxin; in contrast, the mutation of His<sub>367</sub> to Arg retains complement-activating activity but abolishes approximately 99% of the cytotoxicity (2). In a murine model of bronchopneumonia, the H $^+$ /C $^-$  mutant fails to augment T-lymphocyte infiltration after 48 h, while the H $^-$ /C $^+$  mutant impedes neutrophil migration into the lungs (41).

### Inhibition of Deposition of Opsonic C3 Ligands

#### PspA

In vitro, proteins of the PspA family inhibit the deposition of C3b onto pneumococci, as demonstrated when immunoblotting and enzyme-linked immunosorbent assays are used to measure deposited C3b (1, 72). Surprisingly, a PspA $^-$  mutant was just as virulent as the parent strain in factor B-deficient C57BL/6 mice. Although the initial explanation was that PspA interfered with the formation of the alternative pathway C3 convertase, additional in vitro studies of opsonization in murine serum point to a function for PspA in inhibiting classical pathway-mediated C3 deposition (62, 63).

This mechanism was confirmed by additional in vitro studies with nonimmune mouse serum. C3 (including C3b and iC3b) was readily detectable by flow cytometry on the surface of the PspA $^-$  strain, while the PspA $^+$  strain was resistant to C3 deposition, even in the presence of anticapsular antibodies. Antibodies to PspA restored C3 deposition onto the PspA $^+$  strain (62). PspA and pneumolysin act jointly to interfere with complement deposition mediated by both classical and alternative pathways; a *pspA* *ply* mutant was greatly attenuated in both systemic and pulmonary infections in mice (81).

### Perturbation of Ligand-Receptor Interactions in Phagocytosis

In vitro studies have identified an important role for covalently deposited iC3b as the critical ligand for pneumococcal phagocytosis via CR3 (26, 31). Less than one-third of C3 molecules covalently deposited onto serotypes 3 and 4, resistant to phagocytosis, are iC3b.

In contrast, nearly 100% of C3 molecules deposited onto serotypes 6A and 14, more readily phagocytized, are iC3b (31). Using antibody blockade of CR1, CR2, or CR3 to inhibit the phagocytosis of serotype 3, 6A, or 14, Gordon et al. demonstrated that CR3 is responsible for 80 to 100% of phagocytic uptake of strains bearing iC3b (6A and 14) (26). The ligation of CR3 with iC3b stimulates the production of superoxide, myeloperoxidase, and lactoferrin, but the pneumococcal proteins responsible for these effects have not been identified.

### PspA

PspA<sup>+</sup> strains exhibit considerably less iC3b than PspA<sup>-</sup> mutants. In CR1<sup>-/-</sup>, CR2<sup>-/-</sup>, CR3<sup>-/-</sup>, or CR4<sup>-/-</sup> mice with a C57BL/6 background, challenge with a PspA<sup>+</sup> strain (WU2) causes increased mortality for CR3<sup>-/-</sup> or CR4<sup>-/-</sup> mice while the PspA<sup>-</sup> mutant, JY1119, is virulent only in mice deficient in factor D or CR1 and CR2 (61). From the standpoint of ligand-receptor interactions in complement-mediated phagocytosis, these results indicate that CR3 and CR4, which recognize iC3b as the ligand, play major roles in pneumococcal phagocytosis. The virulence of the PspA<sup>-</sup> mutant suggests that proteins in addition to PspA may interfere with the generation of iC3b.

### CppA

The expression of CppA, a surface protein with a molecular mass of 34 kDa, has a similar role. Isogenic *cppA* mutants with the TIGR4 background have substantially increased amounts of iC3b on their surfaces, are killed far more easily in vitro, and in outbred Swiss Webster mice have a 50% lethal dose that is reduced 100,000-fold. IgG antibodies recognizing CppA can be detected in human serum (V. Vellucci and M. K. Hostetter, unpublished observations), and orthologs exist in *Streptococcus pyogenes* and *Streptococcus agalactiae*. These results suggest that CppA also interferes with the binding of factor I to C3b.

### Interactions of Pneumococcal Polysaccharides with Complement Components

Pneumococcal capsular polysaccharides may influence the deposition of opsonic C3b, iC3b, or C3d. The results of quantitation of covalent C3b binding on the basis of serotype ranged from 29,000 molecules on a serotype 3 strain to 155,000 molecules on a serotype 4 strain (31). Patterns of factor-I-mediated proteolysis of C3b into iC3b or C3d also differed by serotype. Serotypes 3 and 4, resistant to phagocytic killing but potentially immunogenic, exhibit covalently bound C3d, while isolates of serotypes 6A and 14, more easily

killed, degrade C3b into iC3b but not into C3d (31). These differing patterns suggest that interactions of opsonically deposited C3b, iC3b, or C3d influence leukocyte recognition. Moreover, the interaction of C3d with CR2 in activating B lymphocytes may provide a partial explanation for the vigorous antibody response to these two capsular serotypes (9).

### IMMUNODEFICIENCIES THAT PREDISPOSE TO PNEUMOCOCCAL INFECTION

Additional evidence that complement-mediated opsonization and phagocytosis are essential to pneumococcal killing is provided by animal and human models of complement deficiency states that are associated with an increased risk of pneumococcal disease.

### Immunodeficiencies Affecting All Three Pathways of Complement Activation

C3 sits at the conjunction of the classical, lectin, and alternative pathways (Fig. 1). Both biochemical and genetic depletion of C3 augments the host's susceptibility to invasive pneumococcal infection. Guinea pigs depleted of C3 by cobra venom factor (CVF), a C3b homolog that amplifies the formation of the C3b convertase and thereby depletes circulating C3, localized approximately half as many <sup>125</sup>I-labeled pneumococci to the liver as normal guinea pigs, while CVF-treated animals had more than twice as many pneumococci in the spleen. As splenic sequestration increased, the number of pneumococci remaining in the bloodstream also increased, whereas the level of hepatic sequestration was inversely correlated with the amount of pneumococci remaining in the bloodstream (11, 13). This pattern suggests that hepatic factors such as CRIg on Kupffer cells may provide protection by enhancing phagocytosis.

Mice depleted of C3 with CVF fail to eliminate pneumococci from the lungs and develop pneumococcal pneumonia and consequent bacteremia (56). C3- or C4-deficient guinea pigs (7, 11, 12, 30) and C3-deficient beagles (5) display increased susceptibility to invasive pneumococcal disease. The challenge of mice made deficient in C3 by targeting the C3 promoter results in a 2,000-fold increase in the incidence of pneumococcal bacteremia compared to that in wild-type mice (17). The C57BL/6 background, which serves as the strain for targeted deletions of C3, factor B or D, and virtually all CRs, may introduce some confounding variables for assessing the relative contributions of innate versus adaptive immunity. In a comparison of the susceptibilities of C57BL/6 (resistant) and A/J (susceptible) mice to *Can-*

*dida albicans*, genome-wide linkage analysis identified the C5 locus and showed additional associations with levels of cytokines tumor necrosis factor alpha, gamma interferon, and interleukin-6 (73).

Humans genetically deficient in C3 have recurrent pyogenic infections ascribable to *S. pneumoniae*, *Streptococcus pyogenes*, and *Staphylococcus aureus*; these manifest either as recurrent bacteremias after 2 years of age or as repeated episodes of erysipelas in adulthood (3, 8). Patients with an inherited factor I deficiency are functionally C3 deficient, because the persistence of C3b generates an unending amplification loop; these patients have pneumococcal and meningococcal infections in childhood and recurrent erysipelas due to *Streptococcus pyogenes* as adults (84).

### Immunodeficiencies Affecting the Classical Pathway

Impaired function of the classical complement pathway in patients homozygous for C2 deficiency, the most common deficiency in Western countries, carries an increased risk for bacterial infections, especially in homozygous C2-deficient subjects who have significantly lower levels of IgG4 and IgA (4). In a Swedish study, homozygosity for the G2M<sup>n</sup> allele, which stimulates antibody responses to polysaccharide antigens, was significantly associated with the absence of severe infections in C2-deficient patients (40). In contrast, the combination of MBL mutations (corresponding to codon 52, 54, or 57; see below) and C2 deficiency exerted a minor effect on increased susceptibility to septicemia or meningitis caused by *S. pneumoniae*.

### Mutations Affecting the Lectin Pathway

The MBL gene encodes a homotrimeric protein with a carbohydrate recognition domain and a collagenous tail; mannose, mannans, and GlcNAc are the preferred ligands (24). Mutations in codon 52, 54, or 57 interfere with the formation of a triple helix in the collagenous tail, which disrupts polymerization and makes the protein susceptible to proteolytic degradation. Persons heterozygous for these codon mutations have a 20% reduction in the concentration of MBL in serum, while the protein is virtually absent (<2% of normal) in homozygotes. A G-to-C change at -221 of the promoter sequence leads to a smaller reduction in concentrations of MBL in patient serum.

A recent case control study found a 2.5-fold increase in the risk for invasive pneumococcal disease in study participants homozygous for mutations in codon 52, 54, or 57 (67). Twelve percent of 229 patients with invasive pneumococcal disease were homozygous for these

mutations, as compared to only 5% of 353 controls ( $P < 0.002$ ); the results were identical for another 787 subjects. Invasive infections with *S. pneumoniae* serotype 14 were overrepresented among the homozygotes. The type 14 pneumococcal polysaccharide has a linear backbone composed of N-acetylglucosamine, with D-galactose and D-glucose as linked monosaccharide side chains (22, 75). The impaired recognition of GlcNAc has clear clinical consequences for patients homozygous for MBL mutations. Although another study found no increased risk for subjects with MBL polymorphisms (47), the different conclusions may relate to the very low prevalence of homozygous MBL mutations in the latter study or to the broader definition of invasive pneumococcal disease (pneumonia, bacteremia, and meningitis) in the former.

### Immunodeficiencies Affecting the Alternative Pathway

Although sera from factor D-deficient mice generated by gene targeting are greatly handicapped in the kinetics of pneumococcal opsonization (79), patients with inherited deficiencies of factor B or D of the alternative complement pathway seem to have no specific predisposition to pneumococcal disease unless there is an accompanying deficiency of C2 (57, 60).

## CONCLUSION

More than a century of experimental evidence has pointed to the primacy of the complement pathways in defense against invasive pneumococcal disease. At least six pneumococcal proteins—pneumolysin, PhpA, Hic, PspC (CbpA/SpsA/PbcA), PspA, and CppA—are now known to interfere with complement-mediated opsonization and phagocytic killing. Expanded-spectrum pneumococcal vaccines containing some combination of these proteins may avert their potent inhibitory effects.

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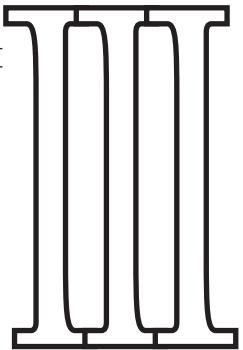
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# *Clinical Disease and Epidemiology*

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Shabir A. Madhi  
Stephen I. Pelton

# 8

## Epidemiology, Diagnosis, and Treatment of Serious Pneumococcal Infections in Children

Invasive pneumococcal disease (IPD) in children persists as a major cause of morbidity and mortality throughout the world, despite the introduction of antimicrobial therapy. Nasopharyngeal colonization on at least one occasion is nearly universal, yet only a small proportion of children develop invasive disease, primarily those less than 3 years of age or those with comorbid illnesses.

The variation in incidence rates for IPD among children across the globe has not been completely defined. Greenwood reported the incidence of IPD in children under 2 years of age to vary from less than 100 cases/100,000 children in Finland to more than 1,000/100,000 in Australian Aboriginal children (Fig. 1). Infection with human immunodeficiency virus (HIV) and malnutrition explain, in part, the high rates of disease observed in developing countries (61, 95, 101). Incidence rates are also higher in indigenous children residing in developed countries and African-American children in the United States (26, 36). However, the majority of cases occur in healthy infants and toddlers, representing an immunologically naïve population susceptible to bloodstream invasion or direct invasion of

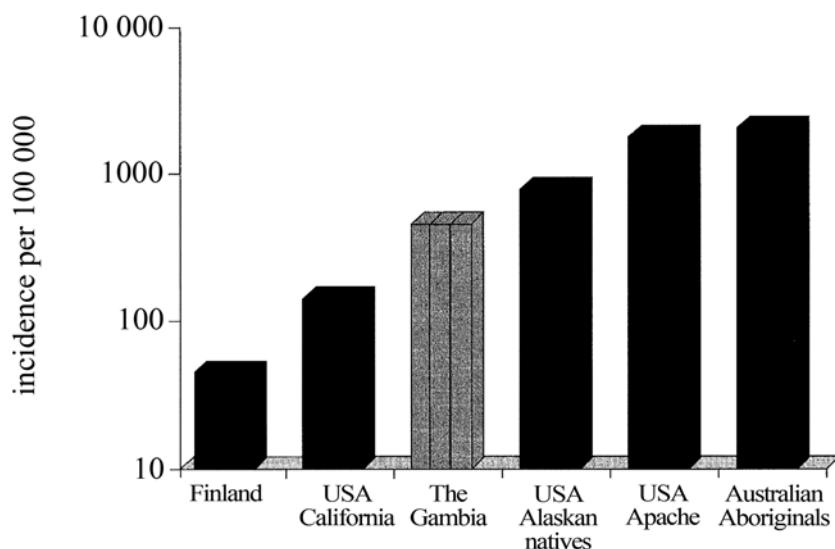
the lower respiratory tract. Colonization rates are also highest in children, peaking in children at approximately 3 years of age and representing an essential part of disease pathogenesis. Following colonization, invasive disease may result from dissemination from a respiratory focus, such as in the case of acute otitis media, sinusitis, or pneumonia, or from dissemination from an unidentified focus to the central nervous system, pleural space, periorbital tissue, bone, or joint. The major clinical syndromes are reflective of the pathogenesis as either (i) bacteremia with or without focal complications or (ii) contiguous spread from the nasopharynx to mucosal surfaces of the lung and middle ear, resulting in pneumonia and acute otitis media, respectively.

### BACTEREMIA

Pneumococcal bacteremia presents with a broad spectrum of clinical manifestations suggesting various diagnoses. Laupland et al. described 254 cases of IPD in Canadian children (84). Bacteremia without a focus, defined by a positive blood culture and no obvious focus

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Shabir A. Madhi, Respiratory and Meningeal Pathogens Research Unit, Medical Research Council/University of the Witwatersrand, and Dept. of Science and Technology/National Research Foundation: Vaccine Preventable Diseases, Bertscham, Gauteng, South Africa. Stephen I. Pelton, Pediatrics and Epidemiology, Boston University Schools of Medicine and Public Health, Maxwell Finland Laboratory for Infectious Diseases, Boston, MA 02118.



**Figure 1** Incidence of IPD in various high- and low-risk populations. Data are from reference 61.

of infection after clinical and laboratory evaluation, was identified in 179 (70.5%) of these cases at an Alberta hospital. Forty percent of those admitted to the hospital and 89% of those not admitted to the hospital did not have a focus of infection. Twenty-nine percent of children had focal infection; 8% had meningitis, almost 10% pneumonia, 5.5% had periorbital cellulitis, and 6% had other clinical syndromes. Few cases were identified in infants in the first 90 days of life, and the majority occurred in children between 3 and 36 months of age. Comorbid illness, primarily cardiac, respiratory, and neurologic conditions, was present in 21.6% of hospitalized children compared to 3.8% of those treated as outpatients. The overall mortality was low, 0.7%.

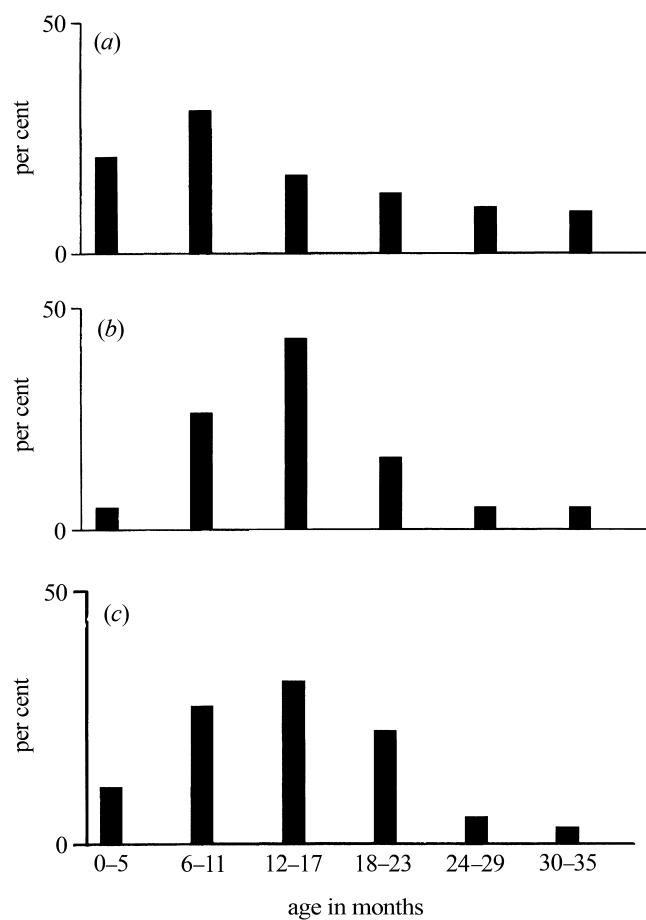
In Chile, Levine et al. described 110 cases of IPD in children of no more than 59 months of age (88). The incidence rate in Santiago was 90 cases per  $10^5$  infants, falling to 18.5 per  $10^5$  toddlers aged 12 through 23 months. Forty-four percent of children had pneumonia, which was complicated by empyema in approximately 25% of cases. Meningitis was present in 32% of all cases. Twenty-two percent of children had underlying chronic illness, and 13 (11.8%) succumbed to their illness. These studies may underestimate the incidence of IPD, as blood cultures are not routinely performed for febrile children in Chile. In one small study of 209 Chilean children 3 to 36 months old with fever of  $\geq 39^\circ\text{C}$  and no focus of infection, 1.8% had invasive pneumococcal infection (88).

In Kenya, Berkley et al. reported *Streptococcus pneumoniae* as the most common bacterial pathogen in chil-

dren under 13 years of age (14). IPD rates peaked in children less than 1 year of age (at 241 cases/100,000) and persisted at rates greater than 100 per 100,000 among children up to the age of 5 years. In The Gambia, pneumonia was the most common clinical presentation and, combined with meningitis, accounted for 95% of cases (108). One of five children with bacteremic pneumonia died, often on the first day of hospitalization. The contrasts between IPD in Canada, Chile, and Africa capture some of the global variation in the epidemiology of bacteremia that is determined partly by differences in disease susceptibility, as well as differences in the diagnosis and treatment of children. Figure 2 details the age distributions in The Gambia and in high- and low-risk communities in the United States. Onset early in life, high rates of severe clinical infections, and substantial morbidity and mortality characterize the disease in the developing world. In contrast, in developed countries, the incidence peaks in children between 6 and 18 months of age and bacteremia without focal infection is the most common clinical syndrome. Overall mortality in developed countries is low compared to that in developing countries, even for cases involving bacterial meningitis.

## OCCULT BACTEREMIA

Unsuspected, or occult, bacteremia in children 3 to 36 months of age has been reported in up to 4% of febrile infants and toddlers without evidence of toxicity or focal infection. In countries where blood cultures are per-



**Figure 2** Distribution of ages of infants and toddlers in The Gambia (1996) (a), among the U.S. White Mountain Apache population (1992) (b), and among generally healthy children in California (1996) (61) (c) at the onset of IPD.

formed for febrile children with mild or moderate toxicity, bacteremia has been identified in studies in emergency or walk-in clinics as well as office practice settings (19). The distribution of bacterial pathogens varies depending on the penetration of *Haemophilus influenzae* type b (Hib) conjugate vaccines. In the United States, *S. pneumoniae* caused 50 to 60% of cases prior to the introduction of Hib conjugate vaccines, and this level increased to 80 to 90% following universal immunization with Hib (7, 79, 158). An age less than 24 months and a temperature greater than 39°C are the features that characterize the majority of children with pneumococcal bacteremia in U.S. studies. Disease is less common in children less than 6 months of age than in children 6 to 24 months of age. Clinically, the risk of bacteremia increases with increasing temperature, with those with temperatures greater than 40°C being at

highest risk. Subtle clinical differences in alertness, color, quality of cry, hydration, and responsiveness of bacteremic children have been described (96, 158). Clinical diagnoses for children with occult bacteremia have included viral syndromes, fever without a source, respiratory distress or asthma, pharyngitis, gastroenteritis, conjunctivitis, and sinusitis (158).

Laboratory studies reveal that elevated white-blood-cell or absolute neutrophil counts and an elevated erythrocyte sedimentation rate are associated with an increased risk of pneumococcal bacteremia, but all these factors have poor predictive values (although their negative predictive value is substantial at 99.5%). The natural history of occult bacteremia (in untreated U.S. children) demonstrates spontaneous clearance in approximately one-third of cases and persistent signs or symptoms of illness in two-thirds (79). However, even among those with persistent clinical illness, most do not continue to be bacteremic. A temperature greater than 39°C at follow-up is the best correlate of whether a given child is likely to have persistent bacteremia or a new focus of infection. Complications such as meningitis, pneumonia, cellulitis, or periorbital cellulitis are most common. Children in whom a lumbar puncture was performed in the initial investigation for bacteremia had a fivefold-greater risk of having meningitis at follow-up (158). The role of antibiotics in the initial management of the febrile child at risk for bacteremia for the prevention of serious focal complications is controversial. Bachur and Harper reported higher rates of persistent clinical illness, persistent bacteremia, and focal infection in those not treated at the visit during which the blood culture sample was drawn than in children receiving oral or parenteral therapy at the initial visit (12). They also recognized that only one of eight patients with meningitis at follow-up returned prior to notification of a positive blood culture, suggesting that the practice of collecting a blood culture sample at the initial visit permits the identification of at-risk patients prior to the clinical progression of disease in some children.

Whether a comparable syndrome exists in developing countries is unclear, largely because of differences in clinical practices due to resource limitations on performing blood cultures for ambulant febrile children. The syndrome of pneumococcal bacteremia without a focus accounts for 61 to 70% of IPD cases in the United States (163); however, a low proportion of IPD cases described in other industrialized (18 to 20%) or industrializing (as low as 1%) countries are characterized as IPD without a focus (85, 108, 134). Consequently, the proportion of IPD cases presenting as meningitis is relatively low (16%) for children in the United States (76)

compared to that observed for children in Europe (32 to 37%) (25), Australasia (32%), and Asia (19%) (64, 161).

Interestingly, some African countries also report a low proportion of IPD cases presenting as meningitis (4% in Mozambique and 16% in The Gambia) (108, 122). This low incidence is likely due to the overwhelming burden of pneumonia in these countries compared to that in industrialized or industrializing countries. Additionally, differences in the severity of illness among cases of bacteremia without a focus in the United States and those in Bangkok, Thailand, have been described. In the United States, some proportion of IPD patients recover even in the absence of antibiotic therapy and there is minimal mortality (71, 89, 142). Conversely, in a hospital-based study in Bangkok, IPD without a focus was associated with 23% mortality, and mortality was especially high among children with comorbid conditions (138). These reports suggest that the syndrome, IPD without a focus, was different in severity and associated complications in Thailand from the syndrome described in the United States. The large proportion of cases involving pneumonia or meningitis, the high death rates during the initial few days of hospitalization, and the earlier age of onset of IPD in developing countries may suggest that host factors such as nutritional status, comorbid conditions, or genetic factors result in rapid progression when pneumococcal bacteremia occurs rather than the slower progression and relatively high rate of spontaneous resolution observed in U.S. children.

## CHANGING EPIDEMIOLOGY OF BACTEREMIA IN THE UNITED STATES AFTER THE INTRODUCTION OF PNEUMOCOCCAL CONJUGATE VACCINES (PCVs)

Herz and colleagues at the Northern California Kaiser Permanente reported that compared with the period from 1998 to 1999, 41.5, 78.3, 91.5, and 84.9% declines in the incidence of IPD were observed during the subsequent 4 years (66). *S. pneumoniae* caused 78% of all bacteremic episodes in 1998 and 1999, but the proportion had declined to 33% by 2002 and 2003.

## PNEUMOCOCCAL MENINGITIS

### Diagnosis and Epidemiology

Although pneumococcal meningitis is one of the less common presentations of IPD (Table 1), it is associated with disproportionately high case fatality rates (CFRs; 6

to 54%) and neurological sequela-related morbidity among survivors (0 to 50%) compared to other manifestations of IPD (61, 114). Furthermore, there appears to be greater consistency in the diagnosis of bacterial meningitis and the detailing of the bacterial etiology than in cases of bacteremia in industrialized and industrializing countries, with the “gold standard” being the identification of abnormalities in cerebrospinal fluid revealed by lumbar puncture. Significant predictors of bacterial meningitis include lethargy or unconsciousness (odds ratio [OR], 5.2; 95% confidence interval [CI], 2.1 to 13), neck stiffness (OR, 29.3; 95% CI, 12.2 to 70.3), a bulging fontanel (OR, 32; 95% CI, 1.2 to 8.5), and reduced feeding (OR, 2.9; 95% CI, 1.3 to 6.7) (155). Using these criteria as proposed by the Integrated Management of Childhood Illnesses guidelines, a sensitivity of 92% and a specificity of 72% for predicting meningitis can be achieved. The examination of cerebrospinal fluid for the presence of bacteria by microscopy, culture, and occasionally antigen detection assays using latex and PCR assays provides an etiological diagnosis in the majority of cases of bacterial meningitis. Additionally, the microscopy and culture of cerebrospinal fluid in cases of bacterial meningitis demonstrate superior sensitivity (70 to 80%) compared to that of blood cultures (5 to 20%) in diagnosing pneumococcal pneumonia (8, 61). Establishing a specific bacterial etiology is further enhanced by antigen detection and PCR assays, especially when preceding antibiotic use has occurred and when other tests are negative (24, 61, 126).

Despite the high sensitivity of cerebrospinal fluid culture for making a specific etiological diagnosis of bacterial meningitis, there remains a paucity of information regarding the burden of pneumococcal meningitis in many parts of the world. The role of *S. pneumoniae* compared to that of other bacterial pathogens, especially *H. influenzae* and *Neisseria meningitidis*, has been reported in numerous cross-sectional studies globally. There are, however, very few population-based studies, especially from industrializing countries, that detail the burden of pneumococcal meningitis in children. This information is pertinent since even if *S. pneumoniae* is found to contribute to a relatively small proportion of meningitis cases, as an example, during epidemics of *N. meningitidis* infection in countries which are part of the “meningitis belt,” the absolute burden of disease being caused by *S. pneumoniae* in such countries may be underappreciated.

In a review by Peltola et al. prior to the introduction of a Hib conjugate vaccine into Africa, *H. influenzae* was identified as the most common cause of bacterial meningitis in children. Nevertheless, *S. pneumoniae* was

**Table 1** Selected studies demonstrating the clinical presentation of IPD in children

Reference	Country or region (type of area or population)	No. of cases of:			
		Bacteremic pneumonia	Meningitis	Bacteremia without a focus <sup>a</sup>	Other presentation of IPD
76	United States (children <2 yrs)	13	16	61	10
76	United States (children 2–12 yrs)	30	10	49	11
70	United Kingdom	31	32	30	7
148	Belgium	30	13	53	2
48	Finland	15	11	69	6
85	France	60	10	20	8
140	Greece	53	13	32	
134	United Kingdom	19	37	18	
33	Israel	39	17	37	3
38	Australia	29	34	30	7
36	United States (Alaska Natives <2 yrs old)	47	13	32	8
108	The Gambia (rural)	77	16	1	6
122	Mozambique	69	4		
95	South Africa (HIV uninfected)	50	28	0	12
	South Africa (HIV infected)	62	33	0	5
138	Bangkok, Thailand	28	26	37	1
28	South Korea	16	19	45	17 (peritonitis)
88	Chile	45	32		
41	Latin America	42	39	8	7
77	Latin America	50	33	11	6

<sup>a</sup>Not all studies reported on whether or not acute otitis media was considered to be a focus of infection among children with bacteremia.

found to be the leading cause of nonepidemic meningitis in Africa and was the most commonly identified pathogen in 40% of studies and the second most common cause of meningitis in a further 38% in a review of 50 publications (Table 2) (114). Also noteworthy is that even in African countries situated in the meningitis belt, many studies in children attributed a greater burden of meningitis to endemic *S. pneumoniae* than *N. meningitidis*, especially during the interepidemic years (111, 136). Selected studies from Asia, Middle Eastern countries, and South America indicate that 17 to 58% of bacterial meningitis cases may be attributable to *S. pneumoniae*. Additionally, the mortality from pneumococcal meningitis in non-African, industrializing countries may be as high as 50% in Asia and 23 to 37% in Middle Eastern and South American countries (5, 6, 11, 29, 47, 124, 133, 143).

In Africa, the proportion of bacterial meningitis cases attributable to *S. pneumoniae* in studies performed from

the mid-1980s onwards ranged between 12% in a study performed in South Africa (Cape Town; 1991 to 1992) to 65% in neighboring Malawi during the period from 1997 to 2001 (67, 102). An additional study from South Africa in Johannesburg between 1997 and 1999 (93) attributed 42% of cases of bacterial meningitis to *S. pneumoniae*. Furthermore, *S. pneumoniae* was the preeminent bacterial pathogen identified in children that were infected with HIV (74%), while it was identified in only 29% of non-HIV-infected children, even prior to the introduction of a Hib conjugate vaccine into the country’s immunization program. Similarly, *S. pneumoniae* was responsible for a higher proportion of bacterial meningitis cases in Malawi among HIV-infected children (52%) than among non-HIV-infected children (32%) (103). These data indicate that HIV-infected children may be more predisposed to developing pneumococcal meningitis than meningitis due to other common bacterial pathogens.

**Table 2** Proportion of purulent bacterial meningitis cases due to *S. pneumoniae* and outcome in children prior to introduction of Hib and *S. pneumoniae* conjugate vaccines (unless specified otherwise) in studies from Africa<sup>a</sup>

Reference	Country(ies) (region or type of area)	Period	Age group	Type of study <sup>c</sup>	% of confirmed bacterial meningitis cases caused by <i>S. pneumoniae</i> (total no. of cases) <sup>c</sup>	CFR(s) for <i>S. pneumoniae</i> meningitis (%)	Comments
15 59	Cameroon Egypt	1982–1983 1983–1988	<15 yrs <13 yrs	R R	43 (174) 24 (282)	39 28	CFRs, 14% and 41% in groups treated and not treated with steroids
160 105 57	Egypt Ethiopia Ethiopia <sup>b</sup>	< 6 yrs 1993–1995 1990–1994	> 4 wks <15 yrs	P P R	30 (228) 36.5 (126) 20 (85)	41 35	Overall CFR greater in children of <1 yr (38%) than in those of >1 yr (15%)
111	The Gambia (semiurban) <sup>b</sup>	1991–1994	<15 yrs	R	41 (269)	37	Among children in first year of life, median age for meningitis was 3 mos
108	The Gambia (rural)	1989–1991	< 5 yrs	P	44 (31)	55	Compared to 1% CFR for bacteremic pneumonia
162 91	Ghana Ghana	1983–1984 1989–1990	>30 days 3 mos–14 yrs	R Both P and R	56 (69) 51 (69)	66 37	<i>S. pneumoniae</i> caused 77–80% of cases during dry season; 83.3% of deaths due to <i>S. pneumoniae</i> meningitis occurred within 24 h of admission
39 106 136	Cote d'Ivoire Kenya Libya <sup>b</sup>	1985–1986 1985–1986 1994–1995	<15 yrs ≤12 yrs 1 mo–10 yrs	R R R	29 (125) 25 (129) 33 (60)	35 34	13% overall CFR; 12% of survivors had sequelae
102	Malawi	1996–1997	<15 yrs	R	35 (174)	46	Median duration of fever, 4.6 days prior to diagnosis; initial diagnosis of malaria delayed diagnosis of meningitis in 23% of cases
104	Malawi	1997–2001	2 mos–13 yrs	P	65 (520)	37	Further 4% died postdischarge; 43% of survivors had hearing loss, which was profound in 18% of cases, irrespective of steroid prescription
113	Mali and Niger <sup>b</sup>	1989–1990	≥2 mos	R	27 (426)	67	Long-acting chloramphenicol similar to intravenous ampicillin used for treatment of purulent meningitis
31 122	Mozambique Mozambique	1989 2001–2003	2 mos–6 yrs < 5 yrs	R P	49 (51) 27 (33)	NA <sup>e</sup> 56	Compare CFRs of 9% for <i>S. pneumoniae</i> pneumonia and 7% for other <i>S. pneumoniae</i> bacteremia
4	Nigeria (Benin City)	1985–1990	1 mo–16 yrs	Both P and R	21 (253)	NA	

3	Nigeria (Maiduguri) <sup>b</sup>	1988-1992	1 mo-14 yrs	R	29 (75)
110	Nigeria (Enugu)	1989-1993	≤15 yrs	R	38 (76)
125	Rwanda	1983-1990	≤15 yrs	R	37 (321)
67	South Africa	1991-1992	1 mo-13 yrs	R	12 (208)
93	South Africa	1997-1999	<12 yrs	R	Overall, 42 (147); among HIV-positive subjects, 74 (62); among HIV-negative subjects, 29 (85)
					Overall, 31; among HIV- positive sub- jects, 37; among HIV- negative subjects, 20
126	Sudan	1985-1986	3 mos-14 yrs	R	16 (44)
90	Tunisia	1993-2001	1 mo-5 yrs	R	24 (119)
45	Zambia	1980-1988	≤15 yrs	R	36 (176)
					NA NA NA

<sup>a</sup>Sections of this table are adapted from Peltola (114).<sup>b</sup>Region in meningitis belt.

R, retrospective; P, prospective.

<sup>c</sup>Numbers in parentheses are total numbers of confirmed bacterial meningitis cases.  
NA, not applicable.

The development of PCVs, albeit including only limited serotypes, has invigorated the scientific agenda of characterizing the burden of IPD, including pneumococcal meningitis, worldwide. A recent review of the burden of pneumococcal meningitis in Western Europe provided valuable burden-of-disease information for that region and explored the factors that may influence burden-of-disease estimates (73). The incidence (per 100,000 children per year) of pneumococcal meningitis in Western Europe for children under 2 years of age ranged from 3.8 cases in Spain to 14.6 in the United Kingdom (Table 3). The review, however, also highlighted the potential impact of study methodology on disease burden estimates, with prospectively conducted studies reporting a 46% higher incidence than retrospectively conducted studies. Additionally, the method of surveillance is important, as demonstrated in a study from Belgium in which the incidence of IPD (59.5 versus 30 cases per 100,000 children per year) and that of pneumococcal meningitis (7.7 versus 4 cases per 100,000 children per year) were found to be twofold greater by a prospectively conducted study than by passive surveillance (148). The latter study emphasizes the importance of standardized, reproducible, national laboratory-based, and clinically based epidemiological studies to truly appreciate the burden of pneumococcal meningitis and to permit meaningful comparisons between studies. The study by Jefferson et al. concluded that differences in the diagnosis of bacteremia without a focus were the most likely explanation for the incidence of IPD in Western Europe (20 to 35 cases per 100,000) being reported as less than that in the United States (160 to 180 cases per 100,000 children per year). The incidence of pneumococcal meningitis in Western Europe was similar to that observed in the United States (8 to 9 cases per 100,000 children per year) prior to the introduction of PCVs (163), indicating that the diagnosis of pneumococcal meningitis is less affected than bacteremia without a focus by differences in medical practices among countries.

Also included in Table 3 are incidence data from selected studies from other regions and continents. Non-standardization in reporting, however, limits direct comparisons between some of the studies. Whereas the incidence rate in the primarily nonindigenous U.S. child population ranged between 6.6 and 10 per 100,000 children per year (130, 139, 163), native Alaskan children were found to have incidence rates more than 30-fold greater than other U.S. children (145 to 216 cases per 100,000 children per year) (36, 129). A much higher proportion of Alaska Natives, however, had underlying conditions (35% of children <2 years old) that may have increased their susceptibility to pneumococcal

**Table 3** Selected studies showing the incidence rates of pneumococcal meningitis in children ≤24 months of age (unless otherwise specified) not immunized with a conjugate pneumococcal vaccine<sup>a</sup>

Region and/or country (reference)	Period	Type of study <sup>b</sup>	Incidence (cases/100,000/yr)	95% CI(s)	Comment
<b>Europe</b>					
Scandinavia					
Denmark (75)	1981–1999	P	17.4 (among children <1 yr old); 12.35 (among children <2 yrs old)	7.2–25.7; 6.3–20.1	
Finland (48)	1985–1989	P	4.85	3.12–6.59	CFR, 3.9%; bimodal seasonal variation—peak during spring and fall
Sweden (34)	1981–1995	R	6.85	3.50–10.21	Overall CFR, 15%
Spain					
(40)	1996–1998	P	3.78	1.73–5.84	Incidence of 3.8 more cases/100,000/yr in children <2 yrs old
(42)	1997–1999	P	7.25	4.29–10.22	
(68)	1981–2001	R	8.35	4.78–11.93	
Other Western European countries					
Switzerland (147)	1985–1994	R	5.64	4.49–6.79	CFR, 8.6%; 1.45-fold-increased risk if resident in a rural area
Austria (120)	1997–1998	P	7.74	4.65–10.85	
Germany (149)	1997–1998	P	7.14	6.22–8.07	
United Kingdom (70)	1980–1999	P	14.64	11.00–18.29	20% mortality; 26% of cases involved neurologic sequelae
Italy (35)	2001–2002	R	5.65	0.11–11.19	
United Kingdom (98)	1996–1998	P	9.98	8.99–10.98	
Scotland (83)	1988–1999	R	11.8		<i>S. pneumoniae</i> disease greatest in winter months, coinciding with influenza virus infection
Belgium (148)	2002–2003	P	16.1	11.3–22.2	Incidence double that determined by passive surveillance; overall CFR for meningitis, 11.4%; neurological sequelae occurred in 28.2% of cases
<b>North America</b>					
United States (130)	1995	P	6.6 (among children 1–23 mos); 15.7 (among children <1 mo old)	NA <sup>c</sup>	Rates of <i>S. pneumoniae</i> meningitis were 2.1–2.6-fold higher in blacks than whites; 21% overall CFR for meningitis
Alaska (37)	1980–1986	P	216 (among natives)	108–386	Rates 30-fold higher than those for other U.S. groups; 67% of cases with comorbid conditions; overall CFR for meningitis, 14%
Alaska (36)	1986–1990	P	145 (among natives)	54–320	
Southern California (163)	1992–1995	P; lab based	10	6–16	CFR, 0%
Virginia (139)	1978–1997	R	2.4 (among children <4 yrs old from 1978–1987); 4.5 (among children <4 yrs old from 1988–1997)	NA	CFR, 8.3%; only 6.7% of cases involved neurological sequelae

New Mexico (53)	1964–1971	P	9 (among children 1 mo–4 yrs old; 12 cases)		Overall CFR, 23%
Australasia					
New Zealand (150)	1984–1992	Both R and P	23.0 (overall); 46.0 (among Pacific Islanders)	NA	Overall CFR, 4.3%; 84% of meningitis cases occurred in children <2 yrs old; 20% of cases involved neurological sequelae
Middle East					
Israel (33)	1988–1990	P	11.1	7–17	51% of all meningitis cases occurred in children in the first yr of life; CFR, 6.2% for meningitis
Africa					
The Gambia (rural) (108) (145)	1993–1995	P	148 (among children <1 yr old)	31–437	CFR, 55% (vs CFR for pneumonia of 1%); 73% of <i>S. pneumoniae</i> meningitis cases occurred in children <12 mos old
Mozambique (122)	2001–2003	P	69 (among children <1 yr old); 40 (among children <2 yrs old)	43–106; 25–60	CFR, 32%; 95% of meningitis cases occurred in children <12 mos old
South Africa (S. A. Madhi, unpublished data)	1998–2003	P	105 (overall); 1,009 (among HIV-positive children); 43 (among HIV-negative children)	12–174; 65–161; 538–1720; 19–85	Imputed from available data CFRs: overall, 34.5%; among HIV-infected children, 46.7%; among HIV-uninfected children, 12.5%
Niger (23)	1981–1994	R	150 (among children <1 yr old); 10.4 (among children 1–4 yrs old)	65–125; 27–46	CFRs, 58% (among children <1 yr old); 57% (among children 1–4 yrs old)
Burkina Faso (112)	2002–2003	P	95 (among children <1 yr old); 36 (among children <5 yrs old)	10–48; 0–41; 9–61	CFR, 52% in <5 yrs old
Mali (24)	2002–2003	P	26 (among children <1 yr old); 9 (among children 1–4 yrs old); 31 (among children 5–15 yrs old)		CFR, 23%
South America					
Brazil (18)			10 (among children <1 yr old)		CFR, 27.5%
Central America					
Costa Rica (144)	1995–2001	R	2.0	NA	CFR, 16% (vs 22% for pneumonia); 74% of patients had sequelae upon discharge or follow-up
Asia					
South Korea (78)	1999–2001	P	10.8	1.9–61.4	Low yield on culture for probable bacterial meningitis; study focus on Hib

<sup>a</sup>Data for Western European studies were adapted from the published review by Jefferson et al. (73).

<sup>b</sup>R, retrospective; P, prospective.

<sup>c</sup>NA, not applicable.

meningitis. The most common underlying factors predisposing Alaskan children to IPD included preexisting anemia (15%), chronic lung disease (7%), congenital defects (7%), and low birth weight or prematurity (7%). Genetic predisposition and socioeconomic conditions may also be additional important predisposing factors. Similar ethnic differences in susceptibility to pneumococcal meningitis have been observed in New Zealand, where children of Pacific Islanders had a twofold-greater incidence of pneumococcal meningitis than the overall child population (46 versus 23 cases per 100,000 children per year), as well as in the Aboriginal population in Australia and the Inuit population in Greenland (30, 63).

Studies of the burden of pneumococcal meningitis in Africa indicate incidence rates much greater than those in Western industrialized countries. The incidence rate among children <2 years of age (40 to 43 cases per 100,000 children per year) (108; S. A. Madhi, unpublished data) is approximately fivefold greater than those reported for Western Europe and the United States. Incidence rates from other areas in Africa may be even greater, as illustrated in The Gambia, where the incidence rate of pneumococcal meningitis in children <1 year of age was twofold greater in a rural region (148 cases per 100,000) than in a semiurban population (69 cases per 100,000 children per year). Similarly high incidence rates have been reported for infants from Niger (150 cases per 100,000 children per year) and Burkina Faso (95 cases per 100,000 children per year). Limited data from South America indicate incidence rates similar to that in Western Europe, and the incidence rates from South Korea (11 cases per 100,000 children per year) are also similar (10, 18).

### Mortality and Neurological Sequelae from Pneumococcal Meningitis

In addition to the higher incidence rates of pneumococcal meningitis in industrializing countries, higher CFRs and more instances of neurological sequelae have been observed in these children than in those from industrialized countries (Tables 2 and 3). CFRs in excess of 50% have been reported in studies from some African and Asian countries, including studies performed during the late 1990s and the first few years after 2000 (23, 108, 112, 122, 125). These rates are even greater than those observed during the 1960s (23%) in the United States (53). *S. pneumoniae* is therefore thought to contribute significantly to the 100,000 to 500,000 annual deaths from meningitis among children in industrializing countries (61). The CFRs for pneumococcal meningitis in de-

veloping countries and indigenous native populations in fact exceed the overall annual incidence rates of pneumococcal meningitis in industrialized countries by 15- to 100-fold (139). Additionally, the reported incidence and CFRs from industrializing countries may in fact be underestimates, as some infected children may not attend health care centers because of fulminant meningitis and death before reaching a hospital, refusal of treatment because of the cost of hospitalization, or cultural reasons (23).

It is unlikely that genetic predisposition to IPD plays a major role in predisposing to pneumococcal meningitis mortality, as despite the higher burden of disease in Alaska Natives, the overall CFR was similar (14%) to others in the United States (21%) (36, 130). The in-hospital CFRs provide a minimal estimate of the true mortality associated with pneumococcal meningitis, as indicated by studies with follow-up beyond the hospitalization period. A study in The Gambia showed that 23% of hospital survivors subsequently died in the community, mainly of causes related to neurological sequelae resulting from the meningitis (60). Late clinical presentation of infected children at a hospital, as well as delays in diagnosing meningitis (e.g., 22% of pneumococcal meningitis cases were initially erroneously misdiagnosed as malaria in a study in Malawi [102]), may contribute to this high mortality. The review by Peltola reported that the mortality rate for pneumococcal meningitis (45%) was greater than that for meningitis caused by *H. influenzae* (29%) or *N. meningitidis* (8%) (114). A meta-analysis on mortality due to bacterial meningitis in developed countries also found higher mortality rates associated with pneumococcal meningitis (15.3% versus 3.8% for Hib meningitis and 7.5% for meningitis caused by *N. meningitidis*) (13).

Similarly, pneumococcal meningitis was more frequently (50%) associated with neurological sequelae among survivors than that caused by *H. influenzae* (40%) or *N. meningitidis* (10%) (13). Additionally, pneumococcal meningitis was more frequently associated with subsequent mental retardation (in 17% of cases versus 6.1% for Hib meningitis and 2.1% for *N. meningitidis* meningitis), spasticity (11.5 versus 6.1 and 0%, respectively), and deafness (27.7 versus 10.2 and 6.4%, respectively). Fortnum computed the incidence of permanent sensorineural hearing loss following bacterial meningitis, due to damage of the eighth cranial nerve or of receptors of the inner ear, to be 31.8% (95% CI, 20.7 to 42.8%) for meningitis caused by *S. pneumoniae*, 11.4% (95% CI, 9.3 to 13.5%) for that caused by Hib, and 7.5% (95% CI, 5.0 to 10.1%) for that caused

by *N. meningitidis* (51). Some of the immediate neurological sequelae from bacterial meningitis improve with time and may resolve completely (116).

The higher mortality rate in industrializing countries cannot, however, be solely attributed to lack of access to health care, but may also be related to the resources available for the management of meningitis and, in particular, the spectrum of antibiotics available. This was shown in The Gambia, where mortality due to bacteremic pneumococcal pneumonia, which too would be fatal if not treated with adequate antibiotics, was found to be 1 to 2% compared to the 32 to 55% mortality rate observed for pneumococcal meningitis (108, 145).

### Management of Pneumococcal Meningitis

In many African and other industrializing countries, despite the emerging prevalence of pneumococcal strains that are nonsusceptible to penicillin and chloramphenicol (55), these are the only antibiotics which are available for the treatment of bacterial meningitis (104). The limitations of using chloramphenicol for the treatment of infections with penicillin-nonsusceptible strains of pneumococci were reported in the early 1990s (54). Friedland and Klugman observed that 80% of children with penicillin-nonsusceptible strains causing pneumococcal meningitis had an unsatisfactory outcome when treated with chloramphenicol compared to 33% of children with penicillin-susceptible types of pneumococcal disease. This finding was attributed to high minimum bactericidal concentrations of chloramphenicol, resulting in borderline cerebrospinal fluid bactericidal activity and frequent treatment failure. The inferiority of chloramphenicol to ampicillin or cefotaxime/ceftriaxone was also shown in Europe (115).

Treatment with cefotaxime or ceftriaxone has been adopted widely in industrialized and some industrializing countries for the empirical management of bacterial meningitis; resistance to these antibiotics has, however, also emerged (10). In a prospective study on pneumococcal meningitis in the United States, the incidence of resistance to cefotaxime was shown to triple over a 3-year period (10). No difference in the outcome of meningitis among cases caused by antibiotic-susceptible and nonsusceptible organisms was, however, demonstrated when cefotaxime and vancomycin were used as initial therapy. The overall CFR in this study was 7.7% (range, 0 to 20% among centers). The cases of meningitis due to intermediately susceptible strains of pneumococci could potentially have been successfully managed with cefotaxime or ceftriaxone alone, as described by Tan et al. (141). Hence, it appears that appropriate antibiotic

therapy is key to minimizing the risk of mortality due to pneumococcal meningitis, even in the face of cephalosporin-resistant isolates (20, 50). The clinical consequence of the emergence of cephalosporin-nonsusceptible strains of pneumococci has been initial empiric treatment of presumed bacterial meningitis with cefotaxime or ceftriaxone in combination with either vancomycin or rifampicin in industrialized countries (17, 80). Recent data, however, suggest that early empiric therapy with vancomycin was not associated with any difference in mortality outcome but was associated with a higher rate of subsequent hearing deficits (21).

Whether the empiric use of cefotaxime or ceftriaxone and vancomycin for the management of bacterial meningitis needs to be continued in countries with a high uptake of PCVs still remains to be determined. The widespread use of PCVs has resulted in a reduction in the transmission of drug-resistant pneumococci, hence reducing the risk of IPD due to these strains of pneumococci (81, 156); however, multidrug-resistant isolates of serotype 19A have emerged as a frequent cause of IPD (49, 62).

The adjunctive use of corticosteroids has been associated with a reduction in neurological sequelae in cases of Hib meningitis (86, 109, 128, 153); its value in the management of pneumococcal meningitis is more controversial (104). A meta-analysis by McIntyre et al. including 178 cases of pneumococcal meningitis reported no significant benefit in relation to hearing or neurological deficits among children treated with dexamethasone (97). There was, however, significant protection observed against hearing loss in four studies in which the steroids were administered before or with the initial dose of antibiotics. In a large study (108 subjects) by Girgis et al., dexamethasone therapy was associated with a reduction in mortality (13.5 versus 40.7%;  $P < 0.002$ ) and hearing impairment (0 versus 12.5%) among individuals treated with ampicillin and chloramphenicol (59). The dose of dexamethasone used in this study differed (8 mg every 12 h for 3 days for children  $<12$  yrs old) from those in other studies, and audiology was not performed for children  $<4$  years of age. Contradictory to these findings were those of a large study from Malawi in which adjunctive dexamethasone therapy was not associated with any difference in mortality (37 versus 44% among placebo recipients) or the occurrence of neurological sequelae (27 versus 12% among placebo recipients). This study was performed in a low-resource setting in which penicillin and chloramphenicol are the mainstay of antibiotic treatment (104). A recent meta-analysis on the use of steroids in

the management concluded that steroids were associated with a favorable outcome in developed countries whereas there was evidence of neither benefit nor harm in children from developing countries (146).

## EPIDEMIOLOGY OF *S. PNEUMONIAE* PNEUMONIA

### Burden of LRTI

Lower respiratory tract infections (LRTI) are the leading cause of death among children under 5 years of age and are responsible for more than 90% of the 1.9 to 2.2 annual childhood deaths due to acute respiratory tract infections. Almost all of these deaths occur in industrializing countries, and 42% occur in Africa (157). Despite the importance of pneumonia in regard to childhood morbidity and mortality, the epidemiology and pathogenesis of pneumonia, particularly in industrializing countries, remain understudied and consequently underappreciated.

Case fatalities due to pneumonia declined 97% between 1939 and 1996 in the United States (44). Although this decline is largely attributable to the discovery and widespread use of penicillin since the 1940s, subsequent declines in the pneumonia mortality between 1966 and 1982 were attributable to better access to health care. Similarly promising strides in reducing childhood mortality from pneumonia, especially in countries with infant mortality rates in excess of 90 per 1,000 live births, are achievable through the implementation of the World Health Organization (WHO) case management strategy for treating LRTI (127). A recent meta-analysis found reductions in pneumonia mortality of 42% (22 to 57%), 36% (20 to 48%), and 36% (20 to 49%) among neonates, infants, and children 0 to 4 years of age, respectively, using the WHO case management strategy.

Fortunately, despite the global emergence of penicillin-nonsusceptible strains of *S. pneumoniae* and the implications thereof for the management of acute otitis media and meningitis, resistance has not had a significant impact on the management of pneumococcal pneumonia (55). The favorable pharmacokinetic-pharmacodynamic profile of beta-lactams for treating pneumococcal pneumonia, coupled with the low number of pneumococcal strains for which MICs are  $\geq 1.0$   $\mu\text{g}/\text{ml}$ , results in penicillin derivatives' remaining the cornerstone of the successful treatment of most episodes of community-acquired pneumococcal pneumonia.

Nevertheless, LRTI remains the leading cause of childhood mortality in industrializing countries, and data on the epidemiology of LRTI in many of these countries are sparse (123, 157). The most recent com-

munity- and hospital-based longitudinal studies aimed at measuring the burden of LRTI in industrializing countries were conducted during the mid-1980s (132), prior to the epidemic of HIV infection. The literature published between 1966 and 2000 on the incidence of LRTI has recently been reviewed by Rudan and colleagues, who estimated the median incidence of LRTI in developing countries at 29 episodes per 100 children per year, equal to approximately 154.5 million new cases each year, 7 to 13% of which were severe enough to warrant hospitalization (123). Rudan et al. estimated that the two regions with the highest burden of LRTI were southern Asia and sub-Saharan Africa. The annual incidence rates in these two regions were 36 and 30 cases per 100 children per year, respectively, which resulted in annual burdens of 61.3 million and 35.2 million episodes of pneumonia, respectively. The burden of LRTI was also found to be greatest among children less than 1 year old. Relative to an incidence of 1.0 in the first year of life, the mean ratios for the subsequent four years of life were 0.58 (year 2), 0.48 (year 3), 0.31 (year 4), and 0.19 (year 5).

These estimates were, however, exclusive of the impact that the epidemic of HIV infection may have had on the incidence of LRTI. Data from South Africa indicated that the incidence of hospitalization for LRTI among children not vaccinated with a PCV was approximately 6.6-fold greater among HIV-infected children (16.7 cases per 100 children per year) than non-HIV-infected children (2.6 cases per 100 children per year) (92). Although the incidence rates observed for HIV-infected children were less than the rate observed by Mofenson and colleagues among HIV-infected children in the United States (24 per 100 children per year) (100), the data from South Africa included only children who were hospitalized for LRTI, as opposed to children who were hospitalized for LRTI and ARI and those who were outpatients in the United States. The incidence of pneumonia in HIV-infected children in the United States was sevenfold greater than historical incidence rates of 3 to 4.2 cases per 100 children per year observed among preschool children in the United States and Finland prior to the epidemic of infection with HIV type 1 (52). In some sub-Saharan African countries, approximately 45% of children hospitalized for pneumonia are HIV infected (94).

## IDENTIFICATION OF *S. PNEUMONIAE* IN THE ETIOLOGY OF PNEUMONIA

A challenge in contextualizing the specific role of *S. pneumoniae* in the important public health concern of childhood pneumonia is the lack of suitable tools with

which a specific etiological diagnosis of bacterial pneumonia can be made. The sensitivity of current diagnostic tools in defining the etiology of LRTI is suboptimal even in countries with abundant resources, such as Finland and the United States (65, 159). The most frequently performed test aimed at detecting the bacterial etiology of pneumonia, i.e., blood culture, has a sensitivity of only 3 to 30% (9, 92, 135). Further complicating the use of blood cultures, however, is the identification of different pathogens in blood samples and in samples from the direct aspiration of the infected lung from the same individual. Additionally, cases of bacteremic pneumococcal pneumonia may differ in their degree of severity; for the majority of children with pneumococcal pneumonia, blood cultures are negative (74). Despite the limited sensitivity of blood cultures for diagnosing pneumococcal pneumonia, bacteremic pneumococcal pneumonia remains the most common manifestation of IPD in industrializing countries (Table 1).

The incidence (per 100,000 children per year) of culture-confirmed pneumococcal pneumonia in The Gambia was 224 (95% CI, 171 to 277) in children 2 to 11 months of age, 139 (95% CI, 93 to 184) in those 12 to 23 months of age, and 82 (21 to 143) in those 24 to 36 months of age (145). The incidence in rural areas of The Gambia and other industrializing countries may be even higher, as was observed for pneumococcal meningitis (Table 3). The impact that HIV has on the burden of bacteremic pneumococcal pneumonia is manifest in a study from South Africa in which the risk was 43 (95% CI, 21 to 90)-fold greater in HIV-infected children (1,233 cases per 100,000 children) than in non-HIV-infected children (29 cases per 100,000) less than 2 years of age (94). The severity of bacteremic pneumonia is detailed in a recent study from Kenya, where at least one-third of deaths in children over 1 year of age are due to bacteremic pneumonia (14). This study also highlighted factors which commonly contribute to the reduced sensitivity of blood cultures in detecting bacterial causes of pneumonia, including a loss of sensitivity of one-third with suboptimal volumes of blood (1 versus 3 ml) as well as a 62 to 73% reduction in yield when antibiotic exposure preceded the venipuncture.

Although lung aspirates in cases of consolidated lung parenchyma provide an alternate and more sensitive means of identifying the bacterial pathogens causing pneumonia, they also have limitations. Foremost is that the spectrum of pathogens identified in such studies may be biased in relation to the type of consolidation in which fine-needle lung aspirates are feasible (usually right-sided peripheral consolidation). Therefore, the yield of pathogens identified in lung aspirate studies may not necessarily represent the spectrum of pathogens

that present with other radiographic manifestations. This is especially important considering that any alveolar consolidation on chest radiographs, as defined by the WHO, occurs in only a third of all cases of pneumonia in large prospective vaccine trials (16, 27, 32, 81). Of further interest is that in some countries, only 37% of pneumococcal pneumonia may in fact present as alveolar consolidation (92). Lung aspirates are also associated with some risk (<2% of cases), such as pneumothorax and hemoptysis (151), that may preclude its routine use in clinical practice and large-scale epidemiological studies, especially in heavily burdened and resource-poor countries. The yield from lung aspirates may be operator dependent and may also be influenced by other factors, such as preceding exposure to antibiotics and subject age. Lung aspirate analysis nevertheless is the gold standard for identifying bacterial infections of the lung when feasible and has a sensitivity of 70% (152).

The diagnostic yield from lung aspirate studies, the majority of which were performed in Africa, has recently been reviewed (151). On average, a bacterial pathogen was isolated from lung aspirates in 52% of cases, compared with a rate of isolation from blood samples of 25% (2 to 45%) in studies in which both procedures were done concurrently (1, 56, 58, 119, 137, 151, 154). Tables 4 and 5 summarize studies that included only children with pneumonia who had not received antibiotics prior to having fine-needle aspiration performed. Except for a small study from South Africa, all the studies confirmed *S. pneumoniae* as the leading isolate among children with pneumonia, although there was a wide range in the frequency of pneumococcus-positive aspirates (18 to 51%) among other studies.

A summary of lung aspirate studies performed during the 1960s in Africa, however, suggested that *Staphylococcus aureus* may also be an important cause of LRTI (accounting for cases in 20% of children) (151). Investigators in Chile and India reported that *S. aureus* was the dominant isolate (25 to 27%), whereas *S. pneumoniae* was isolated from only 2 to 5% of children, among whom 50 to 70% had received antibiotics prior to the performance of the lung aspiration (99). Silverman and colleagues (1977) showed that although the yield of bacteria from Nigerian children with an ill-defined infiltrate (bronchopneumonia) was similar to that from children with lobar consolidation or empyema, the spectra of isolates differed, with 53.6 and 15.9% of isolates being *S. pneumoniae*, respectively (137). Similarly, Mimica and colleagues (1971) in Chile showed that although a bacterium was isolated from 28% compared with 45% of children with lobar consolidation and bronchopneumonia, respectively, the rates of isolation

**Table 4** Bacterial isolates from lung aspirate studies performed since 1970 among children who had not received antibiotics prior to the procedure and had no mentioned underlying illness

Country (reference)	Age range of subjects	No. of samples	% Positive for bacteria	% of positive samples (% of all tests) <sup>a</sup> yielding:				
				<i>S. pneumoniae</i>	<i>H. influenzae</i>	<i>S. aureus</i>	<i>Escherichia coli</i>	Other
United States (119)	2 mos–15 yrs	27	22	50 (11)	0 (0)	0 (0)	0 (0)	50 (11)
South Africa (118)	2 mos–9 yrs	29	17	20 (3)	40 (6)	0 (0)	0 (0)	40 (3)
Nigeria (137)	4 mos–8 yrs	88	79	64 (51)	14 (11)	14 (11)	0 (0)	31 (39)
Zimbabwe (69)	2 mos–11 yrs	40	33	54 (18)	23 (8)	31 (10)	0 (0)	0 (0)
Papua New Guinea (121)	<10 yrs	18	44	88 (39)	13 (6)	0 (0)	0 (0)	0 (0)

<sup>a</sup>More than one bacterium may have been isolated from samples from some children.

of *S. pneumoniae* were much lower among children with bronchopneumonia (1% versus 24%) (99). In most of the previous studies of lung aspirates, conventional bacterial culture techniques have been performed to determine the etiology of pneumonia. The yield may, however, be considerably enhanced by molecular methods, such as nucleic acid amplification techniques, as shown in Finland (152).

The lack of sensitive assays to confirm the etiological diagnosis of bacterial pneumonia has led to the use of other, alternate serological assays to determine the relative contribution of *S. pneumoniae* as a cause of pneumonia. A major confounder in the use of these assays has been, however, the absence of a suitable gold standard that is representative of the full spectrum of pneumococcal pneumonia and against which the assay can be validated for sensitivity and specificity. An example of such assays are those used in Finland in epidemiological studies of pneumonia. These techniques include assaying acute- and convalescent-phase sera for increases in the concentration of immunoglobulin G antibodies to

pneumococcal pneumolysin and C polysaccharide antigen, as well as testing for antigen-antibody immune complexes involving these antigens (74). The use of these assays resulted in a diagnosis of pneumococcal pneumonia for 37% of children, despite only 1 of the 254 children's having a blood culture positive for *S. pneumoniae*. The use of such assays had been previously evaluated with healthy, asymptomatic Finnish children, of whom only 1 to 3% had either changes in concentrations of antibody to pneumolysin or C polysaccharide or the presence of antigen-antibody immune complexes involving these antigens (82, 107). Additional analysis, however, suggested that children with bacteremic pneumococcal pneumonia were more ill and more often had "typical" inflammatory markers of bacterial pneumonia than those diagnosed on the basis of the serological assays (74). Children in whom pneumococcal pneumonia was diagnosed using serological assays were also less likely to have chest radiographic evidence of alveolar consolidation (76%) than those with bacteremic pneumococcal pneumonia (91%) and, conversely, were more

**Table 5** Etiology of pneumonia based on findings from lung aspirate studies<sup>a</sup>

Continent	Age range of subjects	No. of samples	% Positive for bacteria	% of positive samples (% of all tests) <sup>b</sup> yielding:				
				<i>S. pneumoniae</i>	<i>H. influenzae</i>	<i>S. aureus</i>	<i>Escherichia coli</i>	Other
Europe	6 wks–11 yrs	255	66	40 (32)	21 (17)	13 (5)	0.5 (0)	13 (10)
North America	2 mos–15 yrs	240	34	77 (26)	5 (2)	12 (4)	2 (0.1)	15 (5)
South America	1 mo–14 yrs	885	44	22 (10)	14 (6)	44 (19)	3 (1)	22 (9)
Africa	1 mo–12 yrs	949	66	25 (17)	17 (12)	29 (20)	4 (3)	44 (31)
Asia	11 days–14 yrs	674	51	10 (2)	9 (5)	45 (24)	4 (2)	29 (15)
Oceania	1 mo–10 yrs	101	58	57 (34)	71 (42)	1 (1)	0 (0)	39 (23)

<sup>a</sup>Data were derived from the review article published by Vuori-Holopainen and Peltola (151).

<sup>b</sup>Row percentage may exceed 100% since more than one bacterium were isolated in samples from some children.

likely to have an interstitial infiltrate (61 versus 17%, respectively).

The usefulness of these assays is also limited when they are applied individually, and they provide the highest estimate of pneumococcal pneumonia when their results are considered together. The individual sensitivity of each of the assays was 50% compared to the cumulative yield of positive results from all the assays. Additionally, it has been reported that occasionally these assays can be negative even when the individual is bacteremic (72). While the paired-serum antibody assays may be subject to a low positive predictive value for diagnosing pneumococcal infections, perhaps because of coincidental nasopharyngeal colonization with *S. pneumoniae*, this problem is less likely with the immune complex assays, as they are dependent upon the presence of antigen and antibody in the blood (87). For bacteremic pneumococcal pneumonia, the sensitivity of the three serological tests has been reported to be 88% (117).

Findings using the same serological assays were reported for ambulatory children in the United States who had radiographic evidence of pneumonia (159). The prevalence of serologically identified pneumococcal pneumonia in the study was 27%. The prevalence of pneumonia attributable to *S. pneumoniae* was 36% in children aged 0 to 2 years, 26% in children between 3 and 4 years, and 14% in children between 5 and 8 years of age. Among those children with serologically diagnosed pneumococcal pneumonia, 40% in the U.S. study and 49% in the Finnish study had evidence of concurrent viral infection, despite a very low frequency of concurrent pneumococcal bacteremia. The proportion of children with viral infections with concurrent pneumococcal infection in the Finnish study was 25% for respiratory syncytial virus, 28% for parainfluenza virus, and 50% for influenza A/B virus (65). Viral infections predisposing to pneumococcal coinfections are discussed further in chapter 22.

An additional advance made in the diagnosis of pneumococcal pneumonia in adults has been the use of urinary antigen detection assays. These assays, however, are not useful in making an etiological diagnosis in children because of high rates of false-positive results arising from the high prevalence of nasopharyngeal colonization in children (2, 43).

## EMPYEMA AND PARAPNEUMONIC EFFUSION

Empyema and parapneumonic effusion are complications of pneumonia that appear to be increasingly common, at least in parts of the United States and the United

Kingdom (22, 46). In Chile, empyema was reported in 25% of children hospitalized with pneumonia (88). The majority of published series identify *S. pneumoniae* as the principal pathogen in empyema and parapneumonic effusion. Empyema and parapneumonic effusion are part of a spectrum of pleural disease that has been defined as exudative, fibrinopurulent, or organized.

Clinically, the presence of chest pain and/or dyspnea differentiates a child with empyema from one with community-acquired pneumonia. In general, the prototypic child with empyema is older (~6 years) than the peak age for pneumonia. Most children are otherwise healthy, although previous or concomitant illness with respiratory syncytial, influenza, or varicella-zoster virus infection is common. Both respiratory syncytial and influenza viruses have been associated with increased colonization with *S. pneumoniae*, and varicella-zoster and influenza viruses have been associated with the suppression of immune function.

The management of empyema and parapneumonic effusion is controversial and has evolved as experience with the placement of pigtail catheters under radiologic guidance and video-assisted thorascopic surgery has accrued (131). The importance of the evacuation of purulent fluid is widely appreciated, yet the best approach for accomplishing such drainage is debated. Ultrasound and chest computed tomography analyses are useful for the characterization of fluid as exudate, fibrinopurulent, or organized. The need for intrapleural streptokinase or video-assisted thorascopic surgery will depend on individual centers' expertise as well as the presence of loculation and/or organization within the pleural space. Serotype 1 *S. pneumoniae* has been reported as the most frequent serotype identified in children with empyema both prior to and after the introduction of a seven-valent PCV in the United States as well as in the United Kingdom, where immunization with the seven-valent PCV has just been initiated (22, 46). Where reported, the serotype 1 isolates have been clonal and universally susceptible to penicillin (22).

## CONCLUSION

Similarities in the incidence of pneumococcal meningitis between Western industrialized countries suggest that the differences in the overall burden of IPD among these countries are largely attributable to differences in clinical practice. Despite the limited resources and selected subjects that are investigated for IPD in developing countries, the burden of IPD is manifold greater in these countries, especially those in Africa. Additionally, there are select groups of indigenous populations among

industrialized countries that are also at great risk for IPD. Data from Asia are limited, and additional efforts are required to perform burden-of-IPD studies in Asia, which accounts for the largest number of childhood deaths globally. The true burden of pneumococcal pneumonia is largely extrapolated from available data. It is, however, clear that *S. pneumoniae* is a leading cause of childhood pneumonia. Strategies aimed at treating pneumococcal pneumonia early with effective drugs and oxygen therapy, coupled with the attempted prevention by the introduction of new PCVs, will undoubtedly contribute to reducing childhood morbidity and mortality due to pneumonia. Preventative measures such as vaccination will contribute substantially toward attaining the United Nations millennium goal of reducing childhood mortality by two-thirds in 2015 compared to 1990 levels.

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Jeffrey B. Rubins  
David R. Boulware  
Edward N. Janoff

# 9

# Pneumococcal Pneumonia in Adults: Epidemiology, Clinical Features, Diagnosis, and Therapy

Community-acquired pneumonia (CAP) is the sixth leading cause of death in the United States overall and the leading cause of infectious disease deaths (11, 79), accounting for  $\approx$ 4 million cases and 600,000 hospitalizations per year. *Streptococcus pneumoniae* causes up to a quarter or a third of all cases, as well as two-thirds of all bacteremic pneumonias (38, 102). Mortality from pneumococcal pneumonia increases from <2 to 5% among adults treated as outpatients to 12% among hospitalized patients and  $\geq$ 25% among elderly patients with associated bacteremia (48, 107). More predictive of mortality than the presence or absence of bacteremia is the clinical severity at presentation.

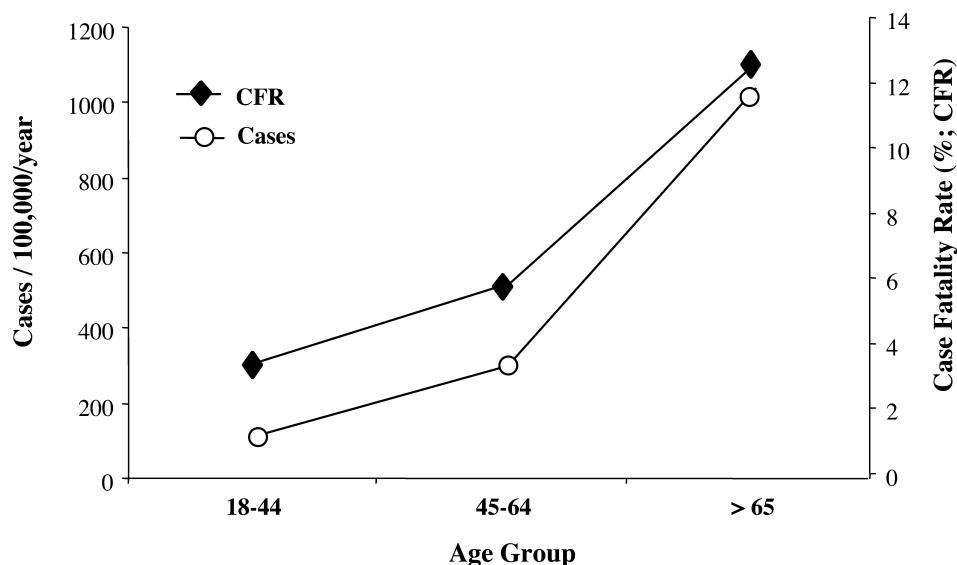
Both rates of and mortality from CAP and other pneumococcal disease increase with age (Fig. 1). The incidence of influenza and pneumonia is  $>$ 50-fold higher among persons  $>$ 85 years of age than among those 55 to 64 years of age (24). Hospital discharge rates for pneumonia are comparable to those for neoplasms and cerebrovascular disease among persons  $>$ 65 years of age (20.7, 21.8, and 21.5/100,000 patient years, respectively) and exceed those for the other syndromes by 1.5-

to 2.5-fold among persons  $>$ 85 years. Clinical presentations of disease are often blunted, diagnoses and treatment are delayed, and mortality is increased due to a presumed attenuation of immune inflammatory responses. Age and comorbidities may each contribute to these changes, both anatomic and physiologic (74). Rates of early mortality, within the first week after the onset of illness, have remained high for over 40 years (9), suggesting that, despite the availability of potent antimicrobials and intensive care support, prevention must be the keystone of the effective control of pneumococcal disease. Defining the efficacy of vaccine and other interventions requires the use of well-accepted case definitions derived from solid clinical and microbiologic evidence.

## CLINICAL PRESENTATION OF PNEUMOCOCCAL PNEUMONIA

Determining whether the clinical syndrome of pneumococcal pneumonia has evolved since the preantibiotic era is problematic. A microbiologic diagnosis is usually

Jeffrey B. Rubins, Division of Pulmonary Medicine, Veterans Affairs Medical Center, and University of Minnesota, Minneapolis, MN 55417. David R. Boulware, Infectious Disease and International Medicine, Dept. of Medicine, University of Minnesota, Minneapolis, MN 55455. Edward N. Janoff, Division of Infectious Diseases, Colorado Center for AIDS Research, University of Colorado at Denver and Health Sciences Center, Denver Veterans Affairs Medical Center, Denver, CO 80220.



**Figure 1** Incidence of hospitalization for CAP and mortality by age, Ohio, 1991 (95).

not sought unless a typical combination of symptoms and signs suggesting pneumonia is present. As guideline recommendations for CAP have shifted from a syndromic to an empiric therapy approach, sputum and blood cultures are less often ordered, particularly for outpatients. Definitive diagnosis of pneumococcal infection usually relies on the isolation of bacteria from a culture of normally sterile body fluid (such as blood or pleural effusion, joint, ascites, or cerebrospinal fluid), although newer methods of detection, as described below, may enhance the sensitivity and accuracy of diagnosis if used in clinical or investigative settings. Thus, reports of the clinical presentation of pneumococcal pneumonia that use blood or body fluid cultures to detect cases are defining a syndrome that may pertain primarily to invasive disease only. Attempts to study the clinical syndrome of noninvasive pneumococcal pneumonia to date rely on sputum culture or serology for defining cases. As discussed below, sputum culture may be falsely positive, as pneumococci are found in the upper airways of some healthy persons. In addition, pneumococci may exist as a pathogen along with other "atypical" pathogens in up to 60% of cases (119). Thus, comparing the clinical syndrome of noninvasive pneumococcal pneumonia with CAP associated with atypical etiologies is challenging. Moreover, identifying cases of noninvasive pneumococcal pneumonia by using sputum culture may lead to substantial underestimates of the true number of cases, especially when no sputum is

sought or available or the prior use of antibiotics reduces the sensitivity of microbiologic tests (85, 98).

## SYMPTOMS AND PHYSICAL FINDINGS

With these caveats in mind, the description of the "typical" presentation of pneumococcal pneumonia seems to have changed little since the preantibiotic era. The syndrome, well described by Heffron in 1938 (64), includes the abrupt onset of a cough productive of purulent and often rust-colored sputum, fatigue, fever, chills, sweats, shortness of breath, and pleuritic pain, which can be severe enough to cause splinting of the affected side of the chest (17, 119). Typical findings upon physical examination include a general appearance of illness, fever, mild tachycardia with a pulse rate of 90 to 110 beats/min, an increased respiratory rate of >20 to 24 breaths/min, tachypnea, and signs of consolidation in the affected lung upon palpation and auscultation (dullness in response to percussion, increased fremitus, crackles or rales, bronchial breath sounds, and egophony). Because of the seasonal prominence of pneumococcal pneumonia in the late fall, winter, and early spring (37, 49, 82, 97, 150), this typical clinical presentation of subjective fever (92%), cough (92%), pleuritic chest pain (79%), chills (77%), shortness of breath (47%), and dyspnea (23%) during high-prevalence seasons may have a reasonable predictive value for pneumococcal pneumonia.

However, many patients with pneumococcal pneumonia do not present with this typical syndrome. Up to 44% of immunocompetent adults with bacteremic pneumococcal pneumonia may have prominent extrapulmonary findings (56). Gastrointestinal symptoms of abdominal discomfort, vomiting, and diarrhea may be seen in 15 to 20% of cases (94, 136), especially with invasive pneumococcal pneumonia. Elderly adults have fewer symptoms overall, tend to have a less well defined history of respiratory illness, and often have lower temperatures upon admission, and each of these factors may obscure and delay the diagnosis of pneumonia (94, 136). Of note, neither human immunodeficiency virus (HIV) infection nor previous immunization with the 23-valent capsular polysaccharide vaccine appears to significantly affect the clinical presentation of pneumococcal pneumonia in adults (16, 72, 73, 109, 131). Similarly, the clinical presentations of pneumococcal pneumonia caused by penicillin-sensitive and -resistant strains do not appear to differ significantly (157, 159).

Overall, clinical symptoms and findings upon physical examination are proposed to have a sensitivity of 47 to 69% and a specificity of 58 to 75% for distinguishing *S. pneumoniae* from other etiologies of CAP. Some authors have reported a nonsignificant trend towards more severe illness, higher respiratory rates, a greater incidence of chills and pleuritic chest pain, higher leukocyte counts, higher levels of urea in serum, and higher creatinine levels in patients infected with *S. pneumoniae* than in those infected with other community-acquired respiratory pathogens (113, 119). However, these reports compared only mean values, and there are no cutoff values for routine clinical tests that reliably identify the pneumococcus as an etiologic agent in pneumonia.

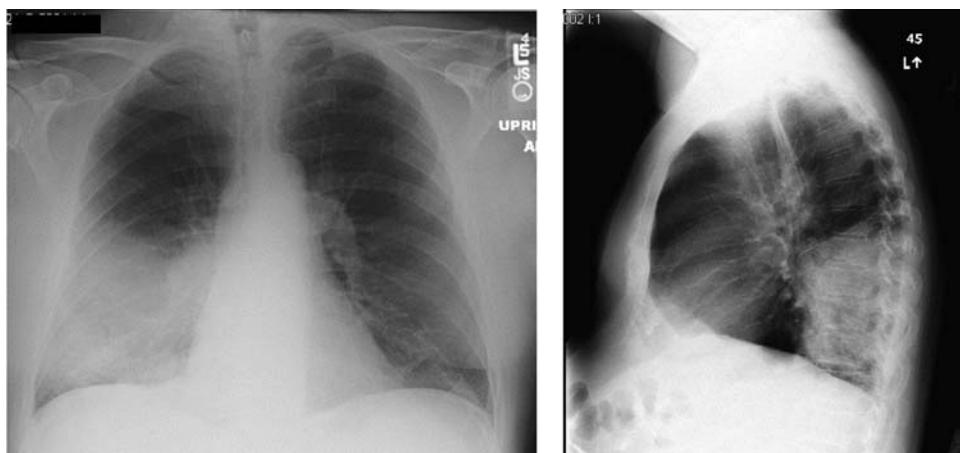
Can clinical presentation identify cases of invasive compared with noninvasive pneumococcal pneumonia? Several clinical factors, including alcoholism, especially in younger adults (49), and asplenia (94), have been associated with increased rates of bacteremic compared with nonbacteremic pneumococcal pneumonia. Increased rates of bacteremic pneumococcal pneumonia in African-Americans compared with those in Caucasians have been reported (12, 18, 62, 107), although these rates were not corrected for possible confounders such as socioeconomic factors, malnutrition, and ease of access to the health care system. On the other hand, pneumococcal vaccination has been independently associated with significantly lower rates of bacteremia (16.3 versus 34.9%) in adults hospitalized with pneumococcal pneumonia (109). Clinical symptoms and physical findings have been variably predictive of invasive pneu-

mococcal pneumonia. A multivariate analysis of 129 adults hospitalized with community-acquired pneumococcal pneumonia in Edmonton, Canada, identified a history of smoking, fever, myalgia, chest pain, altered mental state, abdominal pain, and tachycardia as significant predictors of bacteremia (136). In contrast, several authors have reported no difference in clinical presentations, including histories of chronic lung disease, malignancy, liver disease, neurologic disease, chills, and respiratory symptoms, among adults with and without bacteremic pneumococcal pneumonia (17, 107).

## RADIOGRAPHIC IMAGING

The presence of radiographic findings is key in establishing the diagnosis of pneumonia. Physicians may overdiagnose pneumonia based on patient histories and physical findings alone. In one multicentered Dutch study of 192 patients with clinically suspected pneumonia, patient management changed after chest radiography results for 69% of the patients, leading to a reduction in the proportion of patients receiving antibiotic prescriptions (from 43 to 17%) and more frequent reassurance of the patients (from 8 to 35%) (139). Whereas high-resolution computed tomography (CT) is more sensitive for detecting radiographic abnormalities than chest radiography, in the absence of microbiologic evaluation, the clinical implications of such findings are unclear (141).

Do the patterns of infiltrates on chest radiographs distinguish between pneumococcal and other etiologies of CAP? Chest radiographs are uniformly recommended in clinical guidelines to distinguish pneumonia from bronchitis, to evaluate the extent of pneumonia, to detect an effusion, and to help exclude other possible causes of the clinical presentation. The typical radiographic findings of pneumococcal pneumonia are alveolar consolidation (Fig. 2) accompanied by air bronchograms distributed throughout one or more segments within a single lobe. Although this typical radiographic pattern may be seen in a majority of patients, as reported in one study (107), atypical findings of patchy bronchopneumonia in several lobes, increased interstitial markings, or mixed patterns have been present in the majority of adults in some studies (77, 110). Atypical radiographic findings may be more common among adults with underlying emphysema, congestive heart failure (160), or HIV infection (71, 131). Lobar pneumonia as opposed to bronchopneumonia has been associated with specific serotypes (64) and with invasive pneumococcal infection in some studies (105, 107, 110,



**Figure 2** Right lower lobe consolidation with pneumococcal pneumonia; posterior-anterior (left) and lateral (right) views.

150) but not in others (17). Pneumococcal pneumonia rarely causes necrosis or lung abscess; consequently, such findings should prompt a search for mixed infection with anaerobes or an anatomic abnormality such as bronchial obstruction (from a foreign body or bronchogenic carcinoma) or coexisting pulmonary infarction (83).

In addition to establishing the presence and extent of pneumonia, chest imaging detects pleural effusions, which may require further diagnostic tests. Pleural effusions have been variably reported to occur in 6 to 57% of adults with pneumococcal pneumonia, with increased detection by chest CT compared with that by chest radiography and serial chest radiographs during the course of treatment (56, 68, 119, 136). Effusions are usually unilateral but can be bilateral, especially as detected by chest CT. Effusions typically are small to moderate in size, with only 10% of patients having sufficient pleural fluid for bedside thoracentesis. The detection of pleural effusions has been significantly associated with bacteremic pneumococcal pneumonia and with the persistence of fever and illness longer than 48 h (107, 125, 142). Most effusions are uncomplicated parapneumonic effusions, but empyema is the most common complication of invasive pneumococcal pneumonia, seen in approximately 2 to 8% of adults and associated with a threefold increase in mortality if untreated (1, 9, 17, 56, 68, 105, 107, 119, 125, 136, 146, 159). Consequently, the persistence of fever and leukocytosis for >72 h should be evaluated with chest CT and thoracentesis if a significant pleural effusion is detected. Most radiographic findings, particularly consolidation, resolve by 6 to 10 weeks after presentation (75).

## LABORATORY TESTS

Routine clinical laboratory tests do not distinguish pneumococcal from other etiologies of community-acquired bacterial pneumonia but may identify adults more likely to have invasive pneumococcal disease. Anemia (25, 107, 136), adrenal insufficiency, and elevated bilirubin and decreased albumin levels have more consistently been significantly associated with bacteremic than with nonbacteremic pneumococcal pneumonia in adults (17, 107, 136).

## ETIOLOGIC DIAGNOSIS OF PNEUMOCOCCAL PNEUMONIA

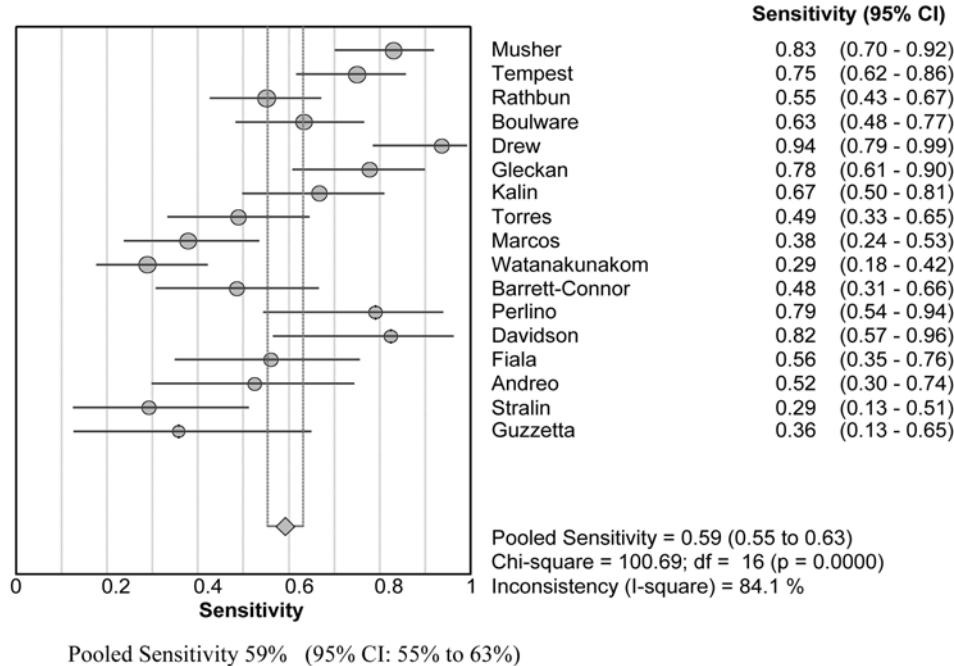
The tools for rapid and accurate diagnosis, particularly of nonbacteremic pneumococcal pneumonia, are limited, lacking a “gold standard.” The diagnosis of pneumonia is based on the constellation of suggestive clinical features and the presence of infiltrate demonstrable by chest radiography or another imaging technique, with or without supporting microbiologic data (90).

With Gram staining and culture of expectorated sputum, rates of pathogen detection in comprehensive epidemiologic studies of CAP are only 30 to 50% (6, 54, 108), often with paired acute and convalescent serology for atypical organisms. In routine clinical practice, the rate of pathogen detection is substantially lower. Etiologic diagnostic tests are neither recommended nor done for most outpatients (90), the majority of whom do well with empiric antibiotic treatment. This approach is less expensive than diagnostic testing. However, microbiologic cultures remain important for ongoing epidemiologic

logic surveillance, including that of the antibiotic resistance patterns in local communities (90).

An adequate sputum specimen shows <10 epithelial cells and >25 white blood cells per low-power field (magnification,  $\times 10$ ) to distinguish a respiratory sample from saliva. The results of Gram staining require interpretation, with differences in the strength of the evidence dependent on whether gram-positive diplococci are the sole bacteria present, the predominant flora, or part of a mixed flora. Among 1,669 hospitalized patients with CAP reported in 2004, an adequate specimen with a predominant morphology apparent upon Gram staining was found for only 14% (54). However, meta-analyses highlight that the selection of the denominator and the data source (e.g., single site versus meta-analysis) greatly affects the performance of sputum Gram staining, with reported sensitivities ranging from 15 to 100% among 12 published studies (123). The yields of sputum evaluation can be quite different for patients who present without prior antibiotic exposure, late in the course of illness with a high burden of pneumococcal disease, and with frank productive, purulent sputum specimens compared with patients with earlier presentations and with nonproductive sputum specimens.

The sensitivity of sputum Gram staining and culture can vary from 31 to 63% and 44 to 86%, respectively, based on whether one includes all patients with bacteremic pneumococcal pneumonia, only those submitting adequate specimens, or those without prior exposure to antibiotics (16, 108, 114). The true performance of sputum culture for the diagnosis of pneumococcal pneumonia is still debated. With results pooled from 17 published studies, the sensitivity of sputum culture for the diagnosis of bacteremic pneumococcal pneumonia is quite variable ( $\approx 60\%$ ) (Fig. 3) (5, 10, 16, 29, 32, 41, 57, 60, 76, 91, 107, 117, 121, 140, 143, 149, 153). Moreover, several studies suggest that culture data have minimal to no effect on clinical management in practice (129, 145). Some caveats are that such studies excluded persons with significant comorbidities and that penicillin-resistant pneumococci were not present. The majority of nonelderly patients without comorbidities and with nonsevere pneumonia can likely be treated as outpatients, and present guidelines do not encourage microbiologic investigation (90). Thus, confounding factors which increase the difficulty in making a diagnosis of pneumococcal pneumonia include (i) the clinical status of the population studied and the timing of presentation; (ii) the quality of expectorated sputum (affected by the



**Figure 3** Sensitivity of sputum culture for diagnosis of pneumococcal pneumonia. Data are listed by first authors of the studies reporting the results (5, 10, 16, 29, 32, 41, 57, 60, 76, 91, 107, 117, 121, 140, 143, 149, 153). 95% CI, 95% confidence interval.

inability to produce sputum; the contamination of sputum with saliva; the rigor of sputum collection by the patient, medical house officer, nurse, or respiratory therapist; and the processing time from sputum collection to culture inoculation) (152); and (iii) the prior use of antibiotics.

## BLOOD CULTURE

The primary source (in 85 to 90% of cases) of bacteremia in adults is pneumonia (23, 40). The value of routine collection of blood for culture is debatable. The goals of blood cultures are (i) to detect bacteremic pneumococcal pneumonia, (ii) to determine antimicrobial susceptibility, and (iii) to detect other causes of bacteremia in critically ill patients. The proportion of pneumococcal pneumonia which is bacteremic is estimated to be 20% (64, 106), although only 30 to 50% of CAP is of known etiology. In large epidemiologic studies, bacteremic pneumococcal pneumonia accounted for 3 to 5% of all CAP (59, 69, 104). In 12 studies, cultures of blood from 11% of almost 3,000 patients with CAP yielded bacteria, two-thirds of which were *S. pneumoniae* strains (11). Among HIV-infected individuals, the rate of pneumococcal bacteremia is much higher (71, 81, 122).

## ALTERNATIVE DIAGNOSTIC METHODOLOGIES

The isolation of *S. pneumoniae* from cultures of specimens from normally sterile sites is relatively insensitive. In The Gambia, despite comprehensive blood culture screening in pneumonia cases, the effectiveness of the conjugate pneumococcal vaccine in children was more than seven times greater against radiologically confirmed pneumonia than against microbiologically confirmed pneumococcal disease defined by blood or lung aspirate cultures (28). This insensitivity extends to patients without, and particularly those with, prior antibiotic therapy (108). Alternative diagnostic strategies hold promise to increase the proportion of CAP cases with a probable etiologic diagnosis.

### Detection of C-Polysaccharide Antigen in Urine

A rapid immunochromatographic test utilizing a colloidal gold-labeled antibody immobilized on a nitrocellulose membrane (Binax NOW) is now approved by the Food and Drug Administration and in clinical use. On a contact test strip, the pneumococcal antigen present binds with conjugated rabbit anti-C-polysaccharide

*S. pneumoniae* antibody, creating an antigen-conjugate complex. Complexes captured by immobilized anti-*S. pneumoniae* antibodies form a colorimetric sample line (indicating positivity) on the test device. We systematically reviewed the performance of the Binax NOW *S. pneumoniae* antigen test for over 4,600 patients in 22 studies from multiple countries. Using blood and/or sputum cultures as the gold standard among patients with CAP, the pooled results showed a mean sensitivity of 75% (range, 58 to 93%) and a mean specificity of 95% (range, 71 to 100%) (5, 16, 19, 21, 31, 36, 55, 59, 63, 66, 69, 78, 84, 91, 104, 116, 124, 130, 137, 140, 151, 155). The positive predictive values (mean, 79%; range, 25 to 100%) and negative predictive values (mean, 93%; range, 74 to 100%) in CAP scenarios exceed those of traditional microbiologic techniques.

In the majority of studies cited above, pneumococcal infection was proposed as the etiology in more cases (mean, 24%; range, 10 to 59%) with the use of the antigen detection system than with other methods. Most likely, significantly more cases of pneumonia are caused by *S. pneumoniae* than conventional microbiologic testing currently confirms. Among more than 2,400 persons with unknown CAP etiology with test results reported in the 16 published studies of antigen detection in urine, 24% had detectable pneumococcal C-polysaccharide antigen in their urine, representing a 54% increase in the diagnosis of pneumococcal infection compared with that by conventional microbiologic techniques for CAP patients. The proportion of CAP patients with a positive urine test for pneumococcal antigen averaged 36% (95% confidence interval, 25 to 47%) across studies. However, even with the increase in etiologic diagnosis, false-negative rates with testing for antigen in urine are ≈25% and range from 15 to 20% for bacteremic pneumonias. Causes for false negatives may include etiologic misclassification, the presence of low levels of C-polysaccharide antigen, or sequestration of the antigen by antibodies in serum or urine as immune complexes.

Finally, the true specificity of testing for antigen in urine is unknown and varies widely across studies, in large part due to the selection of controls and reactivity with C-polysaccharide from other streptococcal species. Studies utilizing nonpneumonia controls report higher specificity (21). The use of patients with nonpneumococcal CAP or CAP of unknown etiology as controls is problematic. At present, it is not possible to determine whether patients with putative false-positive results have pneumococcal infections, mixed infections, or neither. The specificity of testing for *S. pneumoniae* as a cause of CAP is higher for adults than for children, who are more often colonized with the organism. Samples from

healthy children may show reactivity in the absence of a suggestive CAP syndrome.

### Detection of Pneumococcal Surface Adhesin A

Pneumococcal surface adhesin A (PsaA) is a conserved and highly immunogenic protein of *S. pneumoniae* expressed at the cell surface in all 90 pneumococcal serotypes. With an enzyme-linked immunosorbent assay to detect PsaA-specific immunoglobulin G (IgG) in serum (144), epidemiologic studies by the Centers for Disease Control and Prevention showed 70% sensitivity and 98% specificity when using a cutoff of a twofold increase in IgG levels between acute- and convalescent-phase sera from 109 Kenyan adults with pneumococcal pneumonia (135). Among 95 Kenyan adults and children with microbiologically confirmed pneumococcal infection (75% with bacteremic pneumonia, 20% with meningitis, and 4% with empyema), cutoffs of a  $\geq 2.7$ -fold rise in the levels of anti-PsaA IgG in matched acute- and convalescent-phase sera showed sensitivity of 48% and specificity of 97% when the acute-phase sample was drawn at admission (134). Unlike in epidemiologic settings, the use of a single anti-PsaA level in the acute clinical setting at a threshold with high specificity ( $>96\%$ ) showed poor sensitivity (<20%) for acute infection. Nasopharyngeal colonization status did not affect the test specificity.

### PCR Diagnostic Techniques

PCR to detect conserved targets (genes for PsaA, pneumolysin [PLY], and autolysin) is an attractive tool for identifying pneumonia pathogens, since timely and accurate detection does not depend on the microbiologic viability of the organism.

#### Pneumococcal Surface Adhesin A PCR

PsaA PCR analysis of 171 lung aspirates from Kenyan adults with acute pneumonia was evaluated, and the PCR sensitivity was estimated at 83% when results were compared with those for 35 culture-positive lung aspirates (133). However, the diagnosis of pneumococcal infection among all pneumonia cases was increased (61 PCR positive versus 35 culture positive) by the use of PCR. Of these additional diagnoses, three-quarters were confirmed by either blood culture or testing for antigen in urine, supporting a reasonable specificity. Two problems exist. First, interfering substances are problematic for PCR techniques in nearly 20% of samples, and thus, an internal control is necessary to differentiate true negatives from indeterminate results. Second, PsaA is also found in three viridans group streptococcal species, *S. mitis*, *S. oralis*, and *S. anginosus*, with 90 to 95% se-

quence homology (70), although these species are uncommon causes of pneumonia.

#### Pneumolysin (PLY) PCR

Results with the detection of the PLY gene in blood have varied. Salo and colleagues had excellent success using nested PCR for the detection of PLY in sera from 20 patients with pneumococcal bacteremia and 100 healthy control subjects (100% sensitivity and 94% specificity) (127). For eight cases of bacteremic pneumonia, the nested PCR sensitivity was 75% for the buffy coat fractions and 38% for whole blood (126). The specificity was excellent (93%) when the results were evaluated with nonpneumococcal bactemias, with one false positive (involving enterococci). Similarly, for 114 Spanish CAP patients, the sensitivity of a nested PCR assay for PLY was 55% and the specificity was 100% (88). Sensitivity was lower yet for 474 CAP patients in New Zealand (29% for bacteremic patients) (103). The researchers concluded that nested PCR for PLY adds minimally to conventional diagnostic tests for *S. pneumoniae* and is unable to distinguish colonization from infection when respiratory samples are tested. However, more recent investigations have used quantitative PCR assays for the PLY gene on expectorated sputum. In a study using a quantitative TaqMan PCR assay for 129 patients with CAP, the sensitivity was 90% and the specificity was 80% in comparison with conventional microbiologic diagnostic techniques (158). Thus, quantitative PCR holds promise if the expense and technical expertise of a PCR assay can be managed effectively.

#### 16S rRNA PCR

Molecular sequencing of the 16S rRNA gene is a technique used to identify a wide variety of bacteria. Unfortunately, the differentiation of *S. mitis*, *S. oralis*, and *S. pneumoniae*, which show up to 99% homology by 16S rRNA sequencing, is problematic (15). This is a well-known problem of gene sequencing and is a limitation in the use of the GenBank database (26). At present, the costs and quality of databases are major obstacles to the implementation of 16S rRNA gene sequencing in the routine clinical microbiology laboratory, particularly for *S. pneumoniae* (15).

In summary, a range of diagnostic techniques are available to identify pneumococcal pneumonia, but all are limited by the lack of a gold standard and by variability in the results of population studies, collection techniques, cost, availability, and trends to limit diagnostic procedures in clinical practice. Diagnostic tests will continue to be useful for patients with more severe illness to establish a specific etiology and in studies to

monitor the causes of pneumonia and rates of antimicrobial resistance and to characterize the factors in the pathogenesis of infection which determine clinical outcomes.

### CLINICAL PREDICTORS OF MORTALITY ASSOCIATED WITH PNEUMOCOCCAL PNEUMONIA

Clinical predictors of lethal pneumococcal infection in adults have been sought in many studies from the early and more recent antibiotic era. Comparing results from these studies is confounded by the lack of uniform definitions of disease and mortality intervals, the lack of common variables studied, and differences in methodology (Tables 1 and 2), such as the inclusion of both community-acquired and nosocomial pneumococcal bacteremia cases (20, 22, 80, 93, 112, 125, 154) and patients that did and did not receive appropriate antibiotics (49, 58, 125, 154). Such analyses, particularly by multivariate statistical methods, may obscure risks more relevant in community populations or in those treated with appropriate antibiotics. Although a powerful statistical tool, multivariate or logistic regression is designed to identify those variables that best explain the variability of an outcome (in this case mortality) within

**Table 1** Significant characteristics and considerations distinguishing various studies of clinical predictors of mortality and outcome from pneumococcal infection in adults

Characteristic or consideration
Retrospective or prospective design
Definition of cases: bacteremia or pneumonia
Definition of pneumonia (culture and/or serology results and/or testing for pneumococcal antigen)
Age definition of adult (range, 10 to 20 yr)
Inclusion of immunocompromised patients
Inclusion of nosocomial pneumococcal bacteremia
Restriction to hospitalized or ICU patients
Study at single or multiple institutions
Country where study is performed
Academic or community-based practice
Years when cases occurred
Period during which mortality is monitored (7 to 30 days)
Inclusion of cases of early mortality (within 24 h after presentation)
Univariate or multivariate analysis
Inclusion of subjects that did not receive appropriate antibiotics
Outcome variables (e.g., early versus late mortality, time to resolution of symptoms or signs)
Randomization and double blinding (for treatment trial comparison)

a specific study population. Thus, combined variables that incorporate information from other variables (such as pneumonia severity index [PSI] scores and acute physiology and chronic health evaluation [APACHE] scores) will typically emerge from a multivariate analysis as statistically significant, whereas individual variables incorporated into these scoring systems will not. Unfortunately, these results are often misinterpreted as showing that the individual variables are not clinically important.

Given this variability in study design, different studies have derived substantially different clinical predictors of mortality from pneumococcal pneumonia and bacteremia in adults. However, some common clinical findings emerge from these studies. Most importantly, pneumococcal infection is associated with considerable mortality, with a substantial proportion of adults who succumb to pneumococcal pneumonia or bacteremia dying within the first 24 to 72 h. Regardless of the place or study design, 25 to 55% of deaths occur within 24 h and 45 to 83% within 72 h of the index blood culture or hospitalization (56, 82, 94, 111, 120, 150, 159). Overall, case fatality rates attributed to pneumococcal infection over the past 24 years range from 5.4 to 43%, with no clear association between mortality and whether pneumonia is accompanied by bacteremia, which country reports the data, or the years in which cases occurred. Pneumococcal bacteremia appears to be a significant predictor of mortality in more severe CAP but does not necessarily predict lethal infection in adults with pneumonia who are less ill (8, 17, 67, 68, 98, 115, 119, 148). Bacteremia was reported to predict mortality in HIV-infected adults with pneumococcal pneumonia in two studies (65, 118) but not in a third (50). Thus, disease severity, rather than bacteremia, may predict the outcome.

A qualitative review of cases of predominant pneumonia or bacteremia in adults (Tables 2 and 3) reveals that variables examined as predictors of mortality are not standardized from study to study, and most have been evaluated in too few studies to determine whether they are robust predictors of lethal disease (Table 1). Of those that have been evaluated in about half of these studies, decreased systolic blood pressure or clinical shock emerges as the sole risk that reliably predicted lethal pneumococcal infection upon univariate analysis. Underlying malignancy (both pulmonary and extrapulmonary) and the absence of fever also predicted lethal infection in most of the studies in which these variables were examined. Other factors often considered to be predictors of more severe disease, including advanced age, cardiac or pulmonary disease, renal disease, liver disease, alcohol abuse, nosocomial infection, an immuno-

compromised state, increased respiratory rate, leukopenia, and the presence of multilobar infiltrates, predicted lethal pneumococcal infection in approximately half of the studies in which they were examined. Others, such as gender, diabetes, and splenectomy were frequently studied but predicted mortality in only one study each. Smoking status did not emerge as a predictor of mortality in any study. Several studies used multivariate analysis to determine independent predictors of mortality from bacteremic pneumococcal infection, with or without pneumonia. Ultimately, very few clinical risks emerge from multivariate analyses as consistent predictors of lethal pneumococcal infection (Table 4). Age (dichotomized as 48, 50, or 65 years of age), the presence of multilobar infiltrates, an immunocompromised state (variably defined), and shock were identified in three or more of the studies reviewed.

In addition to the host factors (Tables 3 and 4), a few authors have examined whether pathogen-related factors such as serotype or penicillin sensitivity predict lethal infection. Musher et al. (107), in a study of 100 veterans in Houston, found no difference in serotype distribution between bacteremic and nonbacteremic pneumococcal pneumonia but did not correlate serotype with mortality. In contrast, Finkelstein et al. (49), in a study of age-dependent differences in outcomes of pneumococcal bacteremia in 187 adults, described an increased incidence of serotypes 3 and 19 in elderly adults and increased mortality within 14 days from bacteremia with serotypes 3, 8, 4, 9, 23, and 1. Although penicillin resistance has been associated with increased morbidity and the delayed resolution of symptoms in pneumococcal infection, most individual studies have not found that penicillin resistance predicts lethal pneumococcal infection (8, 14, 53, 99, 128, 138, 159). However, a recent systematic review and meta-analysis of 10 studies including 3,430 patients from international sites, restricted to prospective studies of adults with pneumonia, showed a significantly increased risk of mortality from penicillin-resistant isolates, with a relative risk of 1.29 (95% confidence intervals, 1.04 to 1.59) (147).

Although individual clinical risks may have weak and variable significance as predictors of mortality, the combination of multiple risks in any given patient increases the likelihood of a fatal outcome. Consequently, the mathematical summation of risks to calculate pneumonia severity scores may have the greatest applicability in the clinical management of pneumococcal pneumonia. The PSI was derived by Fine et al. and the Pneumonia Patient Outcomes Research Team investigators by logistic regression modeling of potential clinical risk factors predicting lethal CAP for patients in the

MedisGroups Comparative Database hospitals (46, 47). The PSI was subsequently validated as a tool to identify patients with CAP at low risk for mortality, and high PSI scores were shown to be predictive of increased CAP-associated mortality (45). Whereas the PSI has been incorporated into the Infectious Disease Society of America (IDSA) recommendations for the management of CAP (90), the British, Swedish, and Dutch societies favor the more simple CURB-65 severity assessment (*confusion; level of blood urea nitrogen [BUN] in serum, >7 mmol/liter [20 mg/dl]; respiratory rate, >30 breaths/min; low blood pressure [systolic, <90 mm Hg, or diastolic, <60 mm Hg]; and age, ≥65 years*) (86). Because these severity indices were derived from all causes of CAP, and not restricted to pneumococcal infection, recent studies have evaluated whether these severity assessments are applicable to adults with pneumococcal pneumonia. Taken together, these studies have shown that the PSI does not predict which adults with pneumococcal pneumonia have bacteremia (17, 107). However, both PSI and the CURB-65 index predict survival in prospective and retrospective studies of adults with community-acquired pneumococcal pneumonia, especially bacteremic patients (8, 14, 17, 68, 107, 136).

Iochimescu et al. directly compared PSI and CURB-65 scoring systems for 151 adults with infiltrates visible on chest radiographs and sputum or blood cultures positive for pneumococci who were admitted to Danbury Hospital between 1996 and 2000, and were treated with guideline-recommended antibiotics (68). Scores from the two systems were highly correlated, and high-severity-score classes (PSI  $\geq$  III and CURB-65  $\geq$  3) had high negative predictive values of 95 to 100% but lower sensitivity and positive predictive values for mortality (2 to 18%). For this patient population, the addition of information to the CURB-65 score regarding oxygenation status and the type of infiltrate (unilobar or multilobar) better stratified patient risks for mortality, while leaving this severity index still simpler to use than the more complicated PSI. Whether this modified CURB-65 system is a valid predictor of outcome for other adult populations with pneumococcal pneumonia remains to be confirmed.

## THERAPY OF PNEUMOCOCCAL PNEUMONIA

Penicillins remain the mainstay of therapy for pneumococcal pneumonia in most settings. Important clinical questions which have arisen in the subsequent half-century since the seminal report by Austrian and Gold

**Table 2** Summary of studies of clinical predictors of mortality from pneumococcal pneumonia and bacteremia in adults

Authors, yr (reference)	Study design	Age (yr) of subjects	Dates	Type of hospital or research center, location
Aspa et al., 2006 (7)	Prospective, observational	≥18	1999–2000	Multiple centers, Spain
Bonnard et al., 2005 (14)	Prospective	≥18	1995–2000	Academic, France
Porath et al., 1997 (119)	Prospective	≥17	1991–1992	Academic, Israel
Marfin et al., 1995 (92)	Retrospective	≥12	1987–1989	Academic, CA
J. B. Rubins and E. N. Janoff (unpublished)	Retrospective	≥16	1985–1994	Academic, MN
Ortqvist et al., 1988 (111)	Retrospective	≥15	1977–1984	Academic, Sweden
Chi et al., 2006 (27)	Retrospective	≥65	1998–2002	Community, WA
Trampuz et al., 2004 (150)	Retrospective	≥18	1986–2000	Academic, Switzerland
Yu et al., 2003 (159)	Prospective	≥15	1998–2001	Academic, international
Farinas-Alvarez et al., 2000 (37)	Retrospective	>16	1989–1996	Academic, Spain
Mirzanejad et al., 1996 (97)	Retrospective	≥10	1983–1991	Academic, Canada
Afessa et al., 1995 (1)	Retrospective	>16	1980–1993	Academic, FL
Kuikka et al., 1992 (82)	Retrospective	≥18	1976–1979, 1986–1989	Academic, Finland
Bruyn et al., 1988 (20)	Retrospective	≥15	1976–1986	Academic, The Netherlands
Ruben et al., 1984 (125)	Retrospective	≥16	1975–1980	Academic, PA
Finkelstein et al., 1983 (49)	Retrospective	>20	1972–1981	Academic, NY
Potgieter and Hammond, 1996 (120)	Retrospective	>18	1987–1992	Academic, South Africa
Moine et al., 1995 (98)	Retrospective	≥15	1987–1989	ICU, France

<sup>a</sup>Hosp, requiring hospitalization; ICU, requiring treatment in the ICU.<sup>b</sup>P, pneumonia; B, bacteremia.<sup>c</sup>ND, specific interval not defined; usually specified only as “related to infection” and/or during same hospitalization.

(9) include (i) what is the contribution of decreased in vitro antimicrobial susceptibility of *S. pneumoniae* to outcomes of treatment of CAP, (ii) what is the optimal empiric treatment based on clinical severity and are two drugs better than one, and (iii) what is the optimal duration of therapy?

### Role of Antimicrobial Resistance

Evolving rates and the impact of antimicrobial resistance among *S. pneumoniae* strains on a global level are addressed by Dagan and Klugman in chapter 25. The adverse clinical impact of resistance is most clear for meningitis and otitis media. For meningitis, levels of antimicrobials in cerebrospinal fluid are low, few other local host defense mechanisms are active, and the infection progresses quite quickly. In this setting, in which the clearance of infection is directly related to drug levels and sensitivity, elevated MICs of penicillin, expanded-spectrum cephalosporins, or vancomycin may substantially limit the efficacy of these drugs and portend a poor clinical outcome.

As discussed above, the correlation between penicillin resistance and outcomes of therapy for pneumonia and bacteremia suggests a relationship in some (8, 14, 53, 99, 128, 138, 159) but certainly not all (7, 138, 147) studies. Patients with pneumonia caused by *S. pneumoniae* with high-level cephalosporin resistance did show an increased length of stay in the hospital and a prolonged time to resolution but no increase in mortality, intensive care unit (ICU) admission, or intubation compared with matched control subjects (2). However, these outcomes were not adjusted for the therapy received. Mechanisms by which the presence of antimicrobial resistance could be causally related to worse outcomes include the inability of therapy (e.g., because of insufficient levels, local penetration, or activity) to overcome resistance to concordant therapy, discordant therapy, the inherent virulence of the organism based on serotype or other virulence factors, the severity of underlying disease, and the severity of illness among patients with penicillin- or other drug-resistant *S. pneumoniae*. Adjusting adequately for the susceptibility of the

No. of subjects	Severity <sup>a</sup> of illness	Syndrome <sup>b</sup>	Nosocomial infections included	Immunocompromised patients included	Period (days) of monitoring for mortality <sup>c</sup>	Case fatality rate (%)
638	Hosp	P	Yes	Yes	30	15.1
95	Hosp	P	No	Yes	30	31.5
148	Hosp	P	No	No	ND	5.4
102	Hosp	P	Yes	Yes	ND	25
195	Hosp	P	No	Yes	10	17.9
279	Hosp	P	No	No	21	7
200	Hosp	B	No	Yes	30	11
394	Hosp	B	Yes	Yes	ND	25
844	Hosp	B	Yes	Yes	14	16.9
156	Hosp	B	Yes	Yes	ND	33.9
314	Hosp	B	No	Yes	ND	13
293	Hosp	B	Yes	Yes	ND	36
157	Hosp	B	Yes	Yes	30	21
142	Hosp	B	Yes	No	ND	25.9
72	Hosp	B	Yes	Yes	31	43
187	Hosp	B	No	Yes	14	22
63	ICU	P	No	Yes	21	21
132	ICU	P	Yes	No	>14	33

host to pneumococcal infections and adverse outcomes is quite difficult in retrospective and even prospective studies. Patients with comorbidities, who likely have greater exposure to antibiotics and therefore resistant organisms, may show delayed resolution of pneumonia (35). Moreover, many patients receive more than one agent. The observation that, based on antibiotic susceptibility profiles, concordant or discordant therapy does not typically predict outcome supports the lack of a causal relationship in some cases. This “in vitro-in vivo paradox” described by W. Bishai may also be based on the fact that achievable levels of the drugs used for pneumonia and bacteremia, particularly in hospitalized patients, far exceed the defined MICs.

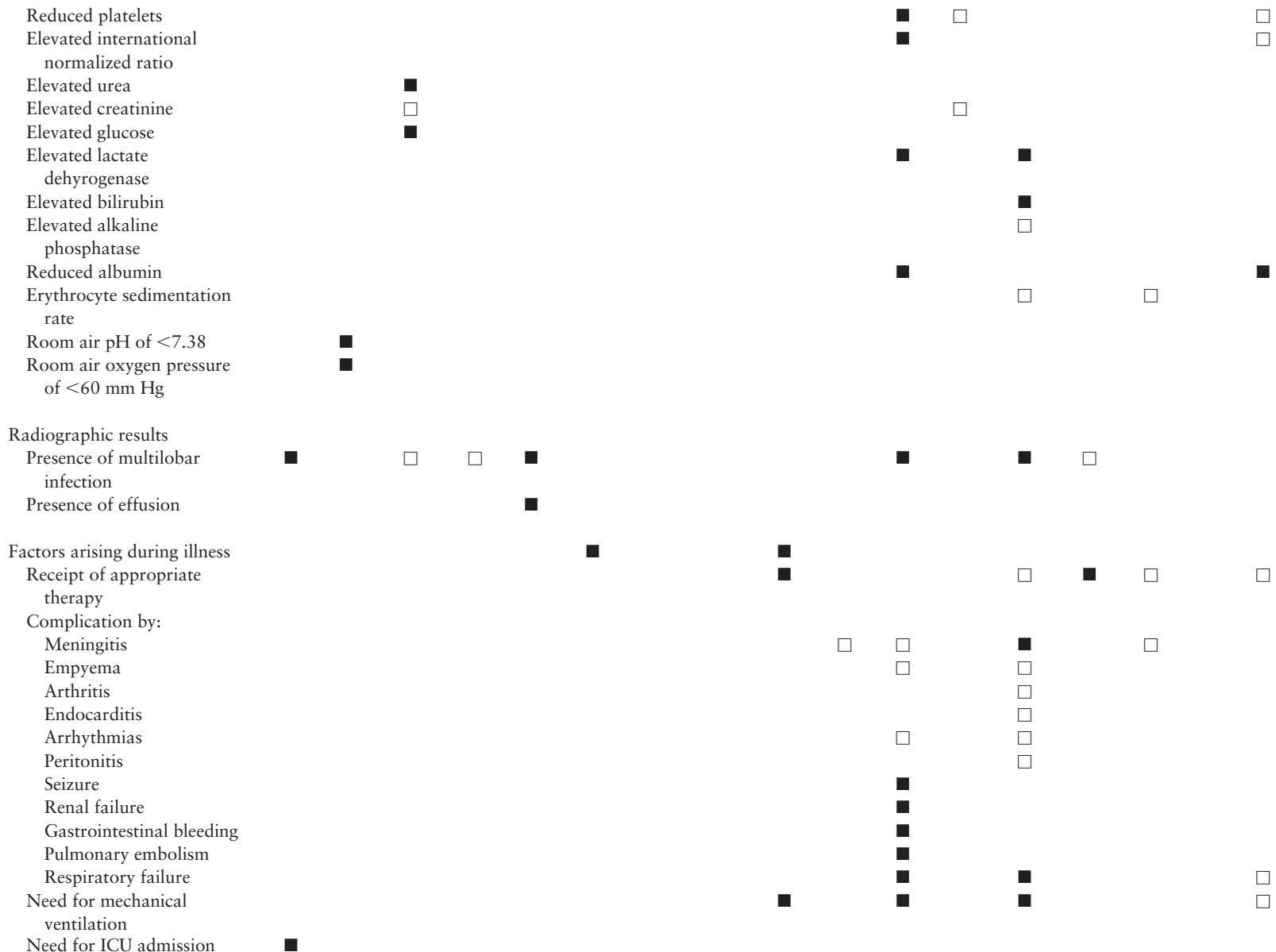
The controversy about in vitro susceptibility results versus treatment outcomes extends to the use of macrolides (3), the second most commonly used class of antibiotics for CAP, and fluoroquinolones (39). Despite increasing rates of in vitro resistance in multiple communities, clinical failure rates are relatively low. However, at least one retrospective case control study does suggest a close relationship between macrolide resistance and clinical failure (87, 132). Similarly, fluoroquinolones show efficacy and safety in the treatment of

CAP. Reported quinolone-associated clinical failures and breakthrough bacteremias during treatment with ciprofloxacin, which has the least activity in this class, and levofloxacin were associated with in vitro resistance before or during and after therapy (4, 30, 52). Risks for resistance included prior exposure to quinolones, particularly within the previous 3 months, nursing home or hospital acquisition, and underlying comorbidities, particularly immunoglobulin deficiencies. Reports of such failures with other newer respiratory quinolones, such as gatifloxacin, moxifloxacin, and gemifloxacin, are few, although these drugs have been in use for a shorter time. The presence of mutations in resistance genes, particularly *parC* and *gyrA*, whether by spontaneous mutation or clonal spread, can affect all agents in the class. As with macrolides, although the great majority of patients do very well on standard regimens, the concern is that the frequency of clinical failures will rise as the frequency of resistance rises.

**What Is the Optimal Empiric Treatment for CAP?** Optimal therapy for CAP is not one question and does not have one answer. A number of variables enter into the decision-making process. These variables include

Table 3 Univariate risks for lethal pneumococcal infection in adults<sup>a</sup>

Characteristic or factor	Relationship to HP reported in reference:						Relationship to HB reported in reference:							Relationship to IP reported in reference 120	
	7	14	119	92	*	111	27	150	37	97	1	82	20	125	49
<b>Admission demographics</b>															
Age	□	■	■	■	□	□	□	□	■	□	■	□	□	□	□
Gender	□	□	□	□	□	■	□	□	□	□	□	□	□	□	□
History of:															
Institutionalization						■									
Splenectomy						□									
Cardiac disease						□									
Lung disease	■			□		□		■		□		□		■	
Nosocomial infection			■	■		□		■		■		□		■	
Chronic renal disease	■		■	■		□		■		□		■		□	
Chronic liver disease		■		■		□		□		□		□		□	
Diabetes mellitus			□	□		■		□		□		□		□	
Alcohol abuse	■			□		□		□		□		□			
Stroke				□		■									
HIV infection or immunocompromised state	□		□	□		□	■	■		□		□			
Malignancy		■		■		■		■		■		□			
Chemotherapy		■				□		■		■		□			
Smoking	□			□		□	□	□	■						
Parenteral nutrition									■						
Indwelling urinary catheter									■						
Intravenous catheter									■						
<b>Clinical signs</b>															
Unclear history or confusion						■									□
Absence of pleuritic pain															
Presence of:															
Respiratory symptoms	■			□		■									
Chills															
Shock	■	■	■				■	■			■	■	■	■	
Absence of fever				■		■			□		■	■	□	■	
Elevated respiratory rate	■	□	□			■					■	■	□	■	
Elevated pulse	■														
Rapidly fatal disease											■		■	■	
<b>Laboratory results</b>															
Leukopenia	□			■		■			□		■	□	□	□	□
Reduced immature polymorphonuclear leukocyte count	□			□							■				



\*HP, pneumonia requiring hospitalization; HB, bacteremia requiring hospitalization; IP, pneumonia requiring admission to the ICU; \*, Rubins and Janoff, unpublished; open square, tested as a risk factor but no significant differences found; closed square, risk factor associated with significant difference.

Table 4 Multivariate risks for death from pneumococcal infection in adults<sup>a</sup>

Characteristic or factor	Relationship to HP reported in reference:			Relationship to HB reported in reference:				Relationship to IP reported in reference 99	
	7	92	*	27	159	37	97	82	20
<b>Demographics</b>									
Age		■	■		■	■	■	■ <sup>b</sup>	
History of:									
Nosocomial infection		■				■			
Cardiac disease						■			
Lung disease		■							
HIV infection or immunocompromised state	■				■	■			
Diabetes mellitus				■			■		
<b>Clinical signs</b>									
Shock	■							■	■
Absence of fever		■							
Impaired alertness		■							■
Renal failure	■								■
Bacteremia									■
Leukopenia							■		■
Thrombocytopenia							■		
Metabolic acidosis							■		
Rapidly fatal disease					■	■		■	
Critical illness		■			■				
Suspected aspiration	■								
Multilobar infection	■			■				■	
Pleural effusion	■			■				■	
<b>Factors arising during illness</b>									
Meningitis								■	
Mechanical ventilation									■
Development of complications							■		
Parenteral nutrition						■			

<sup>a</sup>HP, pneumonia requiring hospitalization; HB, bacteremia requiring hospitalization; IP, pneumonia requiring admission to the ICU; \*, Rubins and Janoff, unpublished; closed square, risk factor associated with significant difference in multivariate model.

<sup>b</sup>Age, >80 years.

the severity of acute illness, which may require treatment as an outpatient or as an inpatient in a hospital or in the ICU, treatment at home versus in the hospital, underlying health status and comorbidities, and the overall severity of illness. Others include risks for infection with less common organisms, such as *Pseudomonas aeruginosa* (e.g., chronic obstructive pulmonary disorder, underlying malignancy, prior use of antibiotics, and rapidly progressive radiographic findings upon more standard therapy) (13) and methicillin-resistant *Staphylococcus aureus*, the setting in which the infection began (outpatient environment, nursing facility, or hospital), the prior use of specific antibiotics within the preceding 3 months, and the prevalence of resistant or-

ganisms in the local community. The IDSA and the American Thoracic Society (ATS), as well as other professional organizations, such as the thoracic societies of Britain, Canada, France and other countries, have comprehensively evaluated the data for each of these factors. The evidence-based IDSA-ATS guidelines for empiric therapy in each setting are presented in Table 5 (90).

In considering the best regimens, most guidelines, reports, and experience suggest that combination therapy is indicated for patients with severe pneumonia admitted to the ICU. Present data suggest that the combination of a  $\beta$ -lactam with a macrolide may be superior to a  $\beta$ -lactam alone for invasive pneumococcal pneumonia

**Table 5** IDSA guidelines for recommended empirical antibiotics for community-acquired pneumonia (2007)<sup>a</sup>

## Outpatient treatment

1. Previously healthy and no use of antimicrobials within the previous 3 months:
  - A macrolide (strong recommendation; level I evidence)
  - Doxycycline (weak recommendation; level III evidence)
2. Presence of comorbidities such as chronic heart, lung, liver, or renal disease; diabetes mellitus; alcoholism; malignancies; asplenia; immunosuppressing conditions or use of immunosuppressing drugs; or use of antimicrobials within the previous 3 months (in which case an alternative from a different class should be selected):
  - A respiratory fluoroquinolone (moxifloxacin, gemifloxacin, or levofloxacin [750 mg]) (strong recommendation; level I evidence)
  - A  $\beta$ -lactam plus a macrolide (strong recommendation; level I evidence)
3. In regions with a high rate (>25%) of infection with high-level ( $MIC \geq 16$  mg/ml) macrolide-resistant *S. pneumoniae*, consider use of alternative agents listed above in item 2.

## Inpatients

For patients without comorbidities (moderate recommendation; level III evidence):

## Inpatients, non-ICU treatment

- A respiratory fluoroquinolone (strong recommendation; level I evidence)
- A  $\beta$ -lactam plus a macrolide (strong recommendation; level I evidence)

## Inpatients, ICU treatment

- A  $\beta$ -lactam (cefotaxime, ceftriaxone, or ampicillin-sulbactam) plus either azithromycin (level II evidence) or a respiratory fluoroquinolone (level I evidence) (strong recommendation)

For penicillin-allergic patients, a respiratory fluoroquinolone and aztreonam are recommended

## Special concerns

If *Pseudomonas* is a consideration:

An antipneumococcal, antipseudomonal  $\beta$ -lactam (piperacillintazobactam, ceftazidime, imipenem, or meropenem) plus either ciprofloxacin or levofloxacin (750 mg)

or

The above  $\beta$ -lactam plus an aminoglycoside and azithromycin

or

The above  $\beta$ -lactam plus an aminoglycoside and an antipneumococcal fluoroquinolone

For penicillin-allergic patients, substitute aztreonam for above  $\beta$ -lactam.

(Moderate recommendation; level III evidence)

If CA-MRSA is a consideration:

Add vancomycin or linezolid (moderate recommendation; level III evidence)

<sup>a</sup>From reference 91. CA-MRSA, community-acquired methicillin-resistant *S. aureus*; ICU, intensive care unit.

(100, 101). However, for 2,453 CAP patients studied (among 8,975 evaluated), the use of ceftriaxone, ceftriaxone with or without a macrolide, or levofloxacin had no impact on the length of stay or mortality (51).

What are the best criteria for determining optimal regimens? Time to clinical improvement (e.g., the resolution of symptoms such as fever, cough, and chest pain and the ability to return to work or usual activity) may be a very relevant but understudied variable. Appropriate criteria for time to clinical stability (including vital signs and the partial pressure of oxygen in arterial blood) have been advanced and validated (34, 61, 96). In this context, quinolones as a class or specific agents may support more rapid resolution of signs and symptoms than a treatment with a  $\beta$ -lactam with or without a macrolide, but with comparable mortality (44, 89, 156).

However, as discussed cogently by File and Tan (43), comparisons of outcomes must include data on the spectrum of disease severity, underlying diseases, the analysis of etiologic agents, the duration of therapy, readmission, and whether macrolides were or were not included in the comparison group, and we would suggest that which macrolide is used may also be relevant. These data are important criteria for optimal patient satisfaction and outcome, cost containment, and determining the duration of therapy. Guidelines are constrained in their recommendations on the duration of therapy due to the paucity of prospective studies which evaluate this variable. There is a trend for decreasing the length from 7 to 14 days to 5 days for responsive cases treated with either new agents or high initial doses of antibiotics (33, 42).

The goal for clinicians and patients is improved survival, the more rapid resolution of disease and thus decreased need for admission and decreased length of hospital stays, and decreased cost. The real goal of understanding CAP is to realize that a substantial proportion of cases are due to *S. pneumoniae*, often as a complication of influenza, and to prevent these infections with more effective pneumococcal and influenza vaccines; more sensitive, specific, and rapid diagnosis and therapy of pneumococcal infections when they do occur; and objective, standardized, and clinically relevant studies to evaluate both preventive and therapeutic interventions.

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William P. Hausdorff, Angela B. Brueggemann,  
Jill G. Hackell, J. Anthony G. Scott

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## Pneumococcal Serotype Epidemiology

Pneumococcal conjugate vaccines are targeted at the polysaccharide capsule of the bacteria. The polysaccharide capsule is a good vaccine antigen because it is the defining phenotype of the pneumococcus: it is the target of the mature human immune response, and it also influences the epidemiology of the infection (176), the transmission of the pathogen (186), and disease virulence. Isolates lacking a capsule rarely cause invasive disease in humans and are greatly attenuated in virulence in animal models of disease (104, 126). There are 91 distinct pneumococcal serotypes, which can be grouped by immunological relatedness into 46 serogroups.

Conjugate vaccines can elicit protective concentrations of anticapsular immunoglobulin G even in infants. However, it does not appear technically or economically feasible to develop a pneumococcal conjugate vaccine formulation containing 91 separate conjugates. Therefore, from the perspectives of those who design, develop, manufacture, or have to decide upon the purchase and implementation of pneumococcal conjugate vaccines, the question regarding pneumococcal serotype epidemi-

ology is straightforward: which are the most important serotypes to include in a pneumococcal conjugate vaccine formulation in order to have a significant public health impact?

The answer, however, must take into account the epidemiological complexity and dynamism of *Streptococcus pneumoniae*. As early as 1939, it was already possible for Roderick Heffron to compile a magisterial tome that analyzed in considerable detail the major variables underlying serotype epidemiology (88). He recognized that pneumococcal serotypes can differ in their abilities to colonize the nasopharynx, and thus to be transmitted from person to person, and in whether they are likely to cause invasive disease (i.e., invade a normally sterile site such as blood or cerebrospinal fluid) once they do colonize (their invasiveness).

As Heffron also noted, serotypes can differ in whom they commonly affect (e.g., children or adults; neonates, infants, toddlers, or older children; and immunocompromised or immunocompetent individuals), what disease manifestation (e.g., conjunctivitis, otitis media,

pneumonia, bacteremia, or meningitis) they are more likely to cause and with what complications, whether they are likely to be resistant to antimicrobial agents, where they are most prominent geographically, and how they cause disease (whether they cause outbreaks or are endemic in a region) (81). Taking these factors into account, this chapter attempts to summarize the relative prevalences of the most common serotypes prior to and following the introduction of the heptavalent pneumococcal capsular polysaccharide vaccine (PCV-7). The chapter concludes with thoughts about the selection of serotypes for future-generation conjugate vaccines.

## WHAT SEROTYPES ARE MOST COMMONLY FOUND IN CARRIAGE STUDIES, AND WHAT IS KNOWN ABOUT THEIR RELATIVE INVASIVENESS?

### Serotype Distribution of Carriage Isolates

A remarkable feature of the global epidemiology of pneumococcal carriage is the consistency of the dominant carriage serotypes in very different environments and at different times. Serogroups 6, 14, 19, and 23 are almost invariably among the five most highly ranked serogroups in collections of nasopharyngeal isolates from healthy young children. This has been true for children in Paddington, London, United Kingdom, in the 1950s (128) and at an American day care center in the 1970s (122); for Aboriginal children living near Alice Springs, Australia, and infants in Alabama in the 1980s (72, 77); and for healthy children in the highlands of Papua New Guinea and in The Gambia, rural Malawi, Central Asia, Vietnam, The Netherlands (Amsterdam), and Finland in the 1990s (14, 56, 58, 78, 121, 137, 157, 194) and Hertfordshire, United Kingdom, and rural Mozambique in the last 5 years (95, 199). These serogroups also collectively account for the majority of all pneumococcal isolates cultured from samples from the nasopharynx. Other serogroups frequently carried among children are 3, 4, 9, 11, 13, 15, 18, and 33.

Since the prevalence of colonization among adults is usually less than half that observed among children, it is harder to precisely characterize the serotype patterns in adults (2, 64, 77, 166, 171). Nonetheless, it appears that the most common serotypes found among adults differ from those among children and that the serotype distribution is broader. For example, among Aboriginal adults in Alice Springs, serogroups 3, 21, 7, 22, 23, and 29 dominated (in that order) and accounted for 52% of carriage isolates, while in Aboriginal children, serogroups

23, 19, and 6 were the most commonly carried and accounted for 48% of the isolates (77). In a small study of 38 isolates from adults attending primary health care facilities in Israel, one-quarter of the isolates were nontypeable and the most common serotypes were 6A and 14. In contrast, among 211 isolates from children attending the same clinics, only 5% were nontypeable and the most common serotypes were 6B, 23F, and 19F (166). The proportion of isolates that were PCV-7 serotypes was estimated in a prevalence survey in Kenya to be 47% among isolates from children <5 years of age and 25% among isolates from older children and adults (2). The switch in serotype patterns occurs at approximately 5 years of age (128), but even children below this age carry more non-PCV-7 vaccine types with each year of age (47).

The interpretation of the serotype distribution data from carriage studies is hampered by several constraints. First, the sensitivity of different detection methods varies from serotype to serotype. Inoculation of the nasopharyngeal sample into the peritoneum of a mouse yields cultures of *S. pneumoniae* approximately twice as frequently as direct plating onto blood agar (92), unless the agar is supplemented with 5 µg of gentamicin/ml. However, either method alone will miss 25 to 35% of the isolates detected by the two methods combined. Furthermore, direct plating favors serogroups such as 14, 19, and 23, which grow poorly in the mouse (41, 64).

Second, it has been estimated that ~30% of all carriers harbor several pneumococcal serotypes simultaneously (71), but it is not always clear which serotypes dominate numerically and are thus detected preferentially. In addition, the duration of colonization varies by serotype and by the age of the host and can range widely.

Finally, the size and duration of carriage studies rarely do justice to the complexity of a system with over 90 different potential serotypes in a highly dynamic environment. The changing prevalence of serotypes was nicely illustrated in a 4-year study of children in an American day care center (122). In the cohort, there was a clear epidemic of serogroup 18 carriage which appeared suddenly, peaked after 2 months, and then declined to baseline after a year. A similar slow epidemic of type 3 carriage has been reported in a longitudinal family study (193).

In light of these technical and methodological considerations, it is clear that any quantitative conclusions from carriage studies must be carefully considered. There are, however, some serotypes that are very rarely detected in any carriage studies. For example, as early as 1915 it was noted that while serotypes 1 and 2 caused

most invasive pneumococcal disease (IPD), they were rarely isolated from the nasopharynges of healthy individuals (50). Serotype 46 caused IPD in Papua New Guinea but is rarely observed among carriage isolates (8, 137). These observations suggest that certain serotypes have a high invasive-disease attack rate and, thus, raise the question of how such highly invasive serotypes are maintained in the community if they are rarely carried.

One possibility is that they are transmitted directly from individuals with pneumococcal pneumonia through coughing. Pneumococcal carriage studies of patient contacts in the middle of the 20th century confirmed that patients with pneumonia are highly efficient in transmitting infection to their close contacts, but pneumococcal carriage due to highly invasive serotypes lasting more than 1 month was rare among these contacts (184, 185). The association of highly invasive serotypes with outbreaks of pneumonia gives more credence to the idea of person-to-person transmission, as does preliminary evidence (48) suggesting that levels of carriage of serotypes 1 and 5 are specifically and greatly increased in children with severe pneumonia. However, most cases of disease due to highly invasive serotypes are sporadic, and it is difficult to identify the primary source of infection. Interestingly, a subset of the population become chronic carriers of invasive types, particularly convalescent persons and patients with chronic bronchitis (43). In 1939, Heffron argued that serotype 1, though accounting for only 1.2% of all pneumococcal isolates from carriers, would be carried by 21,000 of the 4.35 million inhabitants of Massachusetts, a number sufficient to support the observed frequency of disease cases (88). Perhaps even a very low level of carriage of highly invasive serotypes, mainly among ill individuals, is sufficient to maintain those serotypes in the human population.

### Invasiveness of Individual Serotypes and Genotypes

Invasive disease potential, or invasiveness, is a measure of the ability of pneumococci to progress from nasopharyngeal carriage to invasive disease in humans. It differs from virulence in that the latter is often used to describe the ability of a pathogen to cause disease in laboratory animals. Invasiveness is conceptually similar to the attack rate of a pathogen, which measures the risk of disease as a result of pathogen acquisition.

One study used multilocus sequence typing (MLST) to genotypically compare 501 pneumococcal isolates recovered from 1994 to 2001 from children <5 years of age in Oxfordshire, United Kingdom. The bacteria were

isolated either from the nasopharynges of healthy children or from normally sterile sites of IPD patients (19). Importantly, both carriage and invasive isolates were derived from children of the same age group in the same geographic location and at the same time period. The invasiveness was calculated as the ratio of the odds of finding a given serotype or genotype among invasive disease isolates to the odds of finding the same serotype or genotype among carriage isolates.

Several conclusions were drawn from this study. First, with a few recognized exceptions, there was a strong association between the MLST genotype and the serotype—that is, each MLST genotype was associated with a single serotype. In fact, only a few globally distributed MLST genotypes are known to express multiple serotypes. Second, individual serotypes and major genotypes differed markedly in their invasiveness (80- to 120-fold variation). Serotypes 1, 4, 14, and 18C were significantly associated with invasive disease, whereas serotype 23F was significantly associated with carriage. In addition, although the differences were not statistically significant, serotypes 7F, 9V, and 19A were more often associated with invasive disease and serotypes 6B and 19F were more often associated with carriage. Finally, strains with different MLST genotypes but expressing the same serotype had the same invasiveness, suggesting that the capsular serotype may be more important than the genotype in the ability of pneumococci to cause invasive disease. In other words, there is little evidence at present that different clones of the same serotype have markedly distinct epidemiological properties, although further study may reveal such differences (21, 173, 182).

A subsequent meta-analysis incorporated seven diverse, globally distributed data sets from similarly matched pediatric IPD and carriage strains to calculate serotype- and serogroup-specific invasiveness by using an odds ratio. These data supported the conclusion that pneumococcal serotypes differ in invasiveness and that serotypes and serogroups 1, 4, 5, 7, 14, and 18C are associated with invasive disease, whereas serotypes and serogroups 3, 6A, 6B, 15, 19, and 23 are associated with carriage. Moreover, there was a significant inverse relationship between invasiveness and carriage prevalence: the most invasive serotypes were the least commonly carried, whereas the serogroups most prevalent among carriage isolates were the least invasive (18).

One criticism of the cross-sectional studies described above is that their odds ratio estimates may be biased by serotype-specific differences in carriage duration, since serotypes carried for long durations are likely to be sampled more frequently than those carried for short

durations: oversampling among carriage isolates would therefore result in a lower estimate of invasiveness.

To address this potential bias, a recent study calculated serotype-specific attack rates based upon the incidence of invasive disease relative to the incidence of nasopharyngeal acquisition among children <2 years of age (183). The serotypes with the highest attack rates (>20 cases of IPD/100,000 acquisitions) were 1, 5, 9A, 4, 14, 12F, 7F, 8, 9V, 18C, and 19A, and those with the lowest attack rates (<10 IPD cases/100,000 acquisitions) included 38, 3, 23F, 6A, 19F, 16F, 20, 35F, 10A, 15B, and 15C (in addition to other, less common serotypes). Furthermore, such attack rates were significantly correlated ( $P < 0.001$ ) with the invasive odds ratios from the meta-analysis described above (18). Although serotype-specific carriage duration did vary (6 to 20 weeks), in this study the variation was small compared to the variation in disease incidence: therefore, the variation in the serotype-specific duration of carriage cannot explain the serotype-specific variation in invasiveness.

The results of this study supported the use of invasive odds ratios as a reliable method for determining serotype-specific invasiveness. If invasiveness is primarily a function of capsular expression, then repopulating the nasopharyngeal niche with serotypes of low-level invasiveness by using multivalent vaccines may produce a large decrease in the incidence of IPD. Gray and colleagues suggested in 1980 that nature had already achieved just such an adaptation in children. The common serotypes causing disease in the preantibiotic era (e.g., serotypes 1 and 2) were highly invasive compared to the common nasopharyngeal colonizers, serogroups 6, 19, and 23. Gray argued that the tolerance exhibited by young children to prolonged carriage of these serogroups might reduce the total risk of pneumococcal disease by protecting them against colonization with more invasive serotypes (72). The cost of this natural adaptation is disease—but at much lower frequency—caused by the colonizing types themselves.

This idea assumes that there is competition among serotypes in the nasopharynx, for which there is some experimental animal evidence (118). It also assumes that the nasopharyngeal competition is shaped exclusively by capsular characteristics. Using these assumptions, mathematical models of carriage that examine serotype competition in the presence of serotype-specific vaccines suggest that the pneumococcal population in the nasopharynx will shift toward nonvaccine serotypes over time (119). Models have not yet been developed to account for variation in the invasiveness of competing types. Some serotypes that appear to have increased in relative frequency as causes of IPD in the United States

following the introduction of PCV-7 (e.g., 15B, 15C, 22F, and 33F) have attack rates that are lower than those of vaccine serotypes, though others (3, 7F, 19A, and 38) have attack rates that are broadly equivalent to those of the vaccine serotypes (9, 24, 69, 101, 183, 192).

Taken together, the above analyses suggest that serotype replacement in the nasopharynx, which has occurred after widespread use of PCV-7 in the United States, should not necessarily result in commensurate invasive disease replacement in the general population, unless the replacing serotypes are as invasive as the original serotypes. Finally, it should be noted that even serotypes with a low level of invasiveness can and do cause a substantial portion of invasive disease when such serotypes are highly prevalent within the carriage population.

Although there are significant differences in invasiveness among serotypes and genotypes, the same cannot be said for acute otitis media (AOM) disease potential. A Finnish study found that the distribution of pneumococcal serotypes causing AOM was not significantly different from that in the carriage population (75). Furthermore, the differences in the abilities of serotypes or genotypes to cause AOM were small in contrast to the differences in invasiveness estimates. These results suggested that if serotype replacement occurred among carriage isolates following the implementation of the conjugate vaccines, then this effect could result in reduced vaccine efficacy for the prevention of AOM. Consistent with this finding, significant serotype replacement was observed in a study of PCV-7 efficacy against AOM (54). Whether the same replacement phenomenon occurred in the nonbacteremic pneumonia trials is unclear due to the lack of serotype-specific diagnostic capabilities (13, 44, 103).

### Predicting Vaccine Serotype Coverage by Using Nasopharyngeal Isolates

To determine the most effective formulation for multivalent vaccines, we need information on which serotypes are causing disease in many different geographical settings and populations. Therefore, in poor areas with little invasive disease serotype data, can we use samples of nasopharyngeal isolates, which are easy to obtain in cross-sectional studies, to predict the likely distribution of serotypes of invasive disease? The central assumption of this approach is that serotypes are generally equally invasive, which, as discussed above, is not true. However, much of the variation in invasiveness among serotypes is accounted for by the highly invasive outliers, serotypes 1 and 5 (18). For the seven serotypes in PCV-

7, the invasiveness indices are closer, and it is possible that carriage collections may give a reasonable approximation of PCV-7 coverage against invasive disease.

Two centers in Africa recently investigated this question. Nasopharyngeal pneumococci were obtained from 2,063 villagers among a population of nearly 4,000 people in 12 villages in the western region of The Gambia (90). Over the same time period, 463 invasive isolates were collected from patients admitted to the Medical Research Council hospital in Fajara, The Gambia, and serotyped (3). The proportions of PCV-7 or related serotypes among carriage and invasive isolates, respectively, were 0.70 and 0.65 among infants, 0.61 and 0.57 among children aged 1 to 4 years, 0.36 and 0.30 among children 5 to 14 years, 0.30 and 0.31 among young adults, and 0.34 and 0.26 among adults aged  $\geq 40$  years. In a similar Kenyan study, the proportions of PCV-7 serotypes among carriage and invasive isolates from children aged  $< 5$  years were identical (0.41) (1). Therefore, at least based on the results of these two African studies, cultures of nasopharyngeal samples from healthy people may provide a reasonable estimate of PCV-7 coverage against invasive disease. It should be noted, however, that in these settings nasopharyngeal carriage studies would greatly underestimate the disease-preventing potential of higher-valent formulations such as the 10-valent pneumococcal *Haemophilus influenzae* protein D conjugate vaccine (PHiD CV-10) and the 13-valent pneumococcal conjugate vaccine (PCV-13).

## WHICH SEROTYPES MOST COMMONLY CAUSE INVASIVE AND MUCOSAL DISEASE IN CHILDREN AND ADULTS, AND WHAT EPIDEMIOLOGICAL VARIABLES MOST AFFECT THE SEROTYPE DISTRIBUTION?

### Pediatric Pneumococcal Conjugate Vaccination: What Age Group Is Appropriate for Serotype Analyses?

When considering the public health benefit of an infant pneumococcal conjugate vaccination program, one must first decide what age group to study. Some serotype analyses focus solely on children  $< 2$  years old (97), perhaps since most pediatric immunization programs provide two or three doses in the first year of life, followed by a booster dose in the middle of the second year. However, this narrow focus underestimates the direct impact of protein-polysaccharide conjugate vaccines, since they elicit immunological memory that can persist for 5 years or more (15, 61, 124, 151). In addi-

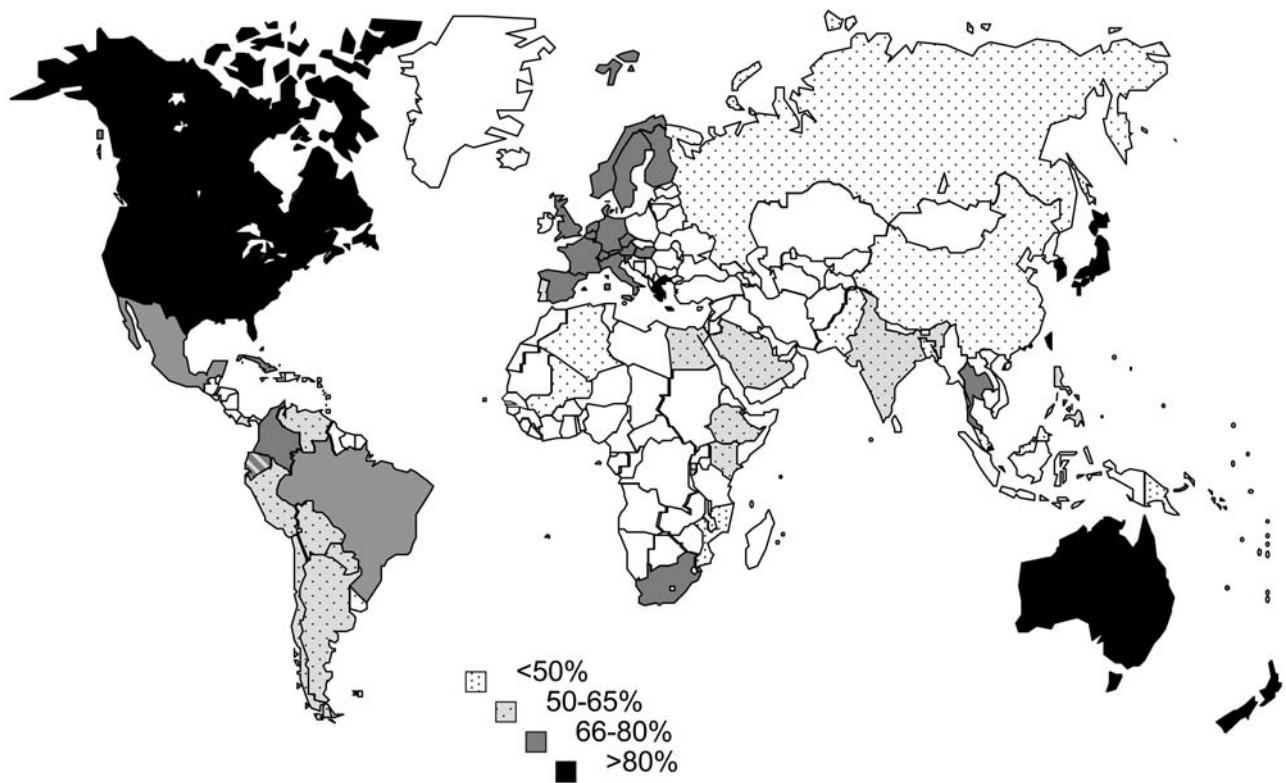
tion, a significant proportion of pneumococcal disease, some of which can be quite severe (e.g., complicated parapneumonic effusions), occurs in children over the age of 2 years (81, 83). Both factors suggest that a more appropriate age group for evaluating disease-causing serotypes is children of 5 or 6 years of age or less.

### IPD Serotype Coverage in Children with Three Vaccine Formulations

At present, one pneumococcal conjugate vaccine formulation is registered, PCV-7 (Wyeth; Prevnar/Prevenar), containing conjugates against serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F, each including CRM<sub>197</sub>, a nontoxic variant of diphtheria toxin. This vaccine also has a convincingly high level of cross protection against invasive disease and AOM caused by serotype 6A (54, 204, 205). A 10-valent conjugate formulation (PHiD CV-10, comprising PCV-7 serotypes plus 1, 5, and 7F) is in an advanced stage of development by GlaxoSmithKline (5a). It is adapted from an 11-valent formulation in which each serotype was conjugated to protein D, an outer membrane protein from *H. influenzae* (163). The 11-valent formulation prevented vaccine-related otitis media and was also shown to elicit antibodies with functional immunogenicity (opsonophagocytic activity) against 6A comparable to that seen with PCV-7 (144). Finally, there is a 13-valent formulation (PCV-13, comprising PHiD CV-10 serotypes plus 3, 6A, and 19A, each conjugated to CRM<sub>197</sub>) under development by Wyeth (5a).

Figures 1 to 3 depict the percentages of IPD due to the serotypes covered by PCV-7, PHiD CV-10, and PCV-13 (in each case including 6A) in children  $< 5$  or  $< 6$  years of age. All data shown are from studies conducted prior to the implementation of PCV-7 infant immunization programs. These averages are based on an extensive review of the medical literature of the past 15 years but are not, due to methodological differences among the studies, mathematical averages of serotype coverage values. When multiple studies existed for a single country, larger and more recent studies restricted to the selected age range were weighted more heavily.

Since our previous attempt to summarize the global serotype landscape in 2000 (79), many more studies have provided information at the serotype level (not just the serogroup), specifically for children in the 0- to 5-year-old age range, and included isolates from both blood and cerebrospinal fluid. However, a number of studies from the most populous countries of the world consist of relatively few isolates collected over a short time period, which limits their validity in representing all IPD.



**Figure 1** Percentages of IPD isolates represented in the PCV-7 formulation and recovered from children  $<5$  years of age. As indicated in the figure, shading refers to the percentages of all IPD isolates identified as serotypes 4, 6A, 6B, 9V, 14, 18C, 19F, and 23F. Data sources are references cited in reference 79 and references 3, 4, 6, 11–12, 16, 17, 23, 25–29, 31–37, 42, 44, 49, 60, 65, 67, 70, 86, 87, 91, 94, 96–98, 100, 102, 103, 105–107, 109–111, 113, 115, 117, 123, 124, 132, 134–136, 138, 142, 143, 153, 154, 156, 158–162, 164, 167–169, 172, 174, 177–179, 188–190, 195–200, 202, 203, 208.

With these caveats in mind, Fig. 1 reveals that while the proportion of serotypes in children potentially covered by PCV-7 varies markedly from region to region and even from country to country, in most places PCV-7 serotypes account for more than 50% of isolates. In Western Europe, at least two-thirds of all isolates are covered by PCV-7, and in the United States, Canada, Australia, New Zealand, and several Pacific Rim countries, PCV-7 covers more than 80% of isolates.

PHiD CV-10 serotypes cover at least two-thirds of all invasive isolates from children aged  $<5$  years in virtually all countries studied and more than 80% of isolates in Western Europe and parts of South America (Fig. 2).

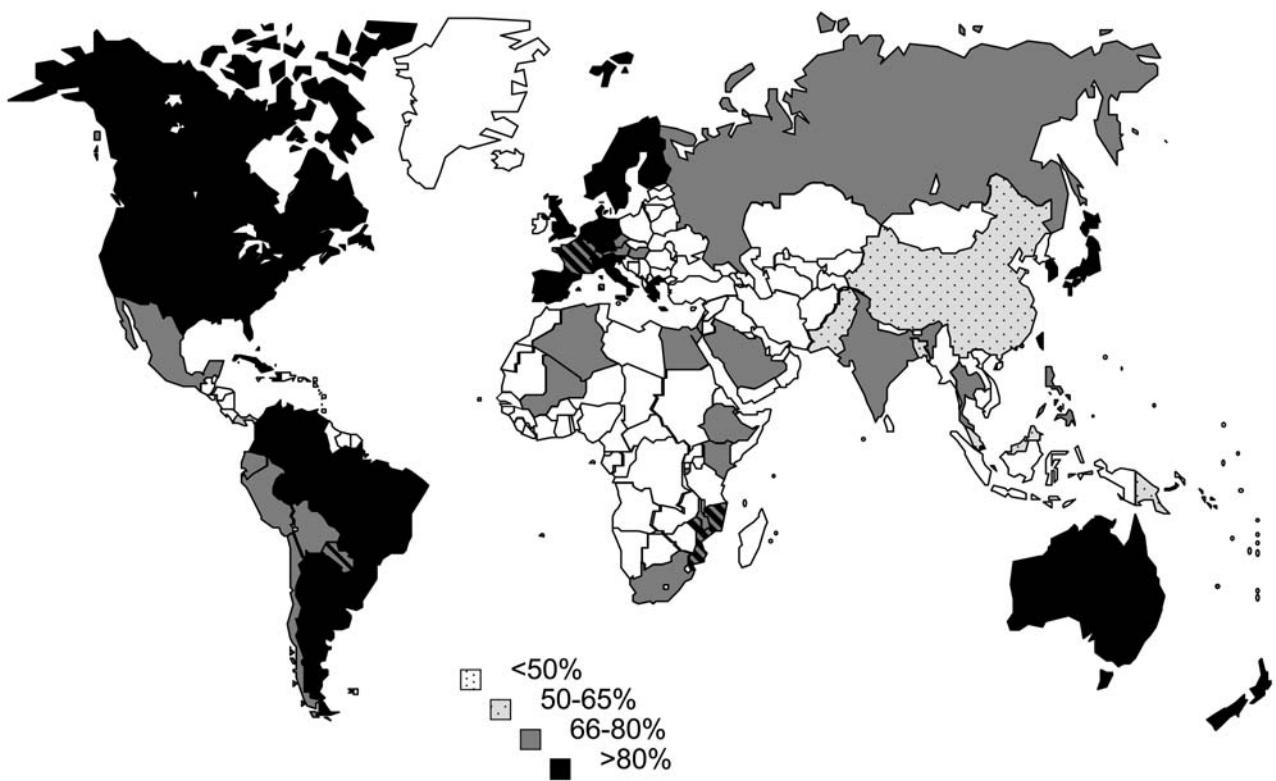
Finally, PCV-13 raises the IPD serotype coverage level to more than 80% among children aged  $<5$  years in almost all regions, with the possible exceptions of the Middle East and South Asia (Fig. 3). However, most

data from these areas come from small studies concentrating on meningitis isolates, which may not be representative of the spectrum of pediatric IPD serotypes in that region.

It should be noted that the global averages presented here do not reflect the likely significant effect of antimicrobial pretreatment on the distribution of serotypes recovered in studies, nor the year-to-year variations in the prominence of certain types (81–83). The impact of PCV-7 on serotype distribution in the United States is discussed later in the chapter.

### Serotypes Causing AOM Among Children

Only a handful of studies describe the serotypes responsible for episodes of otitis media, and most studies are from Europe and the United States. Furthermore, only a subset of those studies are from patients with AOM



**Figure 2** Percentages of IPD isolates represented in the PHid CV-10 formulation and recovered from children <5 years of age. As indicated in the figure, shading refers to the percentages of all IPD isolates identified as serotypes 1, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19F, and 23F. Countries marked with two shades correspond to percentage of serotype coverage exactly intermediate between the percentages represented by the two shades. Data sources are those cited in the legend to Fig. 1.

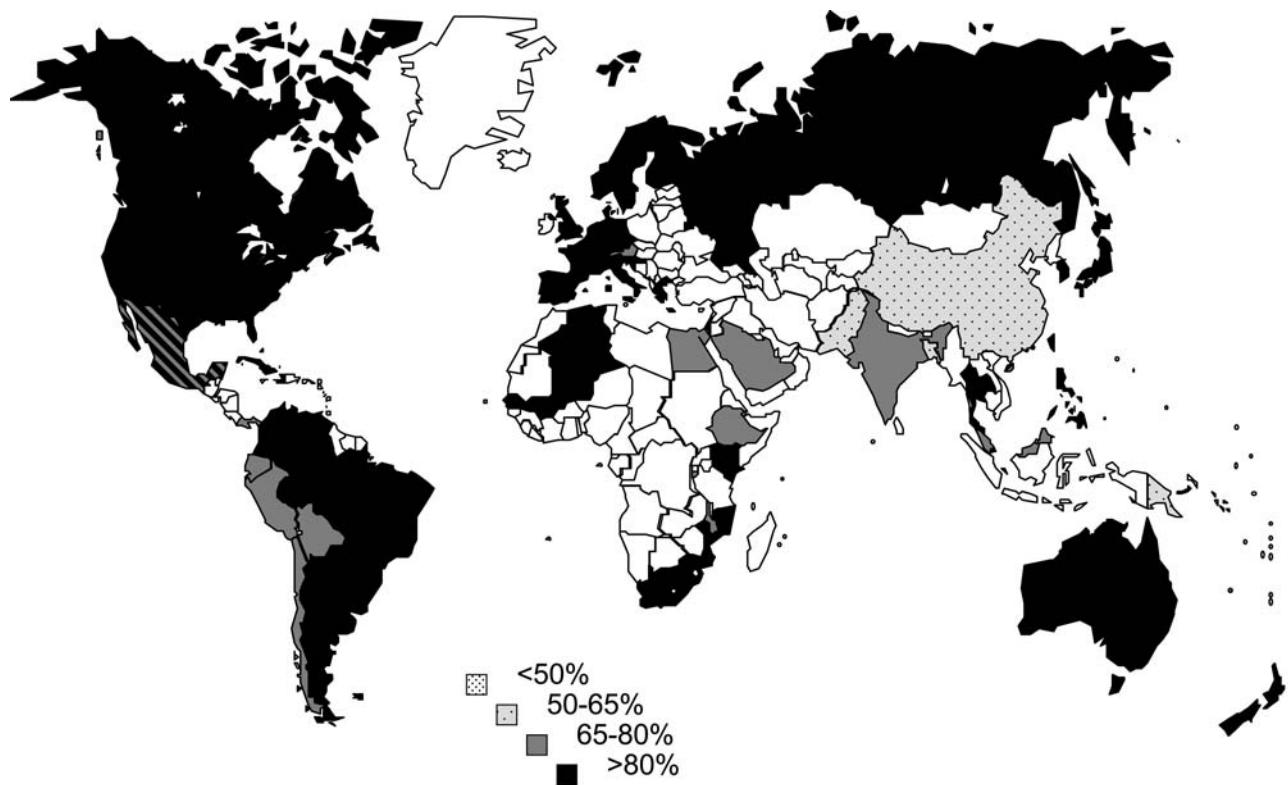
since tympanocentesis and myringotomy are rarely performed in routine clinical practice, and thus, even these studies may be biased toward children with problematic AOM or even otitis media with effusion. Nonetheless, in the absence of pneumococcal conjugate vaccine introduction, the dominant serotypes tend to be those represented in PCV-7, as well as serotypes 19A, 3 (especially in older children), and to a lesser extent, 16F (especially in Australian Aborigines) (7, 54, 68, 85, 113, 127, 130, 152, 162, 170, 178, 180, 208). In one study, serotypes 1 and 5 were major contributors to pneumococcal AOM (170), but in most settings, including places where serotypes 1 and 5 are significant causes of IPD, such as Brazil and Israel, these serotypes account for only a small proportion of AOM cases (16, 178, 180). As a consequence, the PCV-7 and PHiD CV-10 serotypes (including 6A) tend to constitute 60 to 80% of all pneumococcal AOM isolates, while PCV-13 raises the sero-

type coverage by 6 to 10%. In contrast to IPD (see below), there is no compelling evidence that otitis media disease varies in severity by serotype (152).

## Serotypes Causing Nonbacteremic Pneumonia in Children

The only generally accepted way to determine that the etiology of pediatric pneumonia is pneumococcal is to isolate the bacterium from the blood or pleural fluid. Due to the high prevalence of asymptomatic nasopharyngeal colonization with pneumococci in children, neither carriage, urine, nor serological analyses are yet considered to be specific (45, 46, 55, 207).

For the subset of pediatric patients with severe, complicated pneumonia (of increasing concern in a number of countries, including the United States, the United Kingdom, Spain, France, and Taiwan), pleural fluid samples have been routinely obtained and analyzed by culture



**Figure 3** Percentages of IPD isolates represented in the PCV-7 formulation and recovered from children  $<5$  years of age. As indicated in the figure, shading refers to the percentages of all IPD isolates identified as serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F. Data sources are those cited in the legend to Fig. 1.

and molecular detection techniques in some centers. In virtually every one of these studies, serotypes 1, 3, and to a lesser extent, serotype 5 have consistently accounted for a much higher proportion of samples from patients with complicated parapneumonic effusions (empyemas) than from the overall pediatric IPD patient population in the same countries (24, 63, 81, 83, 93, 112, 145, 191).

The most direct way to demonstrate the relevance of pneumococcal conjugate vaccine formulations to the serotypes causing pediatric pneumonias has been through the vaccine-probe method, i.e., the vaccine efficacy studies conducted with PCV-7 in Northern California (13) and with a nine-valent formulation (PCV-9, also containing serotypes 1 and 5) in South Africa (103, 125) and The Gambia (44, 139). In the three studies, 20 to 37% of cases of radiologically confirmed alveolar pneumonia were prevented by vaccination, indicating that at least that fraction of disease is caused by serotypes represented in the vaccine formulations. If the pneumococcus causes only two-thirds (for example) of this type of pneumonia and the true vaccine efficacy against pneumonia caused by vaccine serotypes is closer

to the 57% vaccine type efficacy seen in the otitis media efficacy trial (54), then these results would indicate that  $>60\%$  of pneumococcal serotypes causing radiologically confirmed pneumonia are represented in PCV-9.

#### Other Serotype-Specific Properties To Be Considered

Serotypes 6A, 6B, 9V, 14, 19A, 19F, and 23F tend to predominate as antimicrobial-resistant types in most studies (81). All three vaccine formulations cover these serotypes, with the exception of serotype 19A, which is covered only by PCV-13. In fact, the countries where pneumococci exhibit the highest levels of penicillin resistance, such as those in the Pacific Rim (e.g., Taiwan, Hong Kong, Japan, and Korea), tend to be those with the highest PCV-7 coverage levels (83).

Serotype 19A is also a prominent cause of otitis media in some studies and has the distinction of being the major replacement serotype causing invasive disease in the post-PCV-7 era in the United States. It is often assumed that vaccine pressure by PCV-7 is therefore solely responsible. However, it is also possible that the emer-

gence of serotype 19A is primarily a secular trend and/or is driven or sustained by the continued high level of antibiotic use in the United States (150).

### IPD Serotypes in Adults

There are relatively few studies of the serotypes responsible for disease in adults compared to pediatric disease (79). Serotype information is lacking for large regions of the world, including much of Africa, the Middle East, and Asia (see references 32 and 206 for a few exceptions), as well as most of Latin America, apart from Brazil and Colombia (4, 16). The available data, mainly from the United States and Europe, suggest that adult disease is caused by a broader spectrum of serogroups than is pediatric disease. In the earlier global review, four to five serogroups accounted for half of IPD cases in older children and adults, but two to three serogroups accounted for this proportion of disease in young children (79). As in the pediatric studies, the most prominent serogroups were 6, 9, 14, and 1, but serotypes 3, 8, and 12F also make a significant contribution to adult disease (16, 79, 105). IPD caused by serotype 3 has a particularly high case fatality rate in adults (81, 89, 149), perhaps because this serotype tends to infect older individuals (5). PCV-7 serogroup coverage was estimated to be 30 to 60% of all IPD isolates and to increase to 60 to 70% with the addition of the serotypes found in the higher-valent conjugate formulations (79).

As is the situation with pediatric IPD, the serotypes responsible for adult IPD appear to vary by the patient's age. The highest incidence of IPD and pneumonia is found in elderly adults of  $>65$  years of age, and in some studies the serotypes contained in PCV-7 are more highly represented in  $>50$ - or  $>65$ -year-olds than in younger adult age groups (16, 32, 59, 89).

### WHAT SOCIETAL FACTORS AFFECT SEROTYPE DISTRIBUTIONS?

Societal changes can influence the serotype distribution associated with pneumococcal disease, including shifts in socioeconomic status or living patterns, antibiotic use, the aging of the population (with an attendant increase in chronic disease, immunosuppressive therapies, and/or immune senescence), the prevalence of human immunodeficiency virus (HIV) infection, and other factors that increase the risk for pneumococcal disease.

#### Epidemic or Outbreak Serotypes

In a literature review that considered IPD in the United States in the period from 1928 to 1988, Feikin and

Klugman found that the percentage of IPD cases due to PCV-7 serotypes increased from 15 to 59% for adults and from 53 to 87% for children  $<5$  years of age (57). This shift was accompanied by an overall reduction in the incidence of IPD. The authors explain these findings as a reduction in epidemic pneumococcal disease, and with it a reduction of the serotypes that have been historically associated with epidemics—that is, serotypes 1, 2, 3, and 5 (57).

These serotypes are rarely carried by healthy individuals, although carriage increases during epidemics. Transmission during outbreaks is facilitated by crowding, wood-burning stoves, and other socioeconomic factors (146), but with the improvement of such conditions, large epidemics are less likely. Furthermore, one may predict that as antibiotics decreased the length of illness, they would also decrease the transmission of the organism, further curtailing epidemic disease.

In developing-world populations, in which adverse socioeconomic conditions and limited access to antibiotics still pertain, these serotypes remain prominent causes of disease (12, 81, 111, 169, 187, 196). For example, there is emerging evidence that serotype 1 is a significant contributor to meningitis outbreaks in West Africa (206). Not surprisingly, the serotype distributions may be very different during epidemic compared to intraepidemic periods. For example, among Australian Aborigines  $<5$  years of age, serotype 5 was not isolated at all for an 11-year period, and in the next 12 months it accounted for 39% of all isolates (196).

Similarly, epidemic serotypes have been documented to cause outbreaks in crowded populations in industrialized countries, including nonelderly adults in jails, homeless shelters, and the military (81). It is also increasingly recognized that serotypes 1 and 5 (and probably 12F) can occur in periodic multiyear waves or outbreaks in the general population in industrialized countries and can cause a considerable portion of disease (83, 108, 143, 187). These large secular variations suggest that societal factors such as crowding cannot fully explain the prominence of certain serotypes. One additional factor contributing to this variation may be influenza, since it has long been known that influenza virus infection can predispose individuals to pneumococcal pneumonia (129). There is some suggestion that severe pneumococcal disease following influenza can involve serotypes 1, 3, and 5 (148), but larger studies will be needed to confirm whether these types are preferentially associated with influenza. These observations highlight the necessity of multiyear surveillance studies in order to be able to draw firm conclusions about serotype distribution in a given population.

### Impact of Chronic Illness and Compromised Immune Defenses

The population-wide transmission of certain serogroups, 6, 14, 19, and 23, is likely driven by the high carriage prevalence of these types in children, which, in turn, is attributable to the slow development of immune responses to these types in childhood (51). It may not be surprising, then, that persons with HIV, who have much higher overall rates of IPD than healthy adults, also have a higher proportion of the pneumococcal disease due to these serotypes (42, 59, 62). HIV-positive women are more likely to have pediatric-serotype disease than HIV-positive men, further implicating transmission from young children as the likely source of the pediatric serotypes (22).

### THE EFFECT OF PCV-7 INTRODUCTION ON THE DISTRIBUTION OF SEROTYPES CAUSING INVASIVE DISEASE

The incidence of IPD due to vaccine serotypes has decreased substantially since the introduction of PCV-7 in the United States, in vaccinated children as well as all other age groups, indicating that pneumococcal transmission was interrupted as a result of the reduction in carriage in the vaccinated pediatric population (30, 204). In addition, as most of the antibiotic resistance was associated with these serotypes, the incidence of IPD due to antibiotic-resistant pneumococci has also decreased in all age groups (106).

Increases have been noted in the incidence of IPD due to serotypes that are not in the vaccine, referred to as serotype replacement disease. While this has been seen in all age groups, for most populations there still has been a dramatic net reduction in all IPD (30). However, the exceptions to this rule are instructive.

Lexau et al. (116) compared IPD cases in adults  $\geq 50$  years of age before (1998 to 1999) and after (2002 to 2003) the routine use of PCV-7 in children. In the latter period, a higher proportion of patients had at least one chronic condition that would be an indication for vaccination with the pneumococcal polysaccharide vaccine (i.e., a pneumococcal high-risk condition). This might suggest that patients with chronic conditions benefited less from herd protection than did other patients. Alternatively, this finding may be just an indication of the increased prevalence of chronic illness in the general population over the two time periods compared. However, another explanation is suggested by the finding that proportionately more episodes of IPD were due to nonvaccine serotypes among HIV patients (61%) than were due to those types among other patients (51%).

This trend was also seen in a second study, in which Flannery et al. (62) looked specifically at the HIV-positive population 18 to 64 years of age before (1998 to 1999) and after (2003) the routine use of PCV-7 and noted a 19% overall reduction in the rate of IPD. IPD due to PCV-7 serotypes decreased 62%, but disease due to nonvaccine serotypes increased 44%. Thus, in this immunocompromised population, the decreases in IPD due to vaccine serotypes were offset to a much larger degree than those in the general population by increases in disease caused by nonvaccine serotypes. These results suggest that immunocompromised and other persons at high risk for pneumococcal infection remain disproportionately susceptible to the increased circulation of nonvaccine serotypes.

Additional support is provided by data from the Centers for Disease Control Active Bacterial Core Surveillance (30), which shows changes in projected numbers of IPD cases in the United States by age group and serotype category, for 1998 to 1999 versus 2003 (see chapter 24, Fig. 4, in this volume). Overall, the decrease in vaccine serotype disease ( $-29,599$  cases) far outweighs any increase in nonvaccine serotype disease ( $+4,721$  cases). However, in the age group of  $\geq 40$  years, in which more of the cases are likely to be in individuals with underlying conditions, more serotype replacement disease ( $+4,158$  cases) relative to the reduction in vaccine serotype disease ( $-12,143$  cases) is seen than in the general population.

The surveillance of the high-risk Alaskan native population before and after PCV-7 introduction has revealed some important trends. Overall IPD rates through 2006 were 40% lower in age groups of children under 2 years than those in the pre-conjugate vaccine era, but this result reflected the combination of a 96% decrease in vaccine type disease and a 140% increase in nonvaccine type disease, and the latter appears to have risen substantially in the past few years (181). Increases in the prevalence of serotype 19A are responsible for much of the nonvaccine type increase. These results are consistent with those of pneumococcal carriage studies conducted in a predominantly Native Alaskan population before (1995 to 2000) and after (2001 to 2003) PCV-7 introduction (74). While vaccine serotype carriage decreased significantly in all age groups, overall, pneumococcal carriage rates were stable in children aged  $< 5$  years and actually increased in adults aged  $\geq 18$  years. It is unclear whether this observed increase is due to secular trends and/or whether a reduction in vaccine serotypes resulted in an enhancement of the ability of adults, but not children, to carry nonvaccine serotypes.

Not surprisingly, these cumulative results suggest that populations at very high risk for pneumococcal dis-

ease remain at high risk for nonvaccine serotype disease and may serve as sentinel populations for emerging non-vaccine serotypes. It is worth noting that similar increases in nonvaccine type disease have not (yet?) been observed in two other high-risk, highly immunized Native American populations, the Navajo and Apache (147), raising the question of whether other societal or epidemiological factors play a role in addition to PCV-7 vaccination.

### HOW DO MOLECULAR GENETIC ANALYSES OF PNEUMOCOCCI HELP US UNDERSTAND THE CHANGING EPIDEMIOLOGY OF PNEUMOCOCCAL SEROTYPES?

The genes responsible for capsular biosynthesis are assembled in a cluster, or cassette, on the pneumococcal genome, in gene orders that are similar among all serotypes. The cassette is flanked by the *dexB* and *aliA* genes (these genes are not involved in capsule production), and four genes likely to be involved in the regulation and export of the capsular components precede a central region of serotype-specific genes (10, 66, 99, 201).

The first description of genetic transformation was offered by Griffith, who injected mice with a mixture of live unencapsulated and heat-killed encapsulated *S. pneumoniae* bacteria and found that the mice developed an infection with pneumococci of the same capsular type as the encapsulated pneumococci he had injected (73). Molecular studies have provided sequence-based evidence that transformation can occur at the capsular locus as a result of recombinational events that replace the capsular genes of a recipient pneumococcus with those from a donor pneumococcus of a different serotype (38–40, 141, 165). Although serotype switching may, in principle, occur with any serotype, a survey of the MLST database ([www.mlst.net](http://www.mlst.net)) to identify isolates with identical genotypes but different serotypes suggests that capsular exchange may be most common among certain serotypes (e.g., 6B, 9V, 14, 15B, 15C, 19A, 19F, and 23F). It is possible that this apparent restriction may be due to sampling bias, as these serotypes are among the most prevalent worldwide and are therefore frequently sampled. A study aimed at identifying *in vivo* examples of capsular exchange among pneumococcal isolates colonizing healthy children did not find any evidence of serotype switching (133), but previous estimates of the frequency of switching ranged from 4 to 6% (53, 140). It remains unclear how often capsular exchange occurs spontaneously in a natural environment.

Why are there so few examples of major clones expressing different serotypes? Perhaps the answer is simply that the capsular cassette is prohibitively large (10

to 30 kb in length [10]) for recombination to occur frequently or that the size at least limits the possibility of capsular exchange between certain capsular types. Alternatively, recombinational events at the capsular locus may be occurring reasonably frequently, but new capsular variants may rarely be fit enough to survive, be transmitted, and cause disease and thus be detected as a capsular exchange event.

It is imperative to understand capsular switching in the context of vaccines which target the capsule for protection from disease. Should the capsule of a pneumococcus under vaccine selective pressure be replaced with a nonvaccine serotype capsule, this progeny strain would have the ability to resist the effects of the vaccine. Should this progeny strain also be readily transmitted and capable of causing disease, then this event could ultimately reduce the long-term effectiveness of any limited-valency conjugate vaccines.

Genotypic analyses of isolates recovered in IPD cases occurring after the introduction of PCV-7 in the United States illustrate both serotype replacement and clonal expansion of existing (pre-vaccine introduction) pneumococcal clones (150). In addition, recently described vaccine escape strains which possess an MLST genotype previously associated only with vaccine serotype 4 now express a nonvaccine serotype 19A capsule. The putative recombinational crossover points around the capsular locus have been detected and suggest a limited number of recombinational events (20, 150). For the moment, this finding is encouraging, because it suggests that the recombinational event that resulted in these vaccine escape strains was a rare occurrence. A similar rare recombinational event was observed in a second study (120). Taken together, the observed serotype replacement in nasopharyngeal carriage and in invasive disease and the evidence of recombinational events at the capsular loci suggest that both environmental and genetic adaptations have occurred among pneumococci in the United States, due at least in part to the immunological pressure imposed by PCV-7. Nonetheless, it is important to reiterate that at present the magnitude of IPD due to replacement serotypes remains small in comparison to the magnitude of vaccine serotype IPD prevented (30).

### THE FUTURE: WHICH SEROTYPES SHOULD BE INCLUDED IN FUTURE GENERATIONS OF PNEUMOCOCCAL CONJUGATE VACCINES?

In light of the dynamics of pneumococcal colonization and disease, especially in the PCV-7 era, an expanded conjugate vaccine formulation would most productively

focus on the more prominent nonvaccine serotypes, especially those that are highly invasive. Within specific immunocompromised populations, such as HIV-positive individuals, however, the distinction between invasive and noninvasive serotypes may be less pronounced, as noted earlier.

### Mucosal Disease

For mucosal disease, otitis media and nonbacteremic pneumonia, it is less clear which serotypes it would be most valuable to add since there appear to be less clear-cut differences in invasiveness among serotypes. The only certain way of preventing mucosal disease is to sterilize the nasopharynx with respect to pneumococci. However, against a species with 91 serotypes to target, it is hard to conceive of a sterilizing vaccine, even if the formulation was restricted to the 30 more common disease-causing serotypes—unless perhaps a common pneumococcal protein antigen were used. However, the biological costs of such a strategy are unknown. Competition among species in the nasopharynx is inevitable to some degree, and a colonizing pneumococcus may be less threatening to health than the *Staphylococcus aureus* or group A streptococcus strain that may replace it. In addition, the fact that PCR and antigen-based diagnostic tests are positive for healthy children (46, 52), as well as the commonly observed phenomenon of mild bacteremia in outpatient presentations, suggests that children are subject to intermittent systemic exposure to pneumococci (131). It is possible that this exposure plays an important role in the maturation of the humoral immune system for the future challenge of more-invasive bacteria.

### Extending the Benefits

Despite these caveats, the observed benefits of protection from invasive disease by vaccine-induced immunity toward seven serotypes in the United States or nine serotypes in African vaccine trials provide an alluring incentive to extend the range of serotypes covered, and this goal is what is driving the development of both 10- and 13-valent vaccines. However, at some point the incremental manufacturing costs and complexity associated with the addition of more serotypes will make higher-valent vaccines of only marginal epidemiological value.

Setting the order for the inclusion of additional serotypes also presents some interesting questions. Is the vaccine formulation targeted at children or at adults, or is it targeted at children in order to protect both populations? Is the formulation designed for use in the United States, where the serotype coverage of PCV-7 among children is already very high, or for use in Europe or Africa or Asia, where the vaccine provides less coverage?

The remarkable feature of the present PCV-7 vaccination program in the United States is herd protection. This effect is brought about by reducing nasopharyngeal carriage among the primary pool of vaccine serotype carriers, i.e., infants and young children. For serotypes, such as 1 and 5, of which carriage is uncommon or very transient and for which we are unsure what populations represent the primary vectors of disease, herd protection may not occur and the only certain way to protect an at-risk population may be by direct vaccination of that group. Finally, herd protection is most valuable in developing-country settings, where vaccine coverage is rarely complete. It is ironic, therefore, that the prediction of herd protection is the least certain for serotypes like 1 and 5, which most dominate the developing world.

To conjure a formulation for the future, we need to define the target population and the target disease and make clear our assumptions about direct and indirect protection. Inevitably, too, we need more information on the serotypes that cause pneumococcal disease, their patterns of transmission, and their invasiveness. In particular, we need more information from developing countries, more information on disease in adults, and more information on nonbacteremic pneumonia.

### CONCLUSIONS

Despite this apparent complexity, two themes have emerged from the rapidly growing body of serotype analyses. First, only 10 to 12 serogroups appear to be responsible for the vast majority of invasive disease and mucosal infections in children and adults worldwide. These are largely reflected in the PHID CV-10 and PCV-13 formulations. Second, while some serotypes possess epidemiological properties that indicate a greater public health impact than mere percentages can convey, these serotypes are by and large also included in these vaccine formulations (80, 81, 83).

At the same time, given the global magnitude of morbidity and mortality associated with the pneumococcus, it is clear that even the PCV-7 formulation, which may offer only 50% serogroup coverage in some countries, may nonetheless provide a major public health impact wherever it is introduced (114).

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# *Manufacturing and Product Release Issues*

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IV

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Andrew Lees  
Velupillai Puvanesarajah  
Carl E. Frasch

11

## Conjugation Chemistry

Most bacteria that cause invasive disease, especially those that cause bacteremia, are protected from innate host immunity because they express polysaccharides (PSs) on their cell surfaces. *Pneumococcus* bacteria are a prime example, with their many strains corresponding to over 90 different serological types, each with a chemically and antigenically distinct capsular PS. The presence of bacterial capsule-specific antibodies in the immune system helps protect humans against diseases caused by pneumococci. However, the ability of humans to produce these anticapsular antibodies matures with age. For this reason, most invasive bacterial diseases in young children are due to encapsulated pathogens, including the pneumococcus.

The bacterial capsular PSs are composed of thousands of carbohydrate repeat units resulting in polydisperse polymers that can have molecular masses into the millions of daltons. More than 70 years ago, purified high-molecular-mass pneumococcal PSs were found to induce protective opsonic antibodies when administered to adult humans, leading to the introduction of a 14-valent PS vaccine in 1977, which was replaced by a 23-valent vaccine in 1983. A significant shortcoming was

that the vaccine did not induce protection against pneumococcus infection in young children. Because PSs are T-cell-independent antigens, they are poorly immunogenic in infants, but this limitation was overcome by covalently linking the PS to a protein, converting the PS into a T-cell-dependent antigen. This process was first demonstrated in the 1920s by Goebel and Avery (11). The pioneering work of Robbins, Schneerson, Anderson, and Smith in the 1980s led to the first Food and Drug Administration-licensed conjugate, a vaccine against *Haemophilus influenzae* type b (Hib) (42). The introduction of the Hib conjugate vaccine into the childhood immunization program has led to a marked reduction in the incidence of Hib disease.

The wide-scale manufacture and commercialization of the Hib vaccine made clear that the production of carbohydrate conjugate vaccines presented chemical, immunological, and legal challenges. Therefore, the synthetic approach of each of the manufacturers has been defined as much by access to patents as by manufacturing and/or immunological efficiencies. Multivalent pneumococcal conjugate vaccines present additional complexities with regard to their syntheses, as each serotype

is chemically distinct, effectively requiring the optimization of the manufacture of seven or more individual vaccines. The challenge is to cost-effectively produce conjugates of each serotype, while preserving critical epitopes, and then to formulate the individual conjugates into a vaccine that will induce protective antibodies against each serotype.

### PNEUMOCOCCAL CAPSULAR PSs

*Streptococcus pneumoniae* strains have been classified into over 90 different serotypes based on their capsular PS structures. In the U.S. population, if people of all age groups are considered together, 23 of these strains account for approximately 80% of the pneumococcal disease. Accordingly, the currently available licensed 23-valent pneumococcal PS vaccine is formulated with PSs from the 23 most prevalent strains: 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F. This vaccine is licensed only for use in adults and children over 2 years of age. A seven-valent pneumococcal conjugate vaccine, Prevnar, comprising serotype 4, 6B, 9V, 14, 18C, 19F, and 23F capsular PSs conjugated with nontoxic diphtheria toxin analog CRM<sub>197</sub> (PCV7-CRM), was licensed in the United States in 2000 and has since been licensed in many other countries. The vaccine is indicated for use in infants and young children. Various formulations containing up to 15 capsular conjugates are currently under development.

Details of the technology used in the production of pneumococcal PSs have not been widely reported. Available reports describe alcohol or Cetavlon precipitation as well as the use of nucleases and proteases to remove contaminating proteins and nucleic acids (5a, 19, 33). World Health Organization specifications for the impurity levels in the PSs have been established: <3% by weight for protein and <2% by weight for nucleic acid (50). Purified pneumococcal PS preparations also contain various amounts of cell wall common PS (CPS). Published reports conclude that the capsular PS and the CPS are both attached to the cell wall peptidoglycan via unidentified linkages (44). Even though mechanical manipulations during the purification of the PS disrupt these linkages, a subpopulation of the purified PS may still contain attached CPS chains, about 5% by weight (43, 51, 53). Recently, pulsed amperometric detection has been used to quantitate residual CPS in purified capsular PS (48, 53). Depending on the PS activation, conjugation, and purification methods, residual protein, DNA, and C-polysaccharide impurities may be carried

into the final product and induce undesired antibodies (52, 53). The purity of the PSs to be used in the synthesis of conjugates should meet or exceed the purity requirements for the licensed pneumococcal PS vaccine.

The chemical structures of the 23 serotypes of PSs used in PNEUMOVAX have been summarized by Jennings (13) and Kamerling (15), among others. A majority of the capsules contain negatively charged, acidic components, such as D-glucuronic acid and D-mannuronic acid. Nonglycosidic substituents, such as O-acetyl, pyruvyl, and phosphocholine moieties, are integral components of several of the pneumococcal capsular PSs and are considered to be important immunodominant epitopes. Care must be taken that critical epitopes are not lost or changed by the conjugation process. For example, alkaline conditions can cause the shifting or loss of O-acetyl groups. Recent studies using human and animal sera obtained following vaccination showed that O-acetyl groups in serotype 9V and 18C PSs may not be required for the PS to induce functional antibodies (26, 31). Similar findings, i.e., the noninferiority of the de-O-acetylated conjugate to the native analog, have been obtained in clinical trials with meningococcal group C conjugates (39). However, for other PSs, such as meningococcal group A and *Salmonella enterica* serovar Typhi Vi antigen, O acetylation is necessary in order for the conjugates to induce protective antibodies (4, 46). More work is needed to gain full understanding of their role(s) in immunity, but the O-acetyl groups may actually help protect some pneumococcal strains from phagocytosis (10).

The capsular PSs, in their native forms, are known to be high-molecular-weight polymers, containing well over 1,000 repeat units. While the reduction of size prior to conjugation offers several advantages during conjugate manufacture (e.g., a marked reduction in viscosity and ease of separation of the conjugate from the free carbohydrate), it also entails extra steps and losses and can affect important epitopes. Acid or base hydrolysis with heating, oxidation, sonication, microfluidization, and electron beam fragmentation have all been used to reduce the molecular weights of native PSs. The relationships between the sizes of the carbohydrate moieties and the extent of protective immunogenicity have been the subject of many investigations (2, 12, 23, 24, 35). Most of these studies involved the use of animal models, and the relevance of the results for predicting immunogenicity in humans has not been established. Nevertheless, these studies indicate that oligosaccharides composed of as few as two or three repeat units conjugated to a protein may suffice to evoke an immune response,

though not necessarily optimal. In addition, the nature and the extent of this response may be serotype and (animal) species dependent. Other factors such as carbohydrate and protein loading also play important roles.

A few clinical studies with conjugates that were generated using smaller fragments of pneumococcal capsular PSs have been reported (6, 9, 45). A meningococcal outer membrane protein complex-based seven-valent vaccine used size-reduced PSs, with molecular masses of individual serotype PS fragments varying between 100,000 and 1,000,000 daltons. In another study involving pentavalent conjugate formulations, a PS-based vaccine was found to be more immunogenic than the corresponding oligosaccharide analogs (6). The oligosaccharides used in that study had been generated by acid hydrolysis of the parent PSs, but no information regarding their molecular sizes is available.

## SELECTION OF CARRIER PROTEIN

Various proteins and peptide molecules have been demonstrated, in preclinical studies, to be effective carriers for PSs and oligosaccharides, but only a small number of protein carriers have been investigated in humans (3). Surface-exposed proteins and toxins from human pathogenic bacteria have been used as carriers, as they contain one or more of the T-cell epitopes. Some of these epitopes match the major histocompatibility complex haplotypes present in the human population. In these cases, residual toxicity, cross-reactivity to human tissues, and pyrogenicity associated with copurifying impurities such as lipopolysaccharide and peptidoglycans are some of the areas that need careful attention. Diphtheria toxoid (DT), tetanus toxoid (TT), meningococcal outer membrane protein, and a nontoxic mutant analog of diphtheria toxin, CRM<sub>197</sub>, are all in use as carrier proteins in the various licensed Hib and meningococcal conjugate vaccines. CRM<sub>197</sub> is used as the carrier in Prevnar, a seven-valent pneumococcal vaccine licensed in the United States and other countries. A Sanofi-Aventis 11-valent formulation comprises mixed TT and DT conjugates (38), while a recombinant, nonlipidated protein derived from *H. influenzae*, protein D (1, 41), is used as the carrier in GlaxoSmithKline's 11-valent pneumococcal conjugate vaccine PCV11-PD (34, 37), although additional carriers are expected to be used in their 10-valent vaccine.

The toxicity of the proteins such as DT and TT can be eliminated or greatly reduced by "toxoiding," a process in which the toxins are treated with formaldehyde for an extended period of time. Formaldehyde re-

acts with the lysine and tyrosine groups to form "methylene bridges" and also may form small quantities of aggregates (49). It is critical that the toxoids formed maintain a sufficient number of available sites for conjugation as well as conserve T-cell epitopes needed for recruiting T-cell help. The physical changes imposed by the toxoiding process may also limit the accessibility of functional groups in the proteins for conjugation, and thus the proteins may need spacer arms or bifunctional linkers to link to the PS molecules. For these reasons, native nontoxic proteins or a genetic mutant analog such as CRM<sub>197</sub> may be preferred.

As the conjugate vaccine field has expanded to include more complex multivalent meningococcal and pneumococcal conjugate vaccines, immunogenicity in the target population is no longer the sole requirement for the selection of a potential carrier(s) for conjugation. Interference in the immune response to particular vaccine antigens can result when conjugate vaccines with protein carriers that are also the components in other routine childhood vaccines, such as DT and TT, are administered concomitantly (8) (see chapter 6). Studies comparing the degrees of immunogenicity of octavalent pneumococcal vaccines conjugated with TT and DT indicated that the anti-PS response to both vaccines was satisfactory for serotypes 6B, 14, 19F, and 23F. However, the immune response was stronger with TT as the carrier for the PS of serotype 4, while DT was the superior carrier for the PSs of serotypes 3, 9V, and 18C. Furthermore, it has been suggested that there may be interference between serotypes when the same carrier protein is used for all serotypes in highly multivalent vaccines (22). Clinical trials that are currently in progress involving the 13-valent pneumococcal CRM<sub>197</sub> and the 10-valent protein D conjugate vaccines may provide additional data concerning the use of different carrier proteins and immune interference.

The utility of having a dual role for the carrier protein as an effective carrier in inducing T-cell help and also as an antigen eliciting a protective response against pathogenic bacteria other than the primary vaccine target needs to be examined more carefully. Recent clinical data from studies with an 11-valent pneumococcal protein D conjugate vaccine have demonstrated a significant reduction in acute otitis media due to nontypeable *H. influenzae* (34). Even though a clear correlation between efficacy and enzyme-linked immunosorbent assay (ELISA)-measured titers of antibody against protein D was not established in this study, it is likely that protein D contributed to the induction of protection against *H. influenzae*. It would be worthwhile to conduct similar

clinical studies to investigate the use of pneumococcal proteins shown to provide protection, such as pneumolysin (21, 32), PspA (5), and/or other surface pneumococcal proteins, as carriers to enhance the efficacy of pneumococcal vaccines (47).

## CONJUGATION CHEMISTRY

In order to convert PSs into T-cell-dependent antigens, the protein must be chemically linked to the carbohydrate; that is, there must be covalent links between the two components. The conjugation protocol should be mild so that it does not (i) destroy significant epitopes on either the protein or the PS, (ii) cause undesired depolymerization of the PS, or (iii) introduce any deleterious epitopes. While there are a number of conjugation chemistries available for the synthesis of PS-protein conjugates (7), only a few have been used in licensed vaccines. For proteins, surface-exposed amines (e.g.,  $\epsilon$  amines of lysine residues) and carboxyls (e.g., the carboxyl side chains of glutamic and aspartic acid residues) are the predominant groups used for conjugation. Although PSs of some pneumococcal serotypes have carboxyls (e.g., the PS of serotype 3) or other more reactive chemical groups, manufacturers have preferred chemical approaches that are applicable to all serotypes. The general chemical approaches used by each manufacturer to prepare pneumococcal conjugates are similar to those used for their respective Hib conjugates. This is due partly to access to the needed intellectual property restrictions and partly to the experience gained by mastering the chemistry and manufacturing process for their respective Hib conjugates. Thus, reductive amination is used for all seven serotypes of PCV7-CRM, and a cyanation reaction is employed for all serotypes of PCV11-PD. These synthetic approaches will be discussed in more detail below.

The success of the conjugation reaction depends on bringing two macromolecules into close enough proximity that the reactive groups form a chemical bond, and it is related to many factors, including (but not necessarily limited to) the physicochemical properties of the PSs and proteins, the concentrations of the reactants, the reaction pH and temperature, and the buffer and ionic strength, etc. Protein solubility at the required pH, concentration, and temperature is an important determinant of the suitability of a protein for use in a particular conjugation scheme. TT, for example, is poorly soluble at pH 5, a pH used for some methods, unless specially prepared following toxoiding. Also, since toxoiding blocks amino groups, toxoids will carry more negative charge than the unmodified protein (49). As

many pneumococcal PSs carry a negative charge as well, high-salt buffers (e.g., those with 1 to 2 M NaCl) are used to dampen ionic repulsions. If the reaction conditions are not properly controlled, aggregation of the protein can occur, resulting in precipitation of the protein and leading to poor conjugation yields. Extensive cross-linking may result in a very-high-molecular-weight conjugate, leading to either a precipitate or a gel and making purification and sterile filtration difficult.

The conjugation process can be somewhat arbitrarily divided into the following steps (Fig. 1): (i) preparing the carbohydrate, (ii) preparing the protein, (iii) performing the actual conjugation, and (iv) finishing. Some conjugation schemes combine several steps, whereas in others a particular step may be unnecessary.

### Preparation of the Carbohydrate

- Sizing.** Native capsular PSs have molecular masses in the millions of daltons, and solutions tend to be viscous. Reducing the molecular masses of the PSs reduces their viscosity and makes the solutions easier to manipulate. Fragmentation also increases the number of polymer ends, increasing the number of attachment points for methods that link via the terminal residues. Size fractionation is usually necessary, as there will be preferential linking of lower-molecular-mass oligosaccharides since there are more ends available, on a mass basis, than on larger polymers.
- Activation.** Carbohydrates contain hydroxyl groups, which are relatively inert and need to be converted into a more reactive form (functional group). For pneumococcal PSs, the most common activation methods are cyanylation, using either CNBr or 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP), and oxidation, using

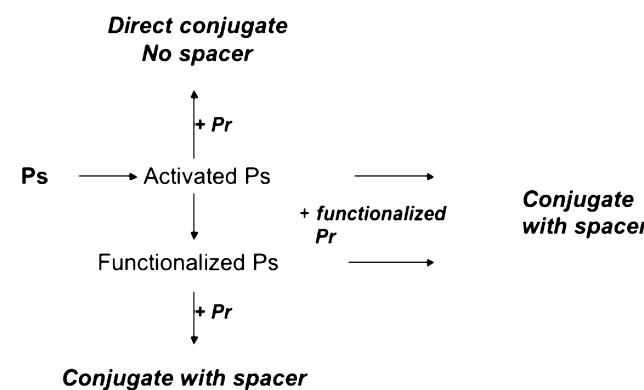


Figure 1 General overview of the protein (Pr)-PS conjugation process.

sodium periodate. Cyanylation converts hydroxyls into cyanoesters, thereby creating reactive groups along the carbohydrate chain, although fragmentation can occur as a result of the alkaline pH needed. Sodium periodate oxidizes diols into aldehydes. Depending on the structure of the PS and the reaction, the reaction may fragment the PS. The activated PS can be either directly conjugated to the protein or further functionalized. The positions of the activated groups determine the positions of the linkages on the PS.

3. *Functionalization.* This process refers to the addition of more-reactive chemical groups to the carbohydrate. More specifically, it refers to the chemical linking of a molecule to the carbohydrate that can subsequently undergo further reaction under milder and/or more specific conditions than the activated carbohydrate. Another purpose of functionalization is to convert the activated PS into a more stable form that is still reactive. Functionalization can involve the addition of a spacer molecule between the carbohydrate and the reactive group. In some cases, functionalization is a multi-step process.

### Preparation of the Protein

Amines (the  $\epsilon$  amines of lysines) and carboxyls (glutamic and aspartic acids) can be used to link directly to the activated or functionalized carbohydrate. However, some protocols rely on the addition of chemical groups that are more reactive and/or more specific in their reaction with the activated or functionalized carbohydrate than the carboxyls and amines of proteins. Functionalization of the protein can also be used to provide a spacer molecule so that the reactive groups on the protein are more accessible to the carbohydrate. Neither PCV7-CRM nor PCV11-PD uses functionalized proteins for synthesis.

### Conjugation

Conjugation is the chemical step that covalently links the protein and carbohydrate and requires that the reactive groups on the protein and the PS be close enough to interact. However, conditions that promote the conjugation, namely, high concentrations of protein and PS and high numbers of reactive groups, also carry the risk of over-cross-linking when both the protein and the PS have multiple reactive groups. Also of concern is the need to ensure uniform mixing and reaction, which can be problematic on a production scale due to the high viscosity of the PSs. The conjugation step is generally the slowest chemical step and risks damage to the com-

ponents. Careful control over the factors relevant to the particular chemistry is key to successful conjugation. These factors include pH, temperature, the ratio of the protein and PS, and the concentration of each. The type of linkage formed will depend on the chemistry and may be reversible or irreversible.

### Finishing

1. *Quenching.* Quenching inactivates any residual groups so that no further cross-linking can occur. This step is usually accomplished with monovalent blocking reagents, such as ethanolamine or glycine.
2. *Locking.* The locking step makes the conjugation linkage essentially irreversible. The condensation of an amine and an aldehyde, the conjugation step used in the synthesis of PCV7-CRM, reversibly forms an imine, which is reduced with a borohydride, making the linkage irreversible.
3. *Purification.* Following the conjugation process, the conjugate needs to be purified to remove the conjugation reagents and to ensure low levels of unconjugated carbohydrate and protein. Unconjugated PS is known to reduce the immune response to the conjugate (36, 40). While excess free protein can be inhibitory, there have been suggestions that the presence of low levels may have little effect (27). Unless the conjugate uses low-molecular-weight oligosaccharides, the purification of the conjugate is usually performed by size exclusion, by using either size exclusion chromatography or membranes with appropriate molecular weight cutoffs. Alternatively, ammonium sulfate precipitation of the conjugate has been used to remove unconjugated components (30).

Table 1 summarizes the details of each of these steps, which are used in the synthesis of PCV7-CRM, PCV11-PD, and PCV11-D-T. The chemistry of the first two vaccines is discussed below. Details of PCV11-D-T have not been published but are likely similar to the approach described for PRP-D (18).

### PCV7-CRM

The synthesis of the conjugates used to formulate PCV7-CRM is based on reductive amination, a process in which amines are condensed with aldehydes to form a reversible imine, followed by reduction to a stable linkage (Fig. 2A). The carbohydrate is first oxidized using sodium periodate to create aldehydes. Although the pH of the oxidation is relatively mild (usually

**Table 1** Comparison of conjugation methods that have been used for PCV

Step	Reactants used for PCV:		
	PCV7-CRM (reductive amination)	PCV11-PD (cyanylation)	PCV-D-T (cyanylation)
Carbohydrate			
Activation	Periodate	CDAP	CNBr
Functionalization	None	None	None
Protein functionalization	None	None	ADH
Conjugation	Schiff base	Isourea	Isourea/amide
Finishing			
Locking	Sodium cyanoborohydride	None	None
Quenching	Sodium borohydride	Glycine	ADH

pH ~5), depending on where these hydroxyl groups are located on the sugar, oxidation can open up the ring and possibly cleave the polymer. The sites of functional groups created on the carbohydrate are not quite random but are difficult to predict, due to preferential accessibility and reactivity of hydroxyl groups on the sugar residues (17). Since each capsular serotype has a different structure, reaction conditions, including concentrations, molar ratios of periodate, oxidation times, and pH, must be optimized for each. The particular hydroxyl residues oxidized are specific to each serotype. Vicinal hydroxyls are usually cleaved first, and at higher concentrations of periodate, *trans* hydroxyls are also cleaved. Following oxidation, activated PSs can be purified using membranes with defined molecular weight cutoffs. The actual conjugation step occurs with the condensation of the aldehyde-carbohydrate with unprotonated amines on the protein to form an imine adduct (known as a Schiff base), which is reduced irreversibly into a secondary amine with sodium cyanoborohydride. Any remaining aldehydes can be reduced with sodium borohydride. Since the pK<sub>a</sub> of ε amines on lysine is greater than 9, alkaline conditions are needed for the amine to be unprotonated, and to further promote the reaction, elevated temperatures are used. The formation of the Schiff base is somewhat slow and reversible. The reaction is allowed to proceed for up to a week in order to maximize conjugation. However, many of the serotypes are less stable in a base, so the reaction conditions are a compromise between high pH to promote the reaction and the stability of the carbohydrate.

Depending on whether the size-reduced PSs are produced with a single or multiple aldehydes, the resulting conjugates are neoglycoproteins or lattices, respectively. In either case, there is a significant increase in the molecular weight of the conjugate over those of the component protein and oligosaccharides, allowing the uncon-

jugated protein and carbohydrate to be removed using membranes with a molecular weight cutoff greater than the molecular weights of the components but low enough to retain the conjugate.

### PCV11-PD

The PCV11-PD vaccine is composed of conjugates of high-molecular-weight PSs. The carbohydrate is activated by cyanylation, (e.g., the formation of a cyanoester), which is subsequently reacted directly with lysines on the protein (Fig. 2B). PCV11-PD uses a novel cyanylating reagent, CDAP, instead of the CNBr used in Hib conjugates. CDAP is considerably more stable and safer to use than CNBr (28). Importantly, in contrast with CNBr, where a high pH is necessary to increase the nucleophilicity of the carbohydrate hydroxyl groups, CDAP exhibits increased stability and electrophilicity of the cyano group, making it more reactive at a lower pH. This allows the activation step to be performed at a lower pH for shorter times (pH ~9.5 for 2.5 min instead of pH 10.5 for 6 min) and with a higher level of activation efficiency than is possible with CNBr (20, 28). The lower pH and shorter time result in less degradation of the PS (e.g., depolymerization and/or de-O-acetylation).

The lower pH also results in greater stability of the activated PS. While a cyanoester may initially form with CNBr activation, the more-alkaline conditions needed with this reagent can cause hydrolysis of the PS, the loss of O-acetyl groups, and other damage. The high pH also results in the hydrolysis of the cyanoester into less reactive linear imidocarbamates, N-substituted imido-carbonates, and carbamates, as well as inert carbamates (20). Thus, CNBr-activated PSs are usually further functionalized with a large excess of a bifunctional reagent (a spacer), typically adipic dihydrazide (ADH). In contrast, CDAP-activated carbohydrate results in higher

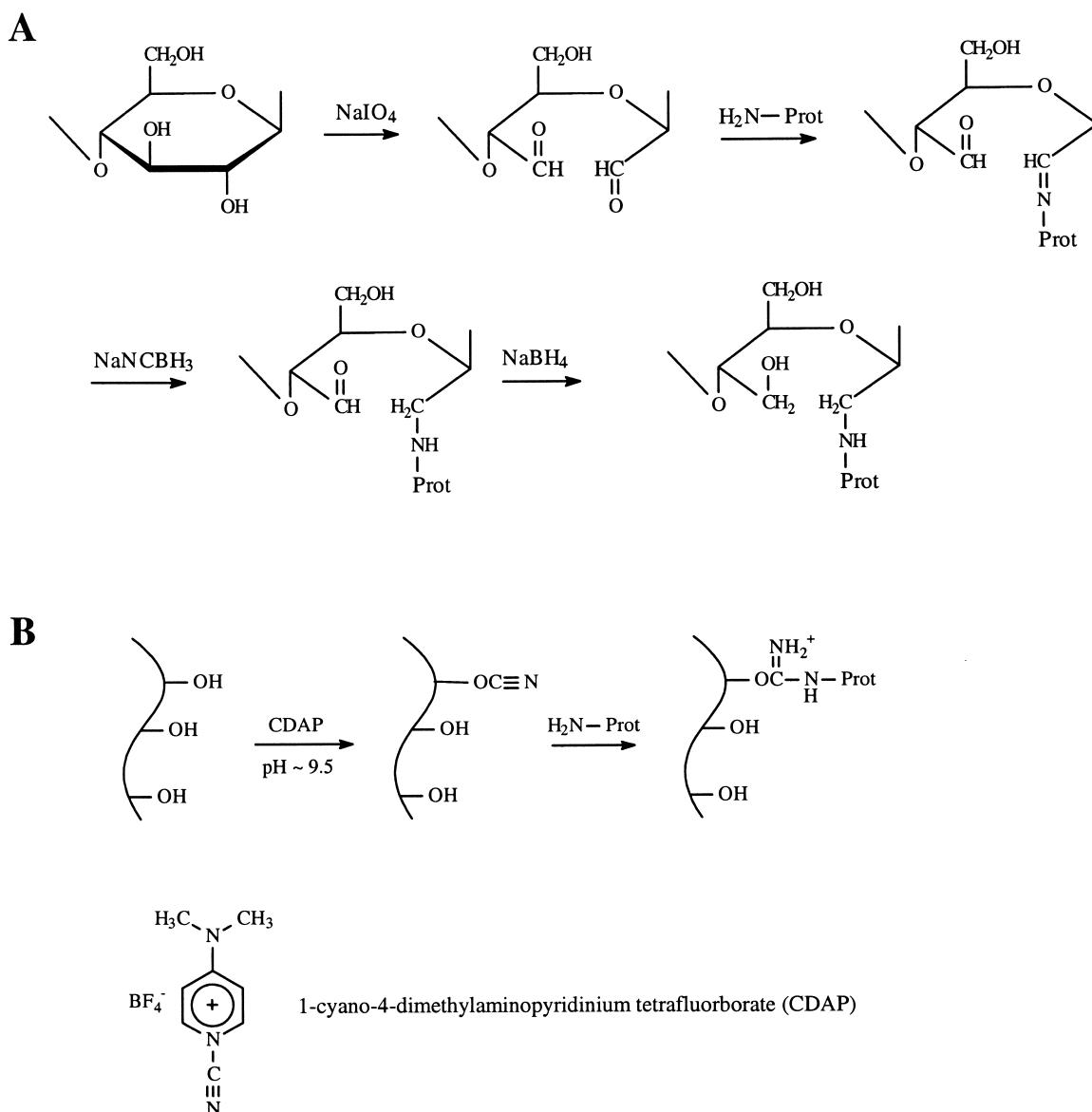


Figure 2 Reaction schemes for reductive amination (A) and cyanylation (B) using CDAP. Prot, protein.

levels of cyanoester on the PS and can be reacted directly with the ε amines of lysines to form a stable O-alkyl-isourea linkage (20, 28).

The overall reaction process is indicated in Fig. 2B. The conjugation step is performed at pH 9 to 9.5 for about 2 h. The alkaline conditions serve to keep the ε-amines of lysines partially deprotonated and hence able to react with the cyanylated PS, forming an isourea linkage. Following the conjugation reaction, the remaining active groups are quenched by the addition of an amine (glycine) at high concentrations. The purification

of conjugates made using native PS can be challenging, because the PS can have a molecular weight much higher than that of the protein and the conjugate can have a molecular weight in the multimillions. Membrane sizing is generally ineffective. Unconjugated protein and PS are removed using a size exclusion column with a very high exclusion limit.

Coupling efficiencies with the use of CDAP in the range of 80 to 90% have been reported (22), depending on the serotype. Since the CDAP activation process creates a multiplicity of reactive sites and the protein has a

large number of amines, a lattice is formed and care must be taken to avoid forming conjugates so large as to precipitate, form a gel, or become too large to be sterile filtered. The unique structures of each serotype mean that the precise activation and conjugation conditions must be carefully controlled and optimized, including the buffer composition, especially ionic strength, pH, and CDAP and PS concentrations, and the protein-to-PS ratios. Additionally, CDAP activation can result in the incorporation of dimethylaminopyridine (a component of the CDAP) into the PS and the protein; dimethylaminopyridine incorporation can be minimized with careful attention to the activation, coupling, and quenching conditions.

Several improvements on reductive amination have recently been introduced for use with meningococcal conjugate vaccines and are in development for use with pneumococcal vaccines. By functionalizing the protein with more-reactive amine groups than secondary amines, each improvement overcomes the slow and reversible condensation of  $\epsilon$  amines with the oxidized carbohydrate, as well as the need for alkaline pH. Significantly, these methods speed up the reaction, increase the yield of conjugated PS, and allow the conjugation reaction to be performed at a lower pH. One approach uses hydrazide-functionalized proteins. Hydrazides rapidly condense with aldehydes to form hydrazone, which are then reduced into secondary amines. Lee and Frasch (25) have described a method in which the carboxyl groups on the protein (glutamic and aspartic acid) are functionalized using hydrazine in a reaction mediated by carbodiimide. The hydrazide-functionalized protein is then condensed with the oxidized PS and subsequently reduced to form a stable secondary amine. This method is currently being used for meningococcal conjugate vaccines (Serum Institute of India, personal communication). Another approach functionalizes the protein with amino-oxy groups instead of hydrazides (29). Amino-oxy groups react rapidly and essentially irreversibly with aldehydes to form oximes, eliminating the need for reduction. Both approaches build on the experience developed with conjugates based on reductive amination but should achieve higher yields under milder conjugation conditions. Each is being evaluated for the synthesis of pneumococcal conjugate vaccines.

## ANALYTICAL CHARACTERIZATION

Complex conjugation products are formed when PSs with random activation sites created by periodate oxidation, cyanylation, or carbodiimide reactions are linked to proteins. A multitude of chemical entities, varying in

molecular size, saccharide loading, and the number and locations of attachment sites in the protein molecule, are formed. Analytical methodologies for fully characterizing such conjugate products do not exist, and animal potency tests have not been established. For these reasons and because of licensing requirements, it is necessary to demonstrate the clinical equivalency of manufacturing lots to those used in pivotal clinical studies. Conjugate vaccine manufacturers will need to rely on a panel of physical and chemical assays to monitor and control those parameters that are critical in making highly immunogenic conjugate constructs. The World Health Organization has published a guidance document containing recommended release assays for the production and control of pneumococcal conjugate vaccines (50) (see also chapter 12).

## Proof of Conjugation

The easiest and most direct way to demonstrate conjugate formation is by performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting analyses. In general, conjugated species either do not enter the gel or appear as high-molecular-weight smears compared to focused bands observed for unconjugated proteins and are easily detected with PS- and protein-specific antibodies. ELISAs can also provide direct proof for conjugation. For example, the ELISA plate is coated with antibodies against one component (e.g., the carbohydrate) and captures both free and conjugated material. After washing away unbound material, antibody against the second component (e.g., the protein) is used to detect conjugated material. Animal studies provide indirect proof since the conjugate induces significantly more antibody than the pure PS and the demonstration of a booster response to the PS upon reimmunization indicates the presence of conjugated species.

## Degrees of Activation and Conjugation

Critical factors associated with the PS and protein characteristics that have an impact on immunogenicity have been discussed in previous sections. As excessive modifications to the PS or protein molecules can have an adverse impact on immunogenicity, it is necessary to have suitable assays to determine and control the extent of these modifications. Generally, residual (unreacted) functional groups in the PSs are “capped off” (quenching) at the end of the conjugation reaction to prevent potential reactions in the tissue during immunization. Neoantigens formed during this capping process may modulate immune responses and should be monitored to ensure product consistency.

With respect to protein modifications, there are two factors that may play an important role: the number and the locations of attachment sites. Overconjugation may result in the reduction or elimination of T-cell epitopes required for eliciting an immune response. In cases in which the conjugation technology involves reductive amination, the average number of attachment sites can be determined by amino acid composition analysis of the conjugate product. The stability of the unique lysine derivatives formed enables the determination of the number of attachment sites on the basis of the number of native lysines recovered. In cases in which the conjugation reactions afford acid-sensitive linkages, the extent of conjugation can be inferred only from the saccharide/protein output ratios after making appropriate corrections to account for free sugar and free protein. The location of the attachment sites in proteins is a cumbersome task. Some preliminary work along these lines has been reported (17).

### Free Saccharide and Free Protein

Conjugate products are often contaminated with various quantities of unreacted PSs and proteins, which are often referred to as free sugar and free protein. Residual amounts of these impurities may ultimately depend on factors such as PS and protein characteristics, conjugation chemistry, and the nature of the purification processes used. Generally, only the conjugated portion of the saccharide will contribute to the induction of protective immunity. For these reasons, the free-sugar levels in conjugates need to be minimized and monitored throughout the product shelf life to ensure product efficacy. Regulatory authorities have considered the potency assay for conjugate vaccines to be a combination of the determination of the PS-to-protein ratio and the estimation of the amount of residual free saccharide.

So far, there is no published literature on direct methods to estimate levels of free sugar. For routine analytical purposes, the free sugar can be separated by any one of the following procedures: the use of various chromatographic resins or immobilized-antibody (anticarrier) columns or the selective precipitation of conjugates with deoxycholate or ammonium sulfate. Following separation, carbohydrate-specific colorimetric or high-performance anion exchange chromatography-pulsed amperometric detection-based assays can be used to quantitate free sugar.

It is difficult to assess the impact of residual free proteins on immune response. Beneficial or detrimental effects of a particular carrier may ultimately depend on the identity and nature of the protein. Nevertheless, assays should be established to assess the free-protein lev-

els to ensure product consistency. Various high-pressure liquid chromatography or capillary electrophoresis techniques can be used for these assessments.

### Conjugates as Well-Characterized Biologicals

The complexity of conjugate products, such as that formed when a PS with multiple activation sites is reacted with a protein, makes it difficult to characterize the products at the level of a well-characterized biological product. In the absence of a meaningful potency assay, the introduction of new conjugates without full efficacy studies or clinical comparison to a licensed pneumococcal conjugate vaccine may depend on further investments in developing additional analytical techniques to better characterize the products. The use of more advanced techniques such as nuclear magnetic resonance, circular dichroism, and/or mass spectrometry will provide further insight (14, 16, 17).

## FUTURE DIRECTIONS

Pneumococcal conjugate vaccines, while effective, are complex and expensive to manufacture compared with protein vaccines. Efforts should be made to improve conjugation efficiencies to levels at which the residual unconjugated components, especially free PSs, do not interfere with inductions of protective immune responses. The use of an efficient, mild conjugation chemistry would allow for higher yields of vaccine. For example, achieving greater than 90% efficiency of coupling of the PS may allow for a simplified purification process. This approach may well be applied first by manufacturers seeking to develop new pneumococcal conjugate vaccines, as they would have less of a financial commitment to an older process. It is also likely that vaccine manufacturers in developing countries will develop new pneumococcal conjugates. Clinical trials for new vaccines will likely be different from the earlier ones, since it is now difficult to conduct additional classic randomized efficacy trials. For ethical and financial reasons, for new pneumococcal conjugates it will be necessary to demonstrate equivalence, that is, the non-inferiority of the antibody response to that elicited by a licensed product, rather than efficacy. This approach should allow new conjugate vaccines to be evaluated at lower costs. It would also be desirable to formulate conjugate vaccines that do not need to be refrigerated.

It is likely that any new vaccine will be designed to include additional serotypes, both for protection against replacement serotypes and to provide coverage for serotypes found outside of the developed nations. Another approach that can be used to extend cross-protection to

those serotypes not included in the vaccine as conjugates is to include as carrier proteins pneumococcal surface proteins known to induce opsonic antibodies. Existing data suggest that the carrier protein can affect the immune potency of a pneumococcal conjugate. Therefore, it is likely that, by careful matching for the best combination of pneumococcal PS and carrier protein, immune responses can be improved and some immune interference that has been observed between immunogens in combination vaccines can be eliminated. Newer adjuvants may also enhance the immune response at lower doses but would require high development costs, as well as the risk of adverse events. As analytical methods improve, it will be possible to better characterize each stage of the conjugation process, as well as the final product. For instance, better methods are needed to evaluate the conformation of the PS in the conjugate in comparison to that of native PS. Precise characterization of PS size and of linkages between the PS and the protein may ultimately lead to the development of conjugates which satisfy the requirements of a well-characterized biological.

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Milan S. Blake

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# Pneumococcal Vaccines: Manufacture and Quality Control for Product Release

## BACKGROUND

The argument for the value of standardizing pneumococcal vaccines dates back to papers by Rufus Cole and H. F. Moore published in 1917, in which they state, "In spite of all that has been written concerning the theoretical principles involved in the preparation of anti-pneumococcal serum, and in spite of all the reports of its therapeutic application which have appeared, it is very difficult to learn from the literature on the subject exactly how these sera have been prepared or standardized. Without this knowledge we can have no accurate starting-point from which to proceed toward improvements in methods of production" (8). Even though we have progressed from the production of passive immune sera to the manufacturing of active immune compounds, their concerns regarding the production of potent and effective antipneumococcal vaccines remain. In 1917, Cole and Moore outlined what by today's standard is a primitive method of master seed production for consistent starting materials, as well as growth medium and pH control in a time when pH was measured by matching colors. Further downstream processing and control of the final product were major worries.

Protocols of final potency testing and rationale are described. Although their writings seem antiquated in the light of our sophisticated technologically based quality control, we must keep in mind the foundations these early investigators set and what came out of these studies. Cole had previously shown that the clinical use of therapeutic horse serum was directed at a type-specific substance and would cause the pneumococcal organisms to agglutinate, thus demonstrating that the type-specific antigen was surface exposed (7). Blake demonstrated that it was a soluble substance and could be ethanol precipitated, which led to methods for purification which are still in use today (6).

It is universally agreed that all manufacturing of material to be used for human use should be carried out using current Good Manufacturing Practice (cGMP) procedures. In addition, it is recommended that all those involved in this manufacturing, especially those culturing and/or fermenting the bacteria, be vaccinated with a licensed pneumococcal vaccine. All assays used either to monitor intermediates or the final product or to evaluate lot release criteria should be well documented and validated. Regulatory authorities should be consulted

and agree on all aspects of the production and testing procedures.

## STRAIN SELECTION, CHARACTERIZATION, AND USE

### Production and Maintenance of Master and Working Seeds

As stated in the 1999 U.S. FDA Center for Biologics Evaluation and Research guidance to manufacturers,

Production of a drug substance, whether by fermentation, cultivation, isolation, or synthesis, usually starts with raw materials. Subsequent steps of the procedure involve preparation, characterization and purification of intermediates eventually resulting in the drug substance. The quality and purity of the drug substance cannot be assured solely by downstream testing, but depends on proper control of the manufacturing and synthetic process as well. Proper control and attainment of minimal levels of impurities depends on appropriate quality and purity of the starting materials, including the seed organisms, and reagents (26).

The first industrially produced pneumococcal polysaccharide (PS) vaccine was made under contract from NIAID to Eli Lilly and Company. The source of the strains for this vaccine was the American Type Culture Collection (ATCC), which in essence generated, characterized, and maintained the master seeds. The ATCC also made these master seeds generally available. The majority of strains used for current industrial vaccine production originally came from research facilities, and in the case of PNEUMOVAX, they came mainly from the laboratory of Robert Austrian at the University of Pennsylvania in the early 1970s. Some of the same strains are now being used for conjugate production. The original cultures were subcultured and subjected to clonal selection, and the selected clones were then cultured for the production of master and stock seeds. Strains that are patented or under patent application must be deposited as seed with the ATCC. Once strains are identified, it may be of some advantage to initially generate premaster clones and examine these for optimal growth and type-specific PS expression, thereby selecting those for master seed development that have high PS-to-optical density ratios relative to other clones. The initiation of seed lot production should always comply with Good Laboratory Practices and take place in controlled environments. A sufficient number of vials should be produced to thoroughly characterize the seed

and determine the stability of the seed over time as well as serve as a source of working seed for the foreseeable lifetime of the vaccine. Standardized protocols and record keeping for the production of master cell banks and working seeds are part of the cGMP compliance of any product. Once a clone has been selected for master seed development, it can be expanded using standard cultivation techniques, the addition of a cryostabilizer like glycerol if needed, and aliquots made in the appropriate storage containers. Many of the early master seeds were maintained as lyophilized stocks. However, now many of the cell banks are kept in secure, monitored liquid nitrogen dewars. Master seeds should be stored in more than one location for security reasons; the ATCC offers a cGMP safe deposit which provides further security for cell bank storage that is compliant with cGMP. Once these master seeds are well characterized (see below), working seed cell banks can be similarly created for routine use in manufacturing.

Many of the original seed stocks of *Streptococcus pneumoniae* were cultivated on blood agar plates and in heart infusion medium usually generated from bovine sources. By the late 1990s, it became clear that bovine spongiform encephalopathy (BSE) could pass from animal products to infect humans. How this affected or would affect the manufacturing of biological products and vaccines was discussed in a July 2000 U.S. FDA Vaccines and Related Biological Products Advisory Committee meeting. Many pharmaceutical companies made a decision at that time to rederive their original master seeds using culture medium that did not contain blood or animal-derived products. When this process proved impossible because of the growth restrictions of the organism, animal-derived products were secured from BSE-free sources. Strategies to rule out the BSE issue for starting reagents should always be a part of current seed lot and vaccine production.

### Characterization

Once seed cell banks have been generated, a statistically significant number of vials should be well characterized for culture purity by standard means and subjected to counts of viable cells. An additional number of vials should also be designated for future stability testing. Pneumococcal identity testing should include analyses of colony morphology, Gram staining, and the ability of the organism to ferment insulin and to be lysed in the bile solubility test, as well as its sensitivity to optochin. Most importantly, the type specificity of the organism should be confirmed. Originally, the Quellung reaction was used for this identification (2). Many facilities still use this technique, while others have developed other

means for confirming type specificity, such as the use of serum-agar plates, inhibition enzyme-linked immunosorbent assays (ELISAs), and nephelometry. The Statens Serum Institut of Denmark has long been the source of pneumococcal type-specific antisera and continues to supply the industry with these valuable reagents.

### Stability Testing

Stability testing of both master and working cell banks is not only necessary for regulatory purposes but is also prudent and economical. The protocol should include the monitoring of the vital characteristics of the cell bank (see above) and of trends, for example, the vitality of the cells over time and the stability of production of PS. These data can be used prospectively to identify when new working seeds will need to be generated and, more importantly, whether a new master seed should be constructed. Specifications to initiate these activities should be written into the protocols.

## PS PRODUCTION PHASES

### Growth and Inactivation

It should always be remembered that *S. pneumoniae* is a biological safety level 2 pathogen associated with particular health hazards of infection by inhalation. This organism should be handled with these hazards in mind, most appropriately in a negative pressure environment relative to the outside, with proper venting and incineration. Personnel involved with production and control should be satisfactorily trained on the standard operating procedures for dealing with emergencies arising from accidental spillage, leakage, or other possible events that may disseminate pneumococcal organisms. All these personnel should maintain records of this training and include records of being vaccinated with a licensed pneumococcal vaccine.

Various growth media have been used to culture the pneumococcus; a number of the early media contained animal extracts, with blood and beef bouillon being mentioned as ingredients among many. All pneumococcal PS and conjugate vaccines currently used or in development are multivalent, with components from 7 to 23 strains of different serotypes per manufacturer, each strain with its own growth characteristics and requirements. An optimal conservative medium for one vaccine strain may not be optimal for all. However, two overriding constraints to be considered are that the media be made with the fewest (BSE-free) animal reagents possible and with nothing that will copurify with the PS. Efforts should be made to minimize the number of expansion

steps between the working seed and the production vessel. Seed train protocols should include measurements of cell density and pH and specifications for each expansion step. Methods to minimize a breach in sterility between expansion steps should be incorporated and scrutinized. Once the culture is in the production vessel, key parameters (e.g., the growth rate and pH, etc.) of the culture should be monitored throughout growth and an optimal time or parameter for termination should be selected. Dochez and Avery (9) were the first to demonstrate that the soluble substance appears early in the culture and maximally in late log phase, at approximately 12 h. The entire cultivation and inactivation process require validation.

Methods to inactivate the culture are numerous, but all require adequate demonstration that the technique is lethal to the pneumococcal organism. The sensitivity of pneumococci to bile salts has been used for inactivation by the addition of deoxycholate, which in turn activates a pneumococcal autolysin. Another process includes the addition of phenol. Each of these additives will necessitate the development of assays to ensure its elimination during the purification process. Once the culture is inactivated, the separation of cellular debris from the culture supernatant containing the PS can proceed. Early investigators used centrifugation, which in some form continues to this day, but other, more modern production methods have been devised to increase the speed of this separation.

### Isolation of PS

Heidelberger and Avery (14) were the first to explain a method of purification of the soluble specific substance and some of the chemical characteristics of this substance. They describe the substance to be very soluble in water and precipitable by acetone, alcohol, and ether. It is in their paper that the soluble specific substance is first described as a PS. There is essentially nothing published on the modern methods of pneumococcal PS production, but many of the newer methods of purification follow the early lead of these investigators and use various fractionational ethanol precipitations and solubility for the isolation and purification of the PS.

Both the sizes and charges of the PSs can be exploited as part of the initial purification from the culture media. If the growth medium consists of only low-molecular-weight ingredients, the high-molecular-weight PS can be retained by an ultrafiltration device, allowing the growth components to pass freely through the filter. The PSs may also be concentrated and the buffer may be exchanged in such a step.

Jones (15) was the first to demonstrate that cetyltrimethylammonium bromide (CTAB; Cetavlon) would precipitate PSs. His major objective in the use of CTAB was an easy method to isolate nucleic acid under mild conditions. However, he noted that like DNA, negatively charged PSs could be specifically precipitated. In his published report, he described his specific demonstration that pneumococcal PS of type II (corresponding to type 2 in the modern nomenclature system) was precipitated by CTAB in saline but was soluble in 0.3 M NaCl. German, Jones, and Nadarajah (11) demonstrated later that, depending on molarity and pH, they could separate various species of PSs from *Mycobacterium phlei*. Gotschlich et al. (13) were the first to exploit this method in the preparation of capsular PS vaccines, namely, those containing meningococcal capsular PSs. The addition of 2% CTAB to a growing culture of meningococci killed the bacteria and specifically precipitated the capsular PS. The contaminating nucleic acids and the CTAB were removed by differential ethanol precipitation in the presence of calcium. In many cases, the optimal fractionation scheme for each pneumococcal type-specific PS in relation to pH, molarity, and fractional ethanol precipitation has been determined.

### Final PS Purification

Further purification when necessary can be obtained using a variety of column chromatography techniques, such as ion exchange, hydrophobic interaction, and hydroxyapatite chromatography, etc. In such instances, the addition of proteases or nucleases is required for final purity. Purified pneumococcal PSs and, when necessary, partially purified intermediates are stored as powders at or below -20°C. A stability protocol should be in place to monitor the structural integrity of the PSs over time under these specific storage conditions.

### Physical and Chemical Characterization of Bulk PSs

Each lot of PS should be vigorously characterized with validated test methods to determine the identity and purity of the PS. Specifications for each of these assays should be stipulated and agreed upon by the national control authorities.

PS identification can take the form of a serological method using type-specific antisera. The assay should not only identify the specific type of the PS but also rule out that the PS reacts with any of the other sera, thus demonstrating specificity. In the past, countercurrent immunoelectrophoresis assays have been used, but inhibition ELISAs are easily automated and validated for

this purpose. Each of the reagents used in such assays should meet strict criteria, and each lot of specific antisera should be tested for conformity.

Detailed characterization of these PSs and subsequent vaccines was emphasized early on by Avery and coworkers (see above). The results of these efforts have been condensed today into a combination of simple wet colorimetric chemical tests that define each PS by the percentages of total nitrogen, phosphorus, uronic acid, hexosamine, methyl pentose, and O-acetyl groups. Specifications for each type can be found in the *European Pharmacopoeia* (10).

Nuclear magnetic resonance is being employed on an ever-increasing scale (for review, see reference 16), not only to identify, characterize, and quantify the particular PS but also to monitor each lot for various impurities (1), such as the most frequent contaminant, the C-polysaccharide (27). Other physicochemical methods, such as high-performance anion exchange chromatography, have also been recently developed to identify and quantify pneumococcal PSs for vaccines (17, 24).

The importance of the molecular weight of the PS for the vaccine was first discovered in clinical studies of meningococcal group A vaccines (22). At the time these vaccine trials were conducted, the instability of the group A PS was unknown, and subsequent analysis demonstrated that the PS had become depolymerized and had a low molecular weight. Subsequent trials with stabilized group A vaccines with high molecular weights established that these stabilized vaccines were highly efficacious. Thus, too, the molecular size distribution of the heterogeneous pneumococcal PSs is important and is a good indicator of consistency of isolation and purification of these intermediates. The size distribution can be determined using methods such as a well-calibrated system of gel filtration through Sepharose CL-4B or CL-6B equilibrated in at least a 0.2 M buffer at a neutral pH. A distribution constant is determined by measuring the molecular size distribution of the PS at the main peak of the elution curve. This molecular size distribution should be obtained for each lot. Recently, size exclusion high-performance liquid chromatography in combination with multiple-angle laser light scattering is beginning to replace the gel filtration methodology (5, 23).

Other characteristics of the PS should be determined, such as the moisture content if the PS is stored at any time as a powder and the protein and nucleic acid impurity contents, as well as the results of the required pyrolyticity tests. A stability protocol should be implemented to determine the stability of the PS in the particular environment in which it is held. The pure PS

is the intermediate that is most often held or stored frozen for several years. The storage or hold time, therefore, requires stability validation either with the actual vessel or an approved surrogate container.

PSs for use in conjugate vaccines will be further processed and chemically modified or activated in preparation for being coupled with a carrier protein. Various methods have been devised and used successfully for the preparation of these conjugate vaccines. Each should be presented to national control authorities for preapproval. Depending on the chemical binding technology used, assays should be developed and validated to demonstrate the consistency of PS activation. Furthermore, since most of these activation procedures cause some depolymerization to occur, the molecular size distribution should be reevaluated (*vida supra*).

## CARRIER PROTEIN AND CONJUGATE PRODUCTION PHASES

### Growth and Inactivation

Many different proteins can be used as carriers for pneumococcal PS conjugate vaccines. However, the protein should be demonstrated to be safe in humans and a PS conjugate using the protein should be able to elicit a T-cell-dependent immune response to the PS. Avery and Goebel were the first to demonstrate that a monosaccharide "haptan" when chemically linked to a protein would elicit specific antibodies to an otherwise nonimmunogenic compound (3). The first pneumococcal conjugate was made with horse immunoglobulin and pneumococcal type III (or type 3) PS (4). Many of the first PS conjugates were constructed using licensed toxoids such as diphtheria and tetanus toxoids that already had an extended human safety record. As with the maintenance of cell banks and the culturing of *S. pneumoniae*, the production of these toxoids has been modified to exclude as much animal-derived material as possible. The toxoids destined for conjugate vaccines must still meet all the relevant requirements of purity, potency, and safety prior to conjugation.

CRM<sub>197</sub> is the carrier protein of the currently licensed pneumococcal conjugate Prevnar. Uchida et al. first described the isolation and characterization of this mutant protein from a culture of a nitrosoguanidine-mutagenized *Corynebacterium diphtheriae*  $\beta$  phage carrying the diphtheria toxin gene (25). Once isolated and reintroduced into *C. diphtheriae*, the phage produced a protein nearly identical to diphtheria toxin, thus the name CRM, for cross-reactive material; however, it

lacked any toxic properties (19). The investigators hypothesized that this mutant protein contained a missense mutation in the enzymatically active fragment A of the toxin, and they demonstrated that an active toxin could be regenerated by replacing fragment A of CRM<sub>197</sub> with an enzymatically active fragment A. This missense mutation was confirmed by Giannini et al. (12). Rappuoli then demonstrated that the often-used lysogenic Park-Williams no. 8 strain of *C. diphtheriae*, which overproduces diphtheria toxin, contains two nontandem copies of the lysogenic  $\omega$  *tox*<sup>+</sup> phage (21) and then proceeded to construct a similar nontandem lysogen of the *tox* mutant CRM<sub>197</sub>-expressing  $\beta$  phage, which was also found to overproduce CRM<sub>197</sub> "suitable for large-scale industrial production" (20).

Master and working seed banks should be made and well characterized for the production of the carrier protein as well as assayed for storage stability similar to the pneumococcal seeds (see above). The production culture should contain as few animal reagents as possible. It should be well controlled and demonstrate consistency of product.

### Isolation of Carrier Protein and Final Purification

Methods for the isolation and purification of the carrier protein will depend on the physicochemical nature of the protein and whether it is a secreted product of the culture or is maintained cellularly. Techniques in molecular biology have allowed the addition of various sequences such as histidine residues for easier downstream processing and purification. If expression systems are used with induction, assays for the inducer should be developed to ensure that the final product is uncontaminated. Likewise, expression plasmids should be well characterized and lack important human antibiotic resistance genes; notably, penicillin should not be used at any time in the manufacturing process. Plasmid copy number and stability throughout the culture and expression should be determined as well as the gene of the product sequenced at the end of production. The final purity of the carrier protein should be greater than 90%.

### Physical and Chemical Characterization of Bulk Carrier Protein

Identification of the carrier protein can be made serologically using specific reagents and controls. The most common method is by Western blotting. Other methods of identification include matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) and proteolytic peptide MALDI-TOF. Further characterization of the protein and purity can be made

using sodium dodecyl sulfate-capillary electrophoresis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, isoelectric focusing, high-performance liquid chromatography, amino acid analysis, or sequencing. Each assay should be validated to demonstrate robustness and characterized regarding the limitations of detection of minor contaminants. Specific assays should be included if culture inducers, plasmid stabilizers, detoxification techniques, or protein modifiers have been used during the production process to rule out copurification. Finally, the sterility, the endotoxin content, and the stability of the carrier protein over time should be established.

## FINAL PRODUCT

### Conjugation and Monovalent Bulks

The methods and control procedures used during the conjugation process depend on the chemistry being used for the chemical linkage. These measures should be designed to ensure a reproducible, stable, and safe product. Techniques should be developed to analyze for specific reaction products at various stages of the coupling process (see Kim et al. [18] for an example). The stability of each of the PSs, especially that of PSs known for their instability, should be monitored during and after subjection to the conditions of conjugation. Monitoring methods may include nuclear magnetic resonance or a serological assay for the maintenance of antigenicity. Monovalent bulk conjugates should be tested for identity, any residual reagents or active groups, including capping molecules, and the amount of unconjugated reagents (both protein and PS) remaining after purification. Various methods have been devised to separate unbound PSs from the conjugate, such as hydrophobic chromatography, acid precipitation, and gel filtration, etc. Each method has its advantages depending on the specific PS in question, but attention should be given to these assays to ensure their validity. It is important to measure the percentage of unconjugated (free) PS in the purified monovalent conjugate. In addition, both the protein and total PS contents should be assessed, and the protein-PS ratio should be determined. The relative molecular size distribution of the PS-protein conjugate should be determined for each bulk by using techniques similar to those described above and should exhibit consistency. The conjugate molecular size is normally monitored over the dating period of the bulk conjugate as one measure of vaccine stability. Specifications for each PS conjugate are unique and should remain stable over

time. Finally, the monovalent conjugate bulks should be examined for sterility, pH, and endotoxin content.

### Formulation and Filling

The final formulation of the vaccine is made by the addition of each monovalent conjugate bulk to a single mixed container. The exact amount added is determined by the PS content of each monovalent bulk and its specifications. Adjuvants and stabilizers, if used, are usually added at this time. The specifications of diluents and the amount of aluminum that can be added to a vaccine in the United States are set forth in the U.S. Code of Federal Regulations, title 21, section 610.15 (21 CFR 610.15). Herein it states, "An adjuvant shall not be introduced into a product unless there is satisfactory evidence that it does not affect adversely the safety or potency of the product." Filling should be conducted using strict cGMP procedures in a well-controlled environment. The filling equipment should be well maintained and monitored often for sustaining sterility, consistency, and precise filling. Final filled containers should be visually inspected for defects, anomalies, and particulates. Sufficient final containers should be set aside for quality testing and stability, as well as retained samples.

### Quality Testing and Stability

The identity and quantity of each serotype conjugate must be determined and fall within the specifications shown to be effective in controlled clinical trials. The specifications usually call for validated serological tests such as ELISAs and rate nephelometry using specific controlled reagents. Final vaccines that are to be maintained lyophilized should be monitored for residual moisture, which on average should be no greater than 2.5%. The container closure should demonstrate maintenance of sterility. The contents of endotoxin, adjuvant, and stabilizer and the pH should be established for each lot. Aluminum adjuvant vaccines should be monitored for the initial absorption of the product and then examined over time for desorption unless sufficient data showing that the immunogenicity of the product is unrelated to this parameter are accumulated. Samples of each lot should be tested for general safety until the consistency of the product has been time honored and is satisfactory to regulatory authorities. The specifications and the validity of all the assays for final release should be agreed upon by the national regulatory authority.

The expiry dating is based on the stability of the vaccine in its final container and under the recommended storage conditions. A stability program should be devel-

oped over an extended period of time to adequately substantiate that the final product remains within its final release specifications over time. The assays should be identical to those used for final release testing of the final product. Accelerated stability studies may offer some assurance of the stability of the product but do not replace those conducted in real time. Any modifications of these tests or extensions of the expiry dating should meet regulatory approval.

### Packaging and Labeling

The packaging of the final product should distinguish it from any other product. The lot number and expiry date should be prominent. Included within the packaging must be a listing of the pneumococcal serotypes contained in the final product, as well as the carrier protein if applicable, the amount representing a single human dose, the recommended storage and transport conditions, and instructions for reconstitution, as well as for storage and stability of the product once reconstituted.

### SUMMARY

The development and production of the current pneumococcal conjugate vaccines may represent the last chapter of a saga that began well over a century ago. The efficacy and effectiveness of these vaccines have been difficult to establish with well-controlled clinical trials and will become more complicated in the future. However, much as Rufus Cole and H. F. Moore pointed out in 1917 (see above), only with a well-characterized and controlled vaccine as a benchmark can future improvements be made and compared.

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Marion F. Gruber  
Douglas Pratt  
Manfred Haase

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# Licensing of Pneumococcal Conjugate Vaccines for Children and Adults: Regulatory Perspective from the European Medicines Agency and the U.S. Food and Drug Administration

*Streptococcus pneumoniae* infections remain a major source of morbidity and mortality among children and adults in both developed and developing countries, in spite of the availability of vaccines (39). Two vaccines are currently available in the United States and Europe and certain other regions of the world to protect against pneumococcal disease. The 23-valent pneumococcal polysaccharide (23vPS) vaccine is licensed in the United States for use in persons  $\geq 50$  years of age and persons  $\geq 2$  years of age with underlying medical conditions. The vaccine is recommended by the U.S. Centers for Disease Control and Prevention for the immunization of adults  $\geq 65$  years of age and individuals at high risk for pneumococcal disease. In the European Union (EU), the

23vPS vaccine is recommended for use in adults  $\geq 60$  years of age as well as persons  $\geq 2$  years of age at high risk for pneumococcal disease, although vaccination programs and immunization schedules differ in the individual member states of the EU. The 23vPS vaccine is composed of the purified capsular polysaccharides (PSs) of 23 pneumococcal serotypes. Available data suggest that the 23vPS vaccine protects adults and the elderly against invasive pneumococcal disease (IPD); however, no consistent vaccine effect on the prevention of pneumonia has been observed (10). In addition, this vaccine is poorly immunogenic in children less than 2 years of age and, therefore, is not recommended for this age group. Conjugation of the bacterial PSs to a protein

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Marion F. Gruber, Office of Vaccines Research and Review, Center for Biologics Evaluation and Research, Food and Drug Administration, 1401 Rockville Pike WOC I 360 North, Rockville, MD 20852. Douglas Pratt, Office of Vaccines Research and Review, Center for Biologics Evaluation and Research, Food and Drug Administration, 1401 Rockville Pike WOC I 308 North, Rockville, MD 20852. Manfred Haase, Division of Bacteriology, Paul-Ehrlich-Institute, P.O. Box 1740, D 63207 Langen, Germany.

carrier can help induce a T-cell-dependent immune response after primary vaccination and may enhance the immune response to the PS in persons who do not normally mount strong immune responses to the PS vaccines, notably, children less than 2 years of age. The seven-valent pneumococcal conjugate (7vPnC) vaccine is composed of capsular PSs derived from seven pneumococcal serotypes, 4, 6B, 9V, 14, 18C, 19F, and 23F, coupled to a nontoxic cross-reacting mutant diphtheria toxin molecule. These serotypes were originally selected because they accounted for approximately 80% of invasive disease due to *S. pneumoniae* in infants in North America. A high level of efficacy in preventing vaccine serotype IPD was demonstrated in a clinical study of infants (2). In the United States, the 7vPnC vaccine is indicated to protect children less than 2 years of age against IPD caused by the seven serotypes included in the vaccine. The EU initially granted an indication for the 7vPnC vaccine for active immunization of children 2 months to 2 years of age. This indication was subsequently revised to include children 2 to 5 years of age. However, recommendations for the use of the 7vPnC vaccine in the EU take into consideration the variability of serotype epidemiology in different geographical areas as well as the impact of invasive disease in different age groups. The introduction of this vaccine in the United States has led to a dramatic decline in invasive disease among children younger than 2 years and has also led to declines in the incidence of IPD among adults and the elderly (30, 38). The impact of the 7vPnC vaccine on IPD in Europe is less clear since not every country has introduced this vaccine into its routine childhood immunization schedule (4, 33). Furthermore, differences in pneumococcal serotype prevalence in other regions of the world have resulted in suboptimal protection against IPD by the available 7vPnC vaccine (24).

In order to increase the protection afforded by pneumococcal conjugate vaccines against other pneumococci prevalent in pediatric and adult populations in the United States, in Europe, and worldwide, vaccine manufacturers are generating new pneumococcal conjugate vaccines that differ from the currently licensed 7vPnC vaccine in the identity and number of serotypes. For the purpose of this chapter, these new pneumococcal conjugate vaccines are referred to as second-generation pneumococcal conjugate vaccines. The clinical development and path to licensure for these products present regulatory challenges, as placebo-controlled clinical end point efficacy studies have become difficult to conduct due to universal recommendations for infants in the United States, parts of Europe, and other countries and the resulting decline in disease incidence across all age groups.

Thus, for these products, alternate pathways to licensure have been considered. This chapter will provide EU and U.S. regulatory perspectives for the licensure of pneumococcal conjugate vaccines indicated for the pediatric and adult populations.

## OVERVIEW OF U.S. AND EU REGULATIONS PERTAINING TO LICENSURE OF PNEUMOCOCCAL CONJUGATE VACCINES

### Applicable U.S. Regulations

In the United States, vaccines are regulated as biological products by the Center for Biologics Evaluation and Research of the U.S. Food and Drug Administration (FDA). Current authority for the regulation of vaccines resides in section 351 of the Public Health Service Act as well as specific sections of the Food, Drug and Cosmetic Act. A single set of basic regulatory approval criteria is contained in Title 21 of the U.S. Code of Federal Regulations (21 CFR) and applies to biological products. These regulations also guide the approval process for pneumococcal conjugate vaccines. In addition, there are numerous guidance documents published by the U.S. FDA that aid in the nonclinical and clinical development of preventive vaccines, including pneumococcal conjugate vaccines. These documents can be found at [www.fda.gov/cber/vaccine/vacpubs.htm](http://www.fda.gov/cber/vaccine/vacpubs.htm). The following will provide a brief overview of the vaccine approval process whereby it is stressed that within the framework of applicable laws and regulations, the regulatory process and licensure strategy for any vaccine are tailored to the specific product, its proposed indication, and the conditions of its use.

A sponsor who wishes to begin clinical trials with a vaccine candidate must submit an investigational new drug application (IND) to the FDA. The requirements for the IND content and format are described in 21 CFR 312.23. Critical aspects of establishing product safety are the establishment of well-controlled manufacturing processes and the development and validation of quality control tests for release and a stability program to ensure that the product can be made in a consistent manner within the established specifications. Preclinical immunogenicity studies in animal models conducted prior to the initiation of clinical trials can provide a qualitative indication of the immunogenicity of the product. In the past, preclinical safety evaluations in animal models have not always been required for pneumococcal conjugate vaccine candidates to proceed to phase 1 clinical trials; however, currently these evaluations may be required, depending on the vaccine com-

position, e.g., the number of serotypes included and/or the addition of a novel adjuvant.

Premarketing vaccine trials are typically done in three phases and are referred to as phase 1, 2, and 3 clinical trials. Phase 1 vaccine trials are designed primarily to evaluate safety and immunogenicity. The clinical development of a vaccine typically starts with phase 1 studies to determine the safety of a product in a small number of healthy adults. Safety evaluations include both local injection site and systemic reactions. Phase 2 studies generally involve more subjects, e.g., several hundred per trial, and are often randomized and controlled. These studies offer opportunities to compare local and systemic reactions in subjects assigned to vaccine and placebo groups to further determine the immunogenicity of the product and to establish the vaccine dose and schedule. Immunogenicity evaluations may serve to define an immune parameter to be used as a primary end point for efficacy studies in cases in which it may be possible to infer efficacy from immunogenicity end points. Phase 3 studies typically enroll thousands of subjects and provide information on the effectiveness and safety of the product to support licensure. Efficacy studies with a well-defined primary clinical end point, e.g., the prevention of morbidity from *S. pneumoniae*, represent the “gold standard” for supporting licensure. If successful, the completion of all three phases of clinical development can be followed by the submission of a biologics license application (22). To be considered, the license application must contain adequate information on the vaccine’s effectiveness and safety to make a risk-benefit assessment and to support the approval of a vaccine. Once licensed, many vaccines undergo postmarketing (phase 4) studies to further assess product safety.

For certain new drugs and biological products to prevent or treat serious or life-threatening illnesses, the FDA has published final regulations under which the agency would accelerate the approval of these products (23). Under these accelerated approval regulations, marketing approval may be granted for a biological product that is intended to prevent or treat serious or life-threatening conditions and that provides meaningful benefit over existing treatment. Adequate and well-controlled clinical trials are necessary to establish that the biological product has an effect on a surrogate end point that is reasonably likely, based on epidemiologic, therapeutic, pathophysiologic, or other evidence, to predict clinical benefit (21 CFR 601.41). A necessary condition of accelerated approval is that data from clinical end point studies confirming clinical benefit must be provided postlicensure.

### Applicable EU Regulations

Since the mid-1990s, a new registration system in the EU has governed the granting of marketing authorizations, which manufacturers must obtain for any medicinal product they intend to market, including a vaccine. Marketing authorizations are granted either directly by the European Commission (centralized procedure) or by the competent authorities of a member state (national, mutual-recognition, decentralized procedure). A marketing authorization granted following a centralized procedure is valid throughout the 27 European Community member states and the states which have signed the European economic area agreement, namely, Norway, Iceland, and Liechtenstein. The document provisions applicable to medicinal products, which also guide the licensing process for pneumococcal vaccines in the EU, include the following: a statement of binding legislation; a notice to applicants for marketing authorization describing the administrative procedures to be followed, as well as the format of the application file; guidelines on conducting the quality, safety, and efficacy studies which must be carried out in support of an application; a detailed guide to manufacturing practices; and a detailed guide to drug monitoring. The texts in force, which are continuously amended, are brought together in a series of volumes entitled *The Rules Governing Medicinal Products in the European Union* and are published by the Office for Official Publications of the European Communities (12). Furthermore, directive 2001/83/EC describes the requirements for the demonstration of the quality, safety, and efficacy of medicinal products, including vaccines (18). Thus, the approaches for conducting tests and studies have been harmonized within the EU. In addition, based on the principles of directive 2001/83/EC, guidelines for biologics in general, and for vaccines in particular, have been prepared. The use of these guidelines is not legally binding; nevertheless, they are intended to provide a basis for harmonization of the approaches by which the individual member states and the European Medicines Agency (EMEA) interpret and apply the detailed requirements for the demonstration of quality, safety, and efficacy contained in the community directives. Documents containing guidelines specifically intended to facilitate the preparation of applications for marketing authorization for vaccines include the following: (i) *Note for Guidance on Pharmaceutical and Biological Aspects of Combined Vaccines* (8), (ii) *Note for Guidance on Preclinical Pharmacological and Toxicological Testing of Vaccines* (9), (iii) notes for guidance on adjuvants in vaccines for human use (5, 6), and (iv) *Note for Guidance on Clinical Evaluation of New Vaccines* (7). Relevant guidelines

prepared within the International Conference on Harmonization or World Health Organization (WHO) processes are recognized equally as valid by all member states and the EMEA. Of note, there are no EU requirements specifically pertaining to pneumococcal vaccines to demonstrate the quality, safety, and efficacy of these products.

The new centralized registration system relies on a new institution, namely, the EMEA in London, United Kingdom. Based on scientific advice, the EMEA prepares a single scientific evaluation of the application. Within the EMEA, the Committee for Human Medicinal Products, named the Committee for Proprietary Medicinal Products until 2004, is responsible for preparing the written opinion of the EMEA on any question relating to the evaluation of medicinal products for human use. A marketing authorization granted under the centralized procedure is valid for the entire community market, which means that the medicinal product may be marketed in all member states (15, 20). However, at the same time, a pharmaceutical company may also submit a marketing authorization application to a competent authority of an EU member state via a national, or mutual-recognition or decentralized, procedure (14, 18).

An IND procedure does not exist in the EU. However, a sponsor who wishes to initiate a clinical trial must comply both with the provisions of the EC directives relating to the implementation of good clinical practices in the conducting of clinical trials of medicinal products for human use and with the principles and guidelines on good manufacturing practices regarding medicinal products for human use and investigational products for human use (13, 17). The guideline on the clinical evaluation of new vaccines covers the design of clinical development programs for new vaccines, including pneumococcal vaccines. Since an IND procedure is not available in the EU, potential applicants should obtain scientific advice from competent EU authorities in areas that do not provide specific and/or concise guidance to cover unique situations that may arise or in situations in which a major deviation(s) from the guideline on the clinical evaluation of new vaccines may need to be considered. The EMEA provides companies with scientific advice on specific questions relating to the quality, safety, or efficacy of their products. Such questions usually arise during the research and development phase, as has been the case for pneumococcal vaccines.

The 23vPS vaccine proprietors had already obtained marketing authorizations in the member states before the new European Community rules on the registration of medical products were set into place in the mid-1990s. For products authorized by the EU, there is a

single, harmonized summary of product characteristics (SPC) that is agreed upon at the European Community level. For products authorized via national procedures in individual member states, the various SPCs for a specific product differ and are rarely harmonized. This is because the SPC represents a consensus position for the medicinal product arrived at during the course of the assessment process of the various national control authorities. In general, the indications granted across the EU for the 23vPS vaccine are the following: active immunization against diseases caused by *S. pneumoniae* types present in the vaccine is recommended for all adults of approximately 60 years of age and older as well as for anyone over 2 years of age in clinical risk groups. Moreover, the use of the 23vPS vaccine is determined in the member states on the basis of official recommendations by national vaccination advisory committees. In general terms, the principle of vaccination with the 23vPS vaccine is accepted by all member states; however, there are considerable underlying differences in vaccination programs, immunization schedules and coverage, and to an even greater extent, the systems of recommendations and surveillance.

For the registration of the 7vPnC vaccine in the EU, the centralized procedure was used. Potential applicants for new pneumococcal conjugate vaccines indicated for children and adults are expected also to use the EU centralized procedure. Therefore, in order to illustrate the licensure pathway for the 7vPnC vaccine in the EU, the components of the centralized procedure will be described in the section below. At the request of an applicant, pneumococcal conjugate vaccines are accepted for consideration under this procedure if the applicant shows that either a new active substance or the medicinal product itself constitutes a significant therapeutic, scientific, or technical innovation or if the granting of community authorization is in the interest of people at the community level.

## PNEUMOCOCCAL CONJUGATE VACCINES INDICATED FOR THE PEDIATRIC POPULATION

### Licensure of Seven-Valent Pneumococcal Conjugate Vaccine in the United States

#### IPD

The application for licensure of the first seven-valent pneumococcal conjugate vaccine, Prevnar, was received by the U.S. FDA on 31 May 1999. The biologics license application was the first vaccine application granted priority review status, a designation that expedites the re-

view process and that the FDA reserves for promising products that are intended to treat or prevent serious and life-threatening conditions (3). A determination that the vaccine held promise was based on reported high levels of efficacy against invasive disease in the large Northern California Kaiser Permanente (NCKP) safety and efficacy trial. The license application included data from early studies conducted under the IND process supporting the choice of the final vaccine formulation. Pentavalent conjugate vaccine formulations containing oligosaccharides or PSs (0.5, 2.0, or 5.0 µg) conjugated to the cross-reactive material (CRM 197) mutant diphtheria toxin carrier protein were evaluated for infants and toddlers prior to the selection of the final vaccine formulation. The seven-valent vaccine was studied initially among adults, and phase 2 studies with infants and toddlers confirmed favorable immune responses, antibody kinetics, and the safety profile.

Two phase 3 studies were designed to demonstrate the efficacy of the 7vPnC vaccine in preventing invasive disease in infants and young children. The NCKP study used automated databases of the health maintenance organization to identify cases of invasive disease due to pneumococci. The second study, conducted among Navajo and Apache Native American infant populations, randomized enrollment by community rather than by individual subject, with the aim of estimating the effects of the vaccine on "herd immunity." In both studies, the planned size of enrollment was chosen to demonstrate high levels of vaccine efficacy ( $\geq 60\%$ ) with confidence. The Navajo and Apache study was still ongoing at the time the FDA received the license application. Thus, all efficacy data for invasive disease and the bulk of the safety data in the application came from the NCKP study.

The NCKP trial qualified as an adequate and well-controlled trial, providing substantial evidence to support a claim of effectiveness, as specified in 21 CFR 314.126. The clinical study was a double-blind, controlled study in which 37,816 infants were randomized (1:1) to receive either 0.5 ml of Prevnar or an unlicensed meningococcal conjugate vaccine at 2, 4, 6, and 12 to 15 months of age. Children received recommended childhood immunizations concurrently. A case of IPD was defined as a positive culture of *S. pneumoniae* from a normally sterile body fluid (e.g., blood, cerebrospinal fluid, or joint fluid) obtained from a child presenting with an acute illness consistent with pneumococcal disease. Cases of invasive disease were included in the primary analysis if they occurred at least 2 weeks after the third vaccine dose and before the fourth dose at 12 to 15 months of age; cases occurring at least 2 weeks after

the fourth dose also contributed to the analysis. Thus, the study was designed to support a four-dose vaccination series, and evidence of effectiveness following three doses was limited to the surveillance period from approximately 7 to 12 months of age.

At the time of the primary analysis (first planned interim analysis), 17 cases of IPD had been observed among fully vaccinated children in the control group, compared to 0 cases in the Prevnar group. Thus, Prevnar was 100% efficacious (95% confidence interval [CI], 81 to 100%) in preventing IPD due to vaccine serotypes. Extended follow-up with children for 8 months beyond the time of the primary analysis confirmed the high efficacy of Prevnar in preventing invasive disease due to vaccine serotypes: 39 cases occurred in the control group versus one case in the Prevnar group among children who received all doses according to protocol. Among children who did not receive all the vaccine doses according to the schedule but who received at least one dose, the vaccine was still remarkably effective: 49 cases in the control group versus 3 in the Prevnar group.

The potential for the replacement of the seven vaccine serotypes with nonvaccine serotypes was investigated in the NCKP trial. In total, nine nonvaccine pneumococcal serotypes were isolated in cases of invasive disease, three from the Prevnar group and six from the control group, accounting for 15% of all isolates, consistent with pretrial estimates that the seven vaccine serotypes accounted for approximately 80% of IPD in young children.

Overall, the prelicensure safety evaluation of Prevnar included six clinical studies in which 18,282 infants and children received a total of 58,888 doses of the vaccine. Most doses were administered to infants and toddlers at approximately 2, 4, 6, and 12 to 15 months of age. In addition, safety and immunogenicity were evaluated in 560 children from four supporting studies, 520 of whom were immunized at 7 months to 9 years of age.

In the prelicensure studies, injection site reactions were evaluated by comparison to local reactions at sites of other concurrently administered vaccines. In general, erythema, induration, and tenderness were milder and less common for Prevnar than for a whole-cell pertussis vaccine (DTP-HbOC), but more frequent for Prevnar than for *Haemophilus influenzae* type b (Hib) vaccine and a diphtheria–tetanus–acellular pertussis vaccine (DTaP). Local reactions at Prevnar injection sites tended to be severe more often among older (3- to 9-year-old) children in ancillary studies.

The evaluation of systemic vaccine reactions and other adverse events was complicated by the use of an unapproved meningococcal serogroup C conjugate vaccine

(MnCC) as a control vaccine in the NCKP study and earlier studies. In the NCKP study, fever and irritability were more common in the Prevnar group than in the meningococcal vaccine control group. In other, smaller studies supporting the consistency of manufacture and manufacturing scale up, fever was also observed more commonly in the Prevnar group than in a control group that did not receive any investigational products, though fever in excess of 39°C was uncommon.

Among other adverse events in the NCKP clinical trial, febrile seizures were observed more frequently in the Prevnar group than in the control group, although this finding was not statistically significant. Among eight seizure events following a dose of Prevnar, seven were considered febrile seizures and seven occurred concurrently with the receipt of the whole-cell pertussis vaccine. Additional assurance of the safety of Prevnar was obtained by comparing rates of rare adverse events to historical rates of events such as sudden infant death syndrome and the occurrence of diabetes mellitus and selected autoimmune diseases.

Overall, the safety profile of Prevnar was characterized by higher rates of low-grade fever and more local reactions than other concurrently administered infant vaccines.

The postmarketing evaluation of the safety of Prevnar conducted by the applicant as a postmarketing commitment included an observational study evaluating the safety of Prevnar in approximately 60,000 infants. Events of interest identified in the prelicensure studies, including febrile seizures, were examined. This large body of data confirmed the relative safety of Prevnar, and the findings were summarized in revised product labeling.

### Otitis Media

In June 2000, the FDA received data from two studies supporting a labeling indication for the prevention of otitis media in infants and toddlers in a supplement to the original license of Prevnar. The Otitis Media Efficacy Trial in Finnish Children was a randomized, double-blind, multicenter study conducted by the National Public Health Institute of Finland among children who received four doses of Prevnar or a control vaccine at 2, 4, 6, and 12 to 15 months of age. The control vaccine was a hepatitis B vaccine. Surveillance for cases of otitis media continued until age 2 years for all children enrolled. Clinical management of otitis media in Finland included myringotomy with aspiration of middle ear fluid, which was cultured to determine microbial etiology. Thus, the trial design allowed the determination of serotype-specific efficacy in preventing otitis media.

Among Finnish children who received at least three vaccine doses, the efficacy of Prevnar was 57% (95% CI, 44 to 64%) in preventing otitis media due to vaccine serotypes and 34% (95% CI, 21 to 45%) in preventing otitis due to *S. pneumoniae*, regardless of serotype. Efficacy against all-cause otitis media, regardless of etiology, was 6% (95% CI, -4 to 16%). An increased risk of otitis media due to nonvaccine serotypes was also observed in this trial, indicating some replacement of vaccine serotypes by nonvaccine serotypes among vaccinees during the study period, though overall benefit was clearly demonstrated (11).

In the NCKP safety and efficacy trial, health care utilization information relating to visits for otitis media was analyzed to provide an estimate of vaccine efficacy against all-cause otitis media. Since myringotomy was not part of the routine clinical management of otitis media, culture confirmation was not available, and estimates of efficacy specific to pneumococci could not be determined. Among children who received at least three doses of the vaccine, efficacy in preventing clinical otitis media was 7.0% (95% CI, 4.1 to 9.7%), a result similar to that observed for the same outcome in the Finnish study. Also, Prevnar reduced the risk of tube placement by 20.3% (95% CI, 1.8 to 35.4%). One notable finding in this trial was that serotype 19F accounted for 65.2% (15 of 23) of all vaccine serotype isolates from ruptured eardrums and all 6 isolates of vaccine serotypes in the Prevnar group.

The data provided to support the indication for the prevention of otitis media were presented and discussed at the public Vaccines and Related Biologics Advisory Committee (VRBPAC), which advised that the data did support the requested indication. The license supplement was approved in October 2002, and the vaccine label indication was amended to state that the vaccine is also indicated for active immunization of infants and toddlers against otitis media caused by vaccine serotypes.

### Licensure of the Seven-Valent Pneumococcal Conjugate Vaccine in the EU

In the EU, the applicant Wyeth Lederle Vaccines S.A., Belgium, submitted on 12 October 1999 an application for licensure to the EMEA for the 7vPnC vaccine Prevnar, an adsorbed pneumococcal saccharide conjugated vaccine, through the centralized procedure. Upon the request of the applicant, this vaccine had been referred to part B of the annex to council regulation no. (EEC) 2309/93 (21). This referral was made because, in the opinion of the agency, the vaccine constituted a significant innovation and was of significant therapeutic interest; i.e., *S. pneumoniae* is considered a major cause of

mortality and morbidity worldwide and in Europe, with the highest incidence of mortality and morbidity in infants. As discussed above, the serotypes contained in Prevenar were originally selected because they account for approximately 80% of invasive disease occurring in infants in North America. However, the epidemiology of pneumococcal serotypes and their roles in disease differ between continents. In addition, individual serotypes may also vary regionally, which is the case in Europe. This led to objections during the dossier evaluation for the Prevenar vaccine by the EU authorities. However, as also discussed, the Prevenar vaccine consists of bacterial capsular PSs conjugated to a carrier protein. There had been a successful exploitation of this same principle in the development of and previous experience with the applicant's monovalent vaccine against Hib (HibTITER). Thus, the extensive experience gained in the EU member states with HibTITER, at that time licensed in all EU member states, certainly contributed to the positive outcome of the licensing procedure for the Prevenar vaccine.

In regard to the quality part of the application for Prevenar (i.e., the chemical, pharmaceutical, and biological documentation), overall favorable conclusions were drawn during the assessment. Pharmaceutical development, the method of preparation, and production processes were adequately described and, whenever required, validated. The applicant used well-known microbiological, protein, carbohydrate, and glycoconjugate chemistry techniques in development and production. The bacterial strains used to produce the pneumococcal PSs and the carrier protein were processed using adequate cell bank systems. The release and in-process controls for both the starting material and the finished product, as well as their specifications, were considered in general to be sufficient. The risk of transmission of viruses or transmissible spongiform encephalitis presented by Prevenar was considered negligible, due mainly to the selection of source materials; i.e., the applicant sourced materials of animal origin in accordance with the appropriate EU guidelines. The proposed shelf life of 24 months when the vaccine vials are stored refrigerated was considered to be acceptable; however, for the syringe presentation, the available data supported a shelf life of only 12 months. Nevertheless, a number of unsatisfactorily addressed quality issues were identified during the assessment. In this regard, the applicant provided a letter of undertaking on the 22 follow-up measures to be fulfilled as requested by the committee.

The production of Prevenar is carried out at two sites in the United States and complies with current EU good

manufacturing practice requirements, as stated in a report generated following the inspection of the manufacturing sites by the United Kingdom Medicines Control Agency on behalf of the EMEA. Official control authority batch release for the EU territory in accordance with article 114 of directive 2001/83/EC, as amended by directive 2004/27/EC, is undertaken by the French Official Medicines Control Laboratory, the Agence du Médicament, Lyon (19). With regards to nonclinical (toxicopharmacological) aspects of the application file of Prevenar, overall favorable conclusions were drawn during the assessment. Generally, the tests performed fulfilled the recommendations laid down in the EU guideline on preclinical, pharmacological, and toxicological testing of vaccines (9). The immunogenicity of Prevenar and of each of its specific serotypes were well demonstrated in studies with rabbits and mice, as was the enhancing effect of the adjuvant, aluminum phosphate, on immunogenicity. The antibody induced in animals is of the immunoglobulin G class and has been shown to have opsonizing activity against several relevant *S. pneumoniae* serotypes in vitro. Also, data suggested the induction of immunological memory. No experiments were conducted with animal models to demonstrate protection against a challenge with the pathogens as requested by EU guidance, but protection was inferred from in vitro studies demonstrating opsonic activity of both rabbit and human antibodies.

Even though sufficient data were provided to demonstrate the nonclinical safety of Prevenar, these safety studies were not always conducted according to good laboratory practices, nor was the final formulation of Prevenar used. However, studies with animals conducted with experimental mono- and multivalent candidate vaccines did not suggest systemic toxicity. Furthermore, there was a lack of reactogenicity, and these vaccines produced only minimal local reactions at the injection sites. Finally, the absence of formal tests of genotoxicity, reproductive toxicity, and carcinogenicity was justified by the applicant and was deemed acceptable in view of the nature of Prevenar, a combined bacterial vaccine. Overall, no major objections were identified as to the results of the preclinical, pharmacological, and toxicological testing program and no follow-up measures have been requested by the committee for Human Medical Products.

The critical evaluation by the committee of the clinical safety and efficacy eventually led to a positive benefit/risk ratio for Prevenar; however, several major objections were raised during the licensing procedure which subsequently led to important changes of relevant parts of the originally proposed SPC. Initially, the marketing

authorization applicant applied for the following indication: Prevenar is indicated for active immunization of infants and children from 6 weeks until 9 years of age against invasive disease, pneumonia, and otitis media caused by *S. pneumoniae* serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F. Right after the receipt of the application, concerns were raised regarding the serotype composition of Prevenar; i.e., the question was whether the company should have developed a vaccine composition that would better fit the European epidemiology. No concern was expressed by the EU as to the vaccine's effectiveness against invasive disease caused by vaccine serotypes in the Kaiser Permanente trial in the United States. However, right from the beginning, concerns were shared with the applicant concerning the moderate effectiveness of the 7vPnC vaccine in the Finnish trial, which was conducted to demonstrate protection against otitis media caused by vaccine serotypes. Even though the 7vPnC vaccine showed some efficacy, the overall impact on the total number of otitis media episodes was small, i.e., approximately 6%. It was thought that protection against otitis media should not be maintained as an indication. Instead, the results of both the Northern California and Finnish trials should be objectively described as an "add-on beneficial effect" with regard to otitis media in the SPC section entitled "Pharmacodynamic Properties."

As pointed out previously, the serotype epidemiology in Europe differs from that in the United States, and therefore, the coverage of vaccine strains is generally lower in Europe (55 to 85%), with less coverage in Nordic countries but high coverage in some countries such as the United Kingdom, France, Belgium, and Spain. The applicant agreed to describe these concerns in the original SPC (October 2000): "Estimates of efficacy against invasive disease were obtained in the U.S. population where vaccine serogroup coverage ranged from 89–93%. In Europe, coverage is lower and varies from country to country. The coverage established for children less than 2 years of age is lower in the Northern part and higher in the Southern part of Europe. Consequently, Prevenar will cover between 71% and 86% of isolates causing invasive pneumococcal disease (IPD) in European children less than 2 years of age. More than 80% of the antimicrobial resistant strains are covered by the serotypes included in the vaccine." Also, EU assessors recommended the use of the vaccine in a more restricted age group, i.e., children from 2 months of age through 2 years of age, because a positive benefit/risk ratio for the age groups  $\geq 2$  years could not be ascertained based on the data included in the dossier. As a result of these findings, the originally granted EU indica-

tion was restricted to active immunization of infants and children from 2 months of age through 2 years of age. Another concern raised during the assessment in the EU was that following vaccination, the circulation of nonvaccine serotypes may increase and these serotypes may replace the vaccine serotypes and cause disease, leading to a reduction in vaccine efficacy. For example, in contrast to the U.S. Kaiser Permanente trial, the Finnish otitis media trial showed such an increase of acute otitis media due to nonvaccine serotypes (11). Therefore, the applicant committed to evaluate and report pneumococcal serotypes causing colonization and invasive disease and shifts among nonvaccine serotypes in ongoing efficacy and surveillance studies. As a condition for licensing, the applicant committed to further evaluate the safety and immunogenicity for previously unvaccinated older children, aged 24 to 36 months, and to submit a final phase 4 observational study report from a trial with older U.S. children. Since marketing authorization was granted by the European Commission in February 2001, these two studies were submitted and constituted the basis for a broadening of the originally granted indication to previously unvaccinated children aged 2 to 5 years. Due to European particularities, it was necessary to add the following wording to the indication: "The use of Prevenar should be determined on the basis of official recommendations taking into consideration variability of serotype epidemiology in different geographical area as well as the impact of invasive disease in different age groups." An important aspect of licensing of any kind of vaccine in the EU is also the concomitant administration of a new vaccine with already licensed vaccines, because the 7vPnC vaccine would probably always be used concurrently with other childhood vaccines. Thus, in order to evaluate the potential interference of Prevenar with concurrently administered childhood vaccines, the applicant studied the immune response to routine pediatric vaccines coadministered with 7vPnC vaccines in seven controlled clinical studies. The antibody responses of the Prevenar group to most of the antigens were similar to those of controls. For a CRM-based Hib conjugate vaccine, an enhancement of antibody responses to Hib and diphtheria toxin in the infant series was observed. Following the booster dose, some suppression of Hib antibody levels was observed, but all children had protective levels. Inconsistent reduction in the responses to pertussis antigens as well as to an inactivated poliovirus vaccine was observed. The clinical relevance of these interactions is unknown. Limited results from open-label studies showed acceptable responses to measles-mumps-rubella and varicella vaccines. The whole-cell pertussis vaccine

and concurrently administered Prevenar were associated with high reactogenicity, in particular with regard to fever and fussiness. Systemic reactogenicity data revealed that Prevenar elicited higher rates of fever than the controls. Therefore, a general statement on antipyretics was included in the SPC. Data on the concomitant administration of Prevenar and Infanrix hexa (a diphtheria–tetanus–acellular pertussis–Hib [polyribosylribitol phosphate-tetanus toxoid]-inactivated poliovirus-hepatitis B vaccine) have shown no clinically relevant interference in the antibody response to each of the individual antigens when the vaccines are given as a three-dose primary vaccination series, but the rate of febrile reactions to the coadministered vaccines is higher than that to the hexavalent vaccine alone.

During the meeting on 17 to 19 October 2000, the Committee for Proprietary Medicinal Products, in light of the overall data submitted and the scientific discussion within the committee, issued a positive opinion for granting market authorization for Prevenar. In the course of the assessment, however, a number of unsatisfactorily addressed efficacy, immunogenicity, and safety issues were identified. In response, the applicant provided a letter of undertaking on the 16 follow-up measures to be fulfilled as requested by the committee. Detailed background information on the centralized procedure can be found in the *European Public Assessment Report* (16).

### **WHO Guidance Regarding Licensure Criteria for Second-Generation**

#### **Pneumococcal Conjugate Vaccines for Infants**

Clinical end point efficacy studies to support the licensure of new pneumococcal vaccines have become increasingly difficult to conduct. Thus, the WHO convened a series of consultation meetings to consider serological criteria for the evaluation and licensure of new formulations and combinations of pneumococcal conjugate vaccines. Consensus was reached at a WHO meeting held in June 2003, and the recommendations were adopted by the Expert Committee on Biological Standardization in November 2003. The WHO recommended that immunoglobulin G antibody concentrations, as measured by an enzyme-linked immunosorbent assay (ELISA), in sera collected 4 weeks after a three-dose primary series should be chosen as the main licensing criterion. The organization further indicated that a single-threshold antibody concentration (0.35 µg/ml) could be used for all pneumococcal serotypes, although noting that this threshold does not necessarily predict protection in an individual subject. This antibody level was based on pooled efficacy estimates from three clin-

ical efficacy trials: the NCKP trial, the American Indian study, and a South African trial which studied a nine-valent CRM conjugate vaccine (2, 29, 32, 36). For example, the pooled efficacy estimate was approximately 90% for vaccine serotypes across the three studies; the threshold level of 0.35 µg/ml was the antibody concentration achieved by 93% of subjects in the serology cohorts enrolled in these efficacy studies. Of note, the threshold antibody response of 0.35 µg/ml was chosen based on results from a second-generation ELISA used in the prelicensure clinical studies of the 7vPnC vaccine. However, a third-generation assay, making use of a second adsorption step (22F adsorption) is the standardized assay now in use. In the WHO consensus meeting, it was further recommended that the percentage of individuals with postimmunization antibody concentrations above this antibody threshold should be a criterion to determine noninferiority and that head-to-head comparisons with a registered vaccine (e.g., the licensed 7vPnC vaccine) is the preferred method for evaluating new vaccine formulations. It was thought that noninferiority to the antibody response for each of the serotypes in the registered vaccine is desirable but not an absolute requirement and that the evaluation of new serotypes not contained in a registered formulation may be based on noninferiority to the aggregate response to the serotypes in the registered vaccine. Of note is that a definition of aggregate response for the evaluation of new serotypes not contained in the registered formulation was not provided. Finally, there was consensus that the measurement of opsonophagocytic antibody (OPA) levels by an opsonophagocytic assay after a three-dose priming series should be required to demonstrate the functionality of antibodies. The OPA titer that would serve as a useful threshold for serotype-specific activity in a well-characterized OPA assay was not discussed. However, additional analyses of data from the three efficacy trials used to establish the antibody threshold level showed that an ELISA-determined antibody level of 0.21 to 0.35 µg/ml correlates with an OPA titer of 1:8 (27). It is important to point out that even though functional antibody as measured by an OPA assay is widely thought to be important for protection against pneumococci, the relationship of OPA to efficacy has not been established in clinical trials.

### **Considerations for Licensure Pathways of Second-Generation Pneumococcal Conjugate Vaccines in the United States**

On 8 March 2001, the VRBPAC was convened to consider alternate approaches for the licensure of second-generation pneumococcal conjugate vaccines

indicated for children less than 2 years of age. VRBPAC recommended the following: noninferiority immunogenicity studies conducted in the United States comparing a pneumococcal conjugate candidate vaccine to Prevnar based on an antibody response quantified by ELISA would be an acceptable approach for inferring efficacy against invasive disease for the candidate vaccine. Of note, the committee did not provide clear advice about whether noninferiority would have to be demonstrated for all seven serotypes contained in Prevnar or whether specific serotypes should be weighed more heavily based on the disease impact of those serotypes. For additional serotypes not contained in Prevnar, immunological parameters may be used to infer the efficacy of the additional serotypes.

VRBPAC discussed that data from an invasive disease efficacy study(ies) performed among a non-U.S. population(s) with a new pneumococcal conjugate vaccine could support licensure of the vaccine in the United States provided that adequate bridging studies of safety and immunogenicity were conducted that would include Prevnar as a control arm to establish comparability of the new product. VRBPAC also advised that efficacy against invasive disease could not be inferred directly from a demonstration of efficacy in preventing otitis media. Similar discussions regarding licensure pathways for second generation pneumococcal conjugate vaccines have not taken place in the EU.

## PNEUMOCOCCAL CONJUGATE VACCINES FOR THE ADULT POPULATION

### Considerations for Licensure

#### Pathways: a U.S. Perspective

*S. pneumoniae* is the most common cause of community-acquired pneumonia (CAP) among persons  $\geq 65$  years of age in the United States, resulting in hospitalizations and deaths (26, 28). The 23vPS vaccine is currently recommended for this population to protect against pneumococcal disease; however, several studies have failed to demonstrate a positive preventive effect of the currently available PS vaccine against pneumonia in the elderly (25). It is possible that pneumococcal conjugate vaccines have improved effectiveness compared to the current standard of care to protect the adult population, in particular the elderly, from pneumococcal disease.

The preclinical and clinical development of pneumococcal conjugate vaccines to protect adults and the elderly from pneumococcal disease falls within the framework of U.S. laws and regulations described above; however, there are some unique considerations regard-

ing their licensure pathways, which are discussed below. As already described, clinical trials demonstrating preventive efficacy for clinical end points among the population for which the product is indicated provide the greatest scientific rigor for evaluating pneumococcal conjugate vaccines and for supporting the licensure of these products. Usually, such studies are prospective, randomized, and well controlled, and the primary efficacy end point is the prevention of pneumococcal disease. The licensure of the 14-valent pneumococcal PS vaccine, the predecessor of the currently licensed 23vPS vaccine, was based on a demonstration that a similar vaccine prevented pneumococcal pneumonia and bacteremia in South African gold miners, a population chosen for the clinical studies because of the high rate of pneumococcal pneumonia in that population (1). In addition, the prophylactic efficacy of the 7vPnC vaccine in protecting children less than 2 years of age against invasive disease was demonstrated in a large field efficacy study that enrolled approximately 38,000 infants who received a four-dose vaccine series (2).

However, clinical end point efficacy trials evaluating the effectiveness of pneumococcal conjugate vaccines in protecting against IPD or pneumococcal pneumonia in adults and in the elderly are complicated because of the low and declining incidence of pneumococcal disease due to serotypes in 7vPnC at least in the United States population. Also, since in the United States the 23vPS vaccine is currently recommended for the immunization of adults  $\geq 65$  years of age and high-risk populations, invasive disease clinical end point studies to support the licensure of a new pneumococcal vaccine would likely need to be designed as noninferiority studies using the 23-valent pneumococcal conjugate vaccine in the comparator group. Vaccine manufacturers may not view such studies as feasible because of the sample sizes, resources, and time required. Alternatively, these trials may be designed using a placebo or an unrelated control vaccine. This scenario would likely require conducting studies with a population for whom the 23vPS vaccine is not currently recommended (e.g., healthy adults aged  $\leq 65$  years).

Clinical trials with pneumococcal conjugate vaccines assessing CAP as a clinical end point would be important for the purpose of public health recommendations and could provide clinical evidence of benefit afforded by a vaccine for licensure purposes. Although sample sizes for an end point of CAP requiring hospitalization would be quite large, by choosing appropriate radiological and clinical criteria and pathogen-specific diagnostic criteria (e.g., sputum culture, PCR detection, and detection of antigen in urine), it may be possible to eval-

uate a clinical end point of pneumonia. In the past, low specificity and sensitivity of diagnostic criteria resulted in uncertain etiological diagnoses, making sample size estimates for such trials difficult to ascertain. For example, false positives in the vaccine group would lower the efficacy estimate as well as increase the sample size needed to show any level of efficacy. Recent development of antigen tests may be helpful for the establishment of pneumococcal etiology in CAP in adults, thus facilitating clinical end point definitions (37). The use of nonspecific inflammatory markers such as C-reactive protein and procalcitonin has also been proposed as a method to increase the specificity of the X-ray diagnosis of bacterial pneumonia (31). Greater specificity in the diagnosis of CAP due to *S. pneumoniae* in vaccine trials would result in more manageable sample sizes and more accurate efficacy estimates for a truly effective vaccine. These trials could be conducted simply, using computerized hospital discharge databases, and with few additional resources for diagnosis and case evaluation.

In certain situations, the effectiveness of a vaccine may be established through an immunological end point, in particular in cases in which there is an accepted immunological correlate of protection. A correlate of protection is a laboratory parameter that has been shown to be associated with protection from clinical disease. An immunological correlate of protection is most useful if it measures a known biological function associated with protection. As described above, a demonstration of inferred efficacy of future pneumococcal conjugate vaccines indicated for children less than 2 years of age may be based on noninferiority immunogenicity studies through antibody responses quantified by ELISA. Notably, this recommendation specifically pertained to inferring efficacy in the prevention of IPD in an infant target population, since a clinical efficacy study using this end point had been conducted. However, such efficacy data are not available for adults.

It is unclear whether serologic parameters currently considered to be protective threshold levels for infants can be used as a benchmark to define protective antibody titers for adults. Antibody levels that correlate with protection in the adult population have not been defined, adults may have already high pretiters, and protective antibody levels may differ by disease, e.g., invasive disease versus CAP. The ability to extrapolate data from younger populations to older populations is problematic because of current gaps in the understanding of immune senescence with increasing age. Since the adult population is composed of diverse risk groups, with differing levels of immunocompetence, a new pneumococcal conjugate vaccine may need to be studied

among several adult subpopulations. Therefore, in licensure pathways for pneumococcal vaccines for adults that are based on studies using immunological end points, measures of functional antibody are probably more relevant than ELISA-determined antibody levels.

The capsular PS is the principal virulence factor enabling the pneumococcus to cause invasive disease. Type-specific antibody is considered protective, and protection is thought to be mediated through antibody binding to the bacterial surface, leading to complement-mediated uptake into phagocytic cells (opsonophagocytosis). Thus, functional antibody likely plays a central role in protection against pneumococcal disease. Therefore, measurements of OPA in vitro provide evidence for in vivo protection; in most assays, the usual minimum detectable opsonic titer is 1:8. However, while considerable progress has been made towards developing standardized and automated assays to assess the opsonophagocytic activity of antipneumococcal antibodies (34), the quantitative relationship of OPA titers (as measured by existing assays) to clinical efficacy in the adult population has not been established.

According to the rule published by the U.S. FDA in 1992, the FDA may grant accelerated approval for a biological product that is intended to prevent or treat serious or life-threatening conditions and that provides meaningful benefit over existing treatment (21 CFR 601.41) (23). For a new pneumococcal conjugate vaccine, protection of adults and the elderly from CAP may be a meaningful benefit over existing treatment, since data show that the currently available 23vPS vaccine does not decrease the risk of CAP in the elderly (25). For pneumococcal conjugate vaccines, vaccine serotype-induced OPA titers (OPA activity) may be considered reasonably likely to predict clinical benefit and, thus, would meet the requirements of a surrogate for efficacy under the accelerated approval regulations (21 CFR 601.41). Thus, a possible pathway to licensure would be to infer efficacy of the product by performing prelicensure noninferiority studies comparing the candidate pneumococcal conjugate vaccine to the current standard of care (the 23vPS vaccine) based on the OPA titer for each of the serotypes that are common to the candidate vaccine and the 23vPS vaccine. Suitable controls and assay validation are important for the interpretation of OPA results. In addition to conducting noninferiority studies using OPA titers as an end point, confirmatory postmarketing clinical studies demonstrating clinical benefit of a new pneumococcal candidate vaccine against CAP would need to be conducted to satisfy the accelerated approval requirements as stated in 21 CFR 601.41 (23). Such studies may support a clear

advantage of that vaccine compared to the current PS vaccine, as the effectiveness of a PS vaccine in protecting against CAP remains uncertain. Furthermore, a postmarketing confirmatory study would allow for assessing product safety with a large number of adult subjects in a controlled setting.

An assessment of the benefit of a new conjugate vaccine comprising fewer serotypes than the 23vPS vaccine is complicated by the loss of protection afforded by the additional serotypes in the PS vaccine. Persons vaccinated with the new conjugate would remain susceptible to serotypes not included in the vaccine. On the other hand, the currently available 23vPS vaccine does not induce immunological memory, and there are data to suggest that subsequent doses of the 23vPS vaccine may induce a less vigorous immune response than that measured after the initial dose (i.e., hyporesponsiveness). There is also evidence that antibody titers and efficacy wane over time following immunization with the 23vPS vaccine (35). Thus, a pneumococcal conjugate vaccine that invokes a T-cell-dependent immune response with the potential for more robust immunogenicity and the induction of functional antibody combined with lessened risk of hyporesponsiveness with subsequent dosing may provide increased benefit over the currently available standard of care.

Concerns have been expressed that a vaccine providing coverage for young children may not provide optimal serotype coverage for the prevention of pneumococcal disease in older adults, particularly as the epidemiology changes as a result of indirect effects (e.g., herd immunity to the vaccine serotypes) of the universal immunization of infants with the 7vPnC vaccine. Thus, serotypes not represented in the conjugate vaccine used in childhood may assume greater prominence as a cause of pneumococcal disease in adults. The potential for this scenario would need to be addressed in postmarketing studies. Indirect effects of vaccination with the 7vPnC vaccine are thought to be due to the prevention of colonization by and carriage of pneumococci in the nasopharynges of infant vaccine recipients, with a resulting reduction in transmission to older adults in the household. Studies demonstrating the prevention of colonization and carriage may be considered part of a body of data demonstrating a vaccine effect.

## CONCLUSION

The use of the 7vPnC vaccine licensed in the United States and the EU has been very effective in reducing IPD. In particular in the United States, the rate of IPD in children under 2 years of age has decreased dramatically

since the 7vPnC vaccine was licensed and has also declined among adults and the elderly. The impact of the 7vPnC vaccine in reducing IPD in Europe is less certain, as vaccination programs and recommendations for the use of this vaccine differ in individual member states of the EU. In addition, despite the impact that the 7vPnC vaccine has had in reducing IPD in the United States, *S. pneumoniae* continues to be the cause of serious morbidity and mortality in other areas of the world. This is due potentially to differences in pneumococcal serotype prevalence in various geographical areas, and to differences in subject populations, the availability of the vaccines, immunization coverage, and recommendations for use. In addition to young children, the elderly are especially vulnerable to *S. pneumoniae* disease. The 7vPnC vaccine is not indicated for this population, and the efficacy of the 23vPS vaccine, in particular in protecting against CAP, remains uncertain. Therefore, in order to increase the protection of children, adults, and the elderly against pneumococcal disease, vaccine manufacturers have proposed vaccination with new pneumococcal conjugate vaccines with increased serotype coverage and indications covering expanded age ranges. As discussed in this chapter, clinical end point efficacy studies to demonstrate clinical benefit from these second-generation vaccines in the prelicensure setting have become difficult to conduct. Thus, new regulatory approaches to guide the path to licensure for these products were necessary. To facilitate the clinical development of new pneumococcal conjugate vaccines, a series of global and national workshops and meetings were held in consultation with the WHO and particular advisory bodies to formulate licensure criteria. These efforts have assisted in regulatory decision making and in the formulation of licensure pathways for these new pneumococcal conjugate vaccines in order to enhance the protection of children, adults, and the elderly from pneumococcal disease.

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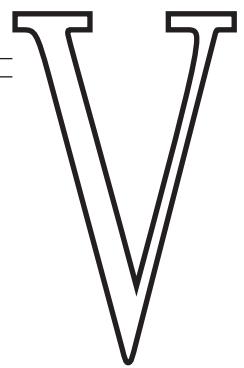
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*Immunogenicity*

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Dace V. Madore  
Sally A. Quataert  
Merja Vakevainen

14

# Quantitation of Anti-Pneumococcal Capsular Antibody in Ligand-Binding Assays

This chapter provides a historical overview of the ligand-binding assays currently recommended by the World Health Organization (WHO) to evaluate immune responses to polysaccharide (PS)-based vaccines against *Streptococcus pneumoniae*. These assays are designed to quantitatively measure serotype-specific antibody concentrations in the sera of subjects participating in treatment and control groups in clinical trials. Additionally, we discuss the utility of other types of pneumococcal immunoassays and provide our perspective on critical parameters for maintaining a bridge to previously established serologic correlates associated with the vaccine efficacy.

The road to licensure of a new pneumococcal vaccine is challenging. Extensive serologic data from the target population are required to demonstrate a consistent immune response that is correlated with and predictive of vaccine efficacy. The clinical serologic data provide a means to compare the immunogenicities of vaccine lots prepared by different manufacturing methods and to confirm that the selected manufacturing process yields a

consistent response across multiple lots. Clinical serologic data are used to determine the optimal dosage and immunization schedule and also to show that the immune responses to individual serotypes are not compromised when additional serotypes are added to the vaccine formulation.

When a protective vaccine is available and efficacy trials are no longer ethical or feasible to perform, the introduction of new and/or improved pneumococcal vaccines must rely on serologic data. Important considerations for the development and/or selection of an appropriate serologic method include whether the assay can characterize the immune response to a variety of potential vaccine formulations, can provide sufficiently reproducible results to support trials spanning many years of clinical testing, and can generate clinically relevant data enabling future vaccine comparisons. Most importantly, the antibody quantitation method must specifically and accurately measure the immune responses to the active ingredients in the vaccine.

## SEROLOGIC METHODS ASSOCIATED WITH PNEUMOCOCCAL VACCINES

The first pneumococcal vaccines were introduced in the 1940s for adults and included serotype-defining capsular PSs purified from four to six serotypes (48). After years of clinical trials evaluating the efficacy of a multi-valent vaccine for the prevention of pneumococcal infections in adults, a 14-valent vaccine was licensed in the United States in 1977 (5, 6, 71, 85), followed in 1983 by the 23-valent vaccine (71, 72), which is still in use globally.

The early methods used to evaluate antibodies to pneumococcal PS (PPS) included quantitative precipitation, mouse protection, and indirect hemagglutination tests (1, 7, 28, 36, 48, 78). In the 1980s, a radioimmunoassay (RIA) was developed and used by Schiffman et al. to measure antibody responses to PPS vaccines (78). The method used <sup>14</sup>C-labeled PPS antigens and the Farr technique (21) for the precipitation of antibody-antigen complexes to determine the total amount of antigen bound. The technique was sensitive and yielded reproducible results but required radiolabeled PPS and was unable to distinguish between the different antibody isotypes and immunoglobulin G (IgG) subclasses. During this time, the enzyme-linked immunosorbent assay (ELISA) was being developed (8, 26, 33, 44, 52, 54, 73). It rapidly became the preferred method because it consumed less serum than the RIA, provided isotype and subclass data, and made radioactive reagents unnecessary, simplifying antigen preparation and disposal as well as reducing hazards in the laboratory.

In the 1980s, new experimental pneumococcal vaccines based on glycoconjugate technology were being developed with the goal of protecting infants from the most invasive pneumococcal strains. During this time, it was noted that the serologic methods were not adequately specific for the quantitation of antibodies to individual serotypes, as antibodies to pneumococcal cell wall PS (CPS) were also detected (42, 82). To support the anticipated years of clinical testing to evaluate vaccines containing multiple PPSs, Quataert et al. further refined and validated serotype-specific ELISAs as well as developed and characterized a standard reference serum, lot 89-S (67). Serologic data generated using these ELISAs and this reference serum supported the licensure of PCV7-CRM, the first seven-valent pneumococcal conjugate vaccine (PCV), for infants in the United States in 2000. This glycoconjugate vaccine provides protection against potential invasive disease caused by seven serotypes of *S. pneumoniae* (4, 6B, 9V, 14, 18C, 19F, and 23F) (10, 80, 99). Subsequent clinical evaluations with other infant populations have supported the

registration and use of this vaccine globally (18, 20, 40, 53, 60, 61).

## ELISA DEVELOPMENT AND STANDARDIZATION

Clinical immunology laboratories from industry, academia, and regulatory agencies began formally sharing methods, reagents, and validation results in 1989 so that they could evaluate and compare the immunogenicities of experimental PCVs with minimal differences among outcomes from different laboratories (82, 98). With the approval and introduction of PCV7-CRM, the WHO sponsored an expert consultation on serologic criteria for the evaluation of PCVs and agreed that the Quataert et al. ELISAs and reference serum (67) were rigorously validated for the testing of sera from infants and were sufficiently robust to provide a foundation for future vaccine evaluations (34). The WHO has published guidelines for quantitating serotype-specific IgG antibodies to *S. pneumoniae* in human sera based on the Quataert et al. assays (100). Additionally, a website is updated regularly with technical information and advice, enabling access to the most current procedures and reagents recommended by laboratory experts consulting for the WHO ([www.vaccine.uab.edu](http://www.vaccine.uab.edu)) (58). Importantly, the clinical data generated by these ELISAs have provided a threshold anti-PPS antibody concentration associated with protection from invasive disease in infants that should be used in noninferiority studies for comparing new PCVs, immunization schedules, or vaccine formulations (see chapter 23) (34, 81).

The demonstrated success of an efficacious pneumococcal vaccine for infants has stimulated interest in expanded vaccine coverage for other populations at risk for pneumococcal disease, including the elderly and human immunodeficiency virus (HIV)-infected individuals. With the abundance of serologic data confirming the consistent performance of Quataert et al. ELISAs in evaluating infant sera (10, 14, 19, 20, 39, 40, 53, 61, 69, 80), we now can reflect on the critical elements of this methodology and address limitations in applying this technology more broadly, as well as make recommendations on migration to new methods and reagents and approaches to interlaboratory comparisons.

### Accurate Antibody Quantitation

The development and validation of the anti-PPS ELISAs requires not just the creation of specialized reagents, such as the standard reference serum and quality control sera, but also the optimization and standardization of the entire testing process. Such laboratory work can-

not be conducted in a vacuum but must involve a thorough understanding of the chemistry of the candidate vaccines and the nature of the human populations to be tested and include a statistical plan for the validation of the assays. Every serologic method needs to be optimized for its intended purpose; acceptance and rejection criteria must be developed to enable replacement with any new reagent, material, or procedure. Once validated,

an assay needs to be continually monitored by testing quality control sera representative of the target population to ensure consistent assay performance. Validation data should demonstrate specificity, linearity, precision, and accuracy and define the assay limits as well as characterize the assay robustness (50). Important performance characteristics of the Quataert et al. assays (65–68) are summarized in Table 1.

**Table 1** Validation of ELISAs for quantification of antibodies to multiple serotypes of *S. pneumoniae*<sup>a</sup>

Characteristic	Example(s) of validation data descriptive of the Quataert et al. ELISA <sup>b</sup>
Specificity	Antibodies do not bind to the plate in the absence of antigen, as shown by optical density readings of less than 0.1 Each lot of anti-human IgG reagent is prescreened to verify that it is isotype specific (>95%) and detects all subclasses, as well as $\lambda$ and $\kappa$ light chains, with equal sensitivity Each lot of PnA blocks the binding of antibodies to CPS by >90% with testing of a 1:50 dilution of serum Homologous PPS antigens inhibit binding of antibodies (from infants at 7 months of age post-third immunization) by >90%; inhibition by heterologous PPS is minimal (<10%) Postimmunization sera do not show increased binding to irrelevant PPS serotypes 1 and 5 that are not represented in the PCV7-CRM vaccine
Linearity	Parallelism of reference standard serum and test specimens ensures that consistent and accurate assignments are given to test specimens. The assay results are linear over a minimum of 3 dilutions when evaluated on a log/log scale of titrations of optical density values versus dilution
Precision	Intermediate precision defines the assay performance over different days and with different analysts and equipment. The QC sera (with high, medium, or low concentrations of IgG antibodies to each serotype) from adults are run with each ELISA and have an average c.v. of 31.2% for the combination of 7 serotypes. Preselected sera (to cover entire anti-PPS concentration range) from infants are run on multiple occasions specifically to define the assay performance by serotype
Accuracy	The sum of serotype-specific IgM, IgA, and IgG in standard reference serum lot 89-S equals total Ig assignments, with a mean ratio ( $\pm$ SD) of the sum to the total value for the 7 serotypes of $0.92 \pm 0.099$ For pre- and postimmunization sera from infants, the sum of the serotype-specific IgM, IgA, and IgG equals the total Ig values in lot 89-S
Assay limits	The minimum concentration for quantitating IgG antibodies was rounded up to 0.01 $\mu$ g/ml for all serotypes. Antibody assignments have a c.v. of $\leq 35\%$ at concentrations of $\geq 0.15$ $\mu$ g/ml for all serotypes except 19F, to which this c.v. applies at $\geq 0.5$ $\mu$ g/ml.
Reproducibility	An interlaboratory study with 10 participants evaluating 5 adult sera demonstrated overall mean c.v.'s for all five serotype anti-PPS determinations of 30% for IgG, 37% for IgM, and 36% for IgA
Robustness	The following critical reagents must be qualified for use whenever a new lot or batch is obtained: PPS antigens, microtiter plates, CPS or PnA, and secondary antibody-enzyme conjugates Antibody quantitation is sensitive to the type of microtiter plate used (specificity is compromised with Maxisorb plates because of increased nonspecific binding) Polyclonal and monoclonal anti-human IgG detecting agents yield equivalent results when screened as described for specificity The antibody binding steps of the assay must be performed at room temperature (20 to 25°C) Water source for all reagent preparation must be pyrogen free Sodium azide must be included in coating buffer if plates are to be stored and not used immediately

<sup>a</sup>Abbreviations: QC, quality control; c.v., coefficient of variation.

<sup>b</sup>References 66 to 68.

### Antigen

Understanding the chemical compositions of the candidate vaccine, its excipients, and any potential contaminants is critical in order to validate that the serologic method measures only antibodies to the vaccine's active ingredient, in this case, serotype-specific PPS. To facilitate ELISA standardization and to reduce interlaboratory variability, a common source of antigen (the American Type Culture Collection) is recommended (58, 67). Even with one common source of antigen, each lot of serotype-specific PPS must be tested to ensure that the laboratory's preestablished purity and performance requirements are met. The use of highly purified water for injection is recommended for buffers to prevent background noise in the ELISAs (50). The use of medium binding (and not high binding) plates is recommended to minimize the binding of proteins, which may be present as contaminants in some PPS antigen preparations. The quality and consistency of antigen preparation and differences in plate performance are frequent reasons for the nonspecific binding of antibodies, contributing to inconsistent antibody assignments among laboratories.

By definition, different serotypes within a serogroup of *S. pneumoniae* strains have common (shared) epitopes (30); various coating methods or antigen modifications may expose these shared epitopes differently (41, 91, 103). Similarly, various vaccine formulations may selectively generate antibodies to serotype-unique and/or shared epitopes (96, 102). Ideally, in order to fully characterize the immunogenicity of a vaccine, the coating antigen should detect all the antibodies elicited by the PPS, irrespective of their avidities or epitope preferences.

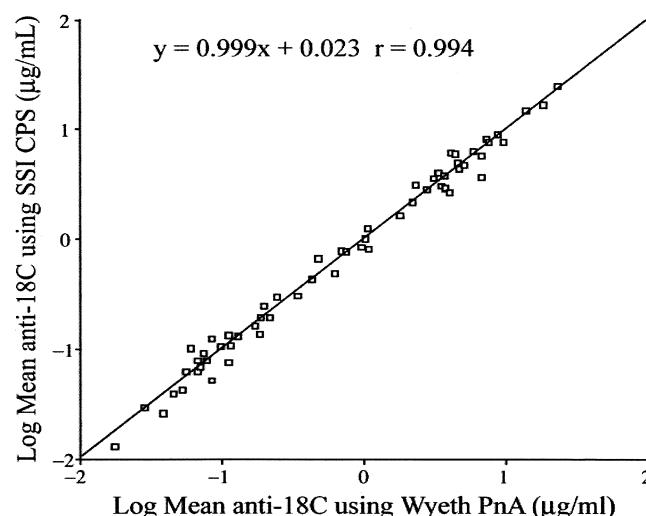
### Specimens

Maintaining the integrity of the serum specimens collected in a clinical trial is critical. If sera are treated (e.g., by heat inactivation or the addition of preservatives), it is critical to validate that the accuracy and reproducibility of antibody quantitation in the ELISA have not been affected. Even when following a previously validated ELISA protocol, the assay performance needs to be assessed, reoptimized if necessary, and revalidated when sera from sociologically different, genetically distinct, and/or at-risk populations (e.g., Navajo, South African, HIV-infected, and elderly subjects) are tested. The Quataert et al. assays were optimized for the quantitation of serotype-specific antibodies in the sera of infants from developed countries; current WHO recommendations (58, 100) further modify these assays to improve their specificity for the testing of sera from other populations. Even when serologic assays are developed successfully for the purpose of evaluating and comparing responses to vaccines, it must be remembered that the

presence of maternal antibodies and other serum components, as well as the immunological maturation status and environmental exposure of the subject, affects antibody quantitation (32, 55, 88); the potential for such factors to affect the immunogenicity of vaccine candidates can be addressed in the trial design (e.g., with appropriate control groups and multiple sampling times postimmunization).

### Absorbent

The accurate measurement of PPS-specific antibodies is challenging because naturally occurring antibodies in sera can bind to CPS as well as to other covalently bound and copurified antigens of *S. pneumoniae*. Antibodies to CPS have been found to be nonprotective and abundant in almost all human sera (11, 23, 42, 57). A consistently prepared and qualified crude pneumococcal absorbent (PnA), consisting predominantly of CPS, was developed and used in the Quataert et al. ELISAs (67, 90). When PnA was compared to a more highly purified CPS preparation (from the Danish Statens Serum Institute) (SSI) in the Quataert et al. ELISAs for five serotypes, similar anti-PPS antibody concentrations were assigned to sera from infants in Finland (Fig. 1) (90).



**Figure 1** Comparison of anti-PPS antibody assignments for sera using two different CPS absorbents (90). Pre ( $n = 32$ ) and post ( $n = 31$ )-immunization sera from infants immunized with an experimental five-valent PCV were tested using either crude Wyeth PnA or purified CPS from the SSI to assess a comprehensive antibody concentration range. A representative comparison is shown here, for anti-PPS serotype 18C. The relatively crude PnA preparation performs equivalently to the purified CPS from SSI in the Quataert et al. assay for all five serotypes: 6B ( $y = 0.919x + 0.091$ ;  $r = 0.981$ ), 14 ( $y = 1.022x + 0.075$ ), 19F ( $y = 0.972x + 0.037$ ;  $r = 0.985$ ), and 23F ( $y = 0.978x + 0.080$ ;  $r = 0.997$ ) (data not shown).

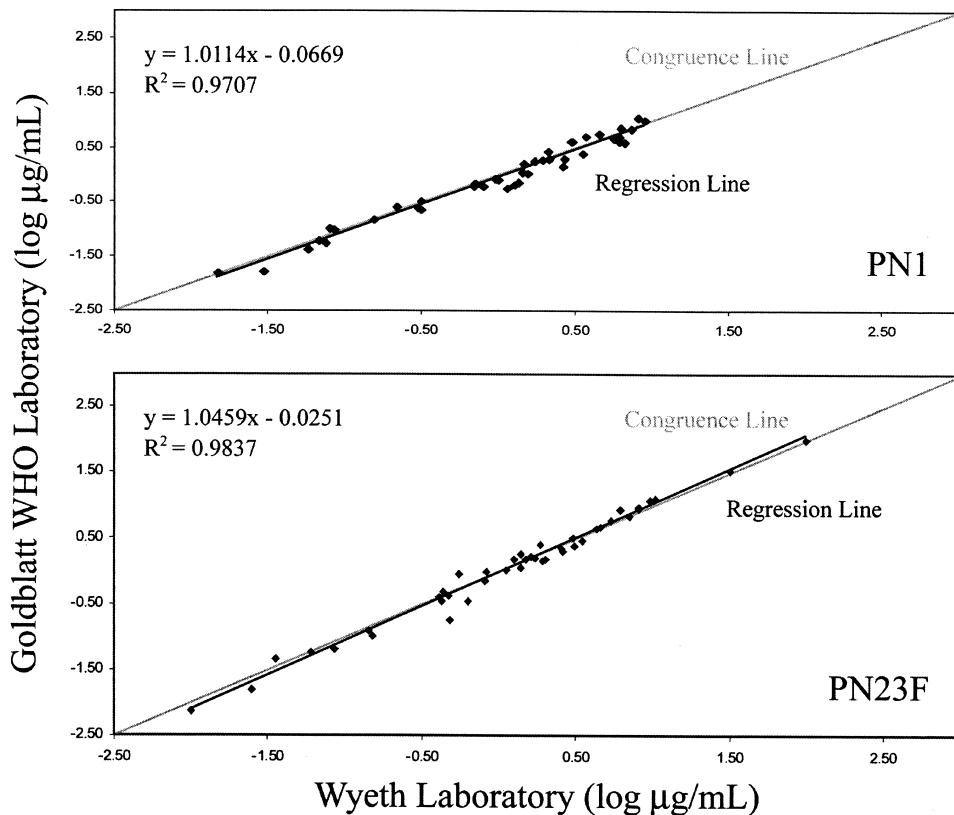
Additionally, the data in Fig. 2 suggest that these two absorbents can be used interchangeably for evaluating sera from adults. Two qualified laboratories used the same protocol and the same acceptance criteria for materials and reagents but used different reagents; the antibody assignments obtained by both laboratories were comparable across a full concentration range for all serotypes tested (1, 4, 5, 6B, 9V, 14, 18C, 19F, and 23).

While CPS or PnA absorption can be adequate to ensure the detection of PPS-specific antibodies in the sera from immunized healthy infants (38, 81, 88), somewhat depending on population (49), these absorbents appear to be inadequate to prevent the binding of other antibodies which bind to copurified contaminants and are commonly found in the sera of many adults and sub-

jects at risk for infection. Therefore, an additional absorbent (PPS 22F) is now recommended to be added to the subject sera (13, 22, 31, 98).

#### Reference Reagent

The critical reagent needed for serologic evaluation of pneumococcal vaccines is a large quantity of standard reference serum with defined serotype-specific anti-PPS IgG antibody assignments. Presently, the only standard reference serum associated with clinically relevant and defined protective thresholds for infants is lot 89-S (34, 67). This standard reference serum is available from the U.S. Food and Drug Association [labeled as lot 89S(F)] for use in assessing PPS-based vaccines in clinical trials (34, 58, 100). Importantly, this reagent enables



**Figure 2** Scatter plot of antibody concentrations assigned by qualifying (WHO-designated) and Wyeth laboratories (102). Both laboratories ran on three separate occasions a panel of adult sera ( $n = 61$ ) covering a broad range of antibody concentrations, using the same protocol but using independently obtained reagents, materials, equipment, and software. The most notable difference is that two different pneumococcal absorbents were used; Wyeth laboratory used PnA while the WHO laboratory used CPS (SSI). Scatter plots (those for serotypes 1 [PN1] and 23F [PN23F] are shown) of the antibody concentrations by serotype as determined by the WHO versus the Wyeth laboratory closely matched the line of congruence, with the regression line very close to 1 and the intercept close to 0. The  $R^2$  value was greater than 0.96 for all serotypes tested (1, 4, 5, 6B, 9V, 14, 18C, 19F, and 23).

comparisons of the clinical immunogenicities of new vaccine formulations to that of an approved, efficacious vaccine for infants, and additionally, it can be used to compare serologic data across time, different laboratories, and alternative methods.

Lot 89S(F) was prepared by pooling plasma (recalified and filter sterilized) from 17 donors who were selected by ELISA for having elevated titers of IgG antibodies to the majority of the serotypes of most clinical interest (67). All the adult donors had been previously immunized with PS vaccines for *S. pneumoniae* (23 valent) and *Neisseria meningitidis* (A/C/Y/W135) and with a *Haemophilus influenzae* type b (Hib) conjugate vaccine. A large number of donors (68) were screened with the intent that one preparation would be sufficiently high and uniform in titer to enable the same dilution schema to be used in ELISAs for 12 different serotypes. Furthermore, using large numbers of diverse donors diminished the influence of distinctive oligoclonal antibody populations in individual adults following immunization with a PPS vaccine (47).

Total Ig and IgG, IgA, and IgM antibody assignments were determined ultimately for 24 serotypes of *S. pneumoniae* (1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F) as well as for CPS (67, 68) by using previously established approaches (76, 104). Briefly, a reference ELISA and the anti-PPS ELISAs were performed in parallel using identical conditions and reagents, except for the antigen and reference sera. In the reference ELISA, the antigen was goat anti-human light chain-specific reagents to capture the Ig molecules in the human reference preparation (USNRP IS 1644, calibrated by the Centers for Disease Control and Prevention); in the pneumococcal ELISAs, the antigen was each respective serotype PS to capture the antibodies in lot 89S(F). Two other laboratories used cross-standardization ELISAs but used different reference reagents and confirmed the Ig and IgG assignments for lot 89S(F) for most of the serotypes they tested (12, 77). Based on the IgG concentrations (67, 68), IgG1 and IgG2 subclass assignments were determined for serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, and 23F (84, 87).

This reference serum does contain some level of non-serotype-specific antibodies, but its use as a calibrator has been validated for accuracy in assessing sera from infants from developed countries (67). The WHO ELISA protocol (58, 100) advises not to add a second absorbent (22F) to lot 89S(F) because the original anti-PPS antibody assignments were determined using only a single absorbent (PnA). The addition of 22F absorption on lot 89S(F) results in lower antibody assignments for al-

most all of the serotypes in the reference serum (Wyeth, unpublished data) because many adult sera contain antibodies to the nonspecific contaminants. Instead of recalibrating the dwindling supplies of lot 89S(F), a new standard reference reagent should be prepared and rigorously quantitated using more universally effective absorbents.

## ALTERNATIVE IMMUNOASSAYS

Although assay standardization efforts have been ongoing, a review of the recent literature on the topic of the immunogenicities of PCVs reveals that multiple variations of ELISAs are used (12, 29, 32, 37, 51, 64, 75, 83, 92, 94, 103). These assays vary in procedure and/or in reagents from Quataert et al. ELISAs, and the data from different methods and laboratories cannot necessarily be compared. Scientifically rigorous and statistically sound approaches for demonstrating the equivalency of reagent and assay performances are needed if the serologic data are to be used for decision making regarding the comparability of the immunogenicity or the protective antibody thresholds of a vaccine candidate to those derived from efficacy studies with a licensed or registered vaccine.

To provide academic, government, and industry laboratories a means to compare new methods and reagents and to generate serologic data that can be assessed with respect to the clinically relevant antibody threshold associated with PCV7-CRM immunization (34, 100), the WHO designated two expert serologic laboratories for *S. pneumoniae* (those led by D. Goldblatt at the Institute of Child Health, London, England, and M. H. Nahm at the University of Alabama, Birmingham). The Quataert et al. ELISAs for serotypes of current interest were transferred to these laboratories; the serotype-specific antibodies measured in adult sera yielded values consistent with the original assignments across a broad concentration range for the serotypes tested (Fig. 2).

One of the most difficult challenges in the migration from an established method or reagent to an alternative is the maintenance of accuracy of the clinically relevant antibody assignments in the new environment. Assay drift can occur subtly with successive changes in reagents or methods; each change may appear insignificant, but cumulatively, over time, the changes can result in a significant difference in the accuracy of antibody quantitation. For this reason, the archiving of two sets of reagents for periodic future testing is critically important. One is the standard reference serum which was used for the establishment of the clinically significant antibody threshold; the other is a panel of sera relevant

to the clinical target of the proven efficacious vaccine which covers a broad range of antibody concentrations and has been thoroughly explored for accuracy. After the licensing or registration of a new pneumococcal vaccine, we recommend that a reference laboratory, identified by an approving regulatory agency, as well as the vaccine manufacturer retain aliquots of such critical and unique reagents.

### Luminex Methodology

While the ELISA is well suited for screening large numbers of specimens against a single analyte, a separate assay is required for each pneumococcal serotype. As more serotypes are included in the new conjugate vaccines, immunogenicity testing by ELISA becomes ever more laborious, time-consuming, and costly. The greatest challenge is having a sufficient volume of sera from all subjects to test in multiple-serotype antigen-coated plates in addition to reserving sera for testing in assays assessing responses to other recommended immunizations (e.g., diphtheria, tetanus, pertussis, polio, measles, mumps, rubella, Hib, and hepatitis B vaccines) to demonstrate the absence of immune interference.

To increase the efficiency of immunogenicity assessment as well as to reduce serum consumption, several laboratories have employed Luminex multiple-analyte (LXA) profiling technology to quantitate anti-PPS antibodies for up to 23 serotypes simultaneously (9, 43, 63). In principle, this microsphere-based flow cytometric system allows testing of a single specimen against as many as 100 different target antigens in one assay well. Coupling of the PPS to microspheres having discrete fluorescence properties permits the simultaneous quantitation of multiple antibody specificities based on detection by a phycoerythrin-labeled secondary antibody. The advantages of the LXA technology over ELISA include rapid reaction kinetics, a large dynamic range requiring fewer serum dilutions, and most significantly, the ability to analyze antibodies to multiple serotypes in one reaction well, resulting in reduced serum consumption. In addition, the greatly reduced requirements of time and labor make the assays cost-effective after the initial investment in equipment.

Although the LXA method for testing anti-PPS antibodies needs to be developed and validated for each serotype individually, this technology appears to produce specific, sensitive, and reproducible data (43, 63). The antibody assignments obtained by the LXA assay for multiple serotypes correlate well with assignments obtained by ELISA and reference serum 89S(F) when using sera from adults (43, 63); the application of this technology to the testing of sera from infants has been

initiated (62). However, one needs to be cautious before transitioning from a well-validated method, such as the pneumococcal ELISAs, to a new system. The coupling of PPS to microspheres may affect the epitope configuration of the antigen (91, 103) and thus, the populations of antibodies that bind may be different from those in the WHO-recommended ELISA, in which antigens are passively adsorbed onto a solid surface; such differences will vary by serotype (Wyeth, unpublished data). The serological correlates of efficacy that have been defined are based on data from the ELISA (34, 81) and may be invalid if there are differences in epitope presentation and/or antibody binding to contaminants. Like the ELISA, the LXA methods for the measurement of anti-PPS antibodies need to be well controlled and validated for each serotype of interest; additionally, consistent preparation of PPS-coupled microspheres needs to be demonstrated (79) before the method can be standardized across laboratories.

### Approaches to Avidity Assessment

Antibody avidity is the strength with which an antibody binds to a complex antigen and has been observed to correlate with the establishment of B-cell memory following the maturation of immunity and/or immunization with conjugate vaccines developed for Hib, *N. meningitidis* group C, and pneumococci (3, 17, 25, 45, 70, 101). Several methods based on ELISA or RIA have been developed for determining the avidity of anti-PPS antibodies (2, 24, 56, 74, 91, 95). In ELISA techniques, the binding of antibody to the coated antigen in solid phase may be prevented by competitive inhibition with the same soluble antigen in the buffer (56) or by elution of the antibody from the antigen with a dissociating agent, such as thiocyanate (2, 74). Calculation of the avidity constant value (representing a median value for the antibody population in each specimen) is feasible by using competitive inhibition but not by using the elution technique, which only ranks the antibodies by their relative avidities. Using the RIA, the avidity constant can be obtained by evaluating the capacity of antibodies to bind radiolabeled antigen at different molar concentrations (27, 91, 95).

Due to their simplicity and the fact that ELISA is the method of choice for antibody concentration measurements, avidity methods based on ELISA and thiocyanate elution are frequently used to measure the relative avidities of anti-PPS antibodies. In one approach, bound antibodies are eluted with increasing concentrations of thiocyanate while using a single serum dilution (24, 74, 89). The results are expressed as serotype-specific avidity indexes which correspond to the molar con-

centration of thiocyanate required to reduce antibody binding by 50%. In another approach, a single concentration of thiocyanate is used to elute the bound antibodies in assay wells containing various serum dilutions (2, 17). The avidity index is expressed as the percentage of antibodies that remain bound to the antigen after thiocyanate treatment. Although the two elution methods have been found to rank anti-PPS antibodies in similar orders according to their relative avidities, no efforts have been made to standardize the assays used in different laboratories. To date, avidity measurements characterize differences in immune responses to different vaccines over time but have not been shown to predict protective efficacy. Previous studies assessing different formulations of Hib conjugate vaccines show that vaccines can be efficacious even though they stimulate populations of antibodies that differ in their avidity characteristics (46).

### RELATIONSHIP OF ANTIBODIES MEASURED BY ELISAS AND OPA ASSAYS

The capacity of pneumococcal capsular antibodies to opsonize bacteria for phagocytosis is considered to be the basis of their protective activity against *S. pneumoniae* (93). Several in vitro opsonophagocytosis assays (OPAs) have been developed to measure the opsonic activity of anti-PPS antibodies (see chapter 15). The correlation between IgG antibody concentrations as measured by ELISA and OPA titers has varied from weak to relatively strong depending on the immune statuses, ages, and health of the subjects, the pneumococcal serotypes tested, and the methods used to measure these laboratory parameters. Sera obtained from immunized children, the most carefully studied group, or healthy young adults show good correlations, while sera from unimmunized individuals show a poorer correlation (4, 59, 74). Further optimization of the ELISAs with second absorbents has improved the correlations (86, 97), but the ELISA results still correlate poorly with OPA titers when sera from the elderly (15, 16, 74) or HIV-infected individuals (49) are tested, suggesting that these populations may have more nonspecific or nonfunctional capsular antibodies that are measured in the ELISA despite double absorption with CPS and 22F PPS.

### INTERPRETATION OF ELISA-DERIVED SEROLOGIC DATA

The WHO has developed serologic criteria for the evaluation of new PCV formulations (34, 100). The primary end point for the demonstration of the noninferiority

of a new vaccine to the licensed or registered vaccine (PCV7-CRM) is the proportion of infants who achieve an IgG antibody concentration of  $\geq 0.35 \mu\text{g/ml}$  after a three-dose primary series as measured by the WHO-recommended serotype-specific ELISAs. Secondary end points include showing that the elicited antibodies are functional, as measured by OPA, and are associated with a boostable response indicative of memory.

The basis for choosing the IgG antibody concentration as the primary end point is well grounded since this isotype represents the desired immune response and the bridge to efficacy data for infants has been established (10, 35). The quantitation of anti-PPS antibodies by ELISA is a suitable surrogate assay for assessing protection from pneumococcal disease for this population. It has been well validated based on data from infants, and it has been standardized among laboratories, including two WHO-designated reference laboratories. It is easy to perform and correlates well with the OPA when testing postimmunization sera from infants.

### CONCLUSION

This chapter reviews the origin and evolution of the currently recommended serologic ligand-binding assays used to evaluate PPS-based vaccines. It is recognized that both the development of vaccines and the methods used to assess immune responses are dynamic. Presently, we are in the fortunate position to have well-defined and -validated assays and reagents, as well as accurate antibody assignments associated with a highly efficacious pneumococcal vaccine for infants. The challenge for the future is to introduce more-efficient serologic methods and new reference reagents without compromising this well-validated serologic predictor of vaccine effectiveness. The success of PCVs for infants has created the hope that protection can be extended to other at-risk populations and include additional serotype coverage. The current ELISAs provide a solid foundation for the future development, validation, and interlaboratory standardization of new ligand-binding assays for such applications.

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Moon H. Nahm  
Sandra Romero-Steiner

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## Functional Assays for Pneumococcal Antibody

Given the success of the seven-valent pneumococcal conjugate vaccine with the CRM<sub>197</sub> carrier (PCV7-CRM), new or modified conjugate vaccines are actively being developed to increase the number of serotypes included in the conjugate vaccine and/or to use in combination with other vaccines. Protective efficacy of the first conjugate vaccine was established through a clinical trial. However, placebo-controlled efficacy trials are no longer feasible to evaluate these new or modified vaccines since an effective pneumococcal vaccine is available. Thus, the development of a laboratory test(s) that can accurately reflect the protective immunity induced by pneumococcal vaccines is highly desirable. While the level of antibodies to pneumococci has long been used for this purpose, various limitations have been found to this method of measuring protective immunity. In addition, animal studies of active and passive immunization are neither surrogates of human clinical trials nor practical to perform due to the increased number of serotypes included in conjugate vaccines. Consequently, many studies have focused on laboratory measures of antibody function, which reflect the protective immunity induced by pneumococcal vaccines.

Assays for antibody avidity as well as an in vitro assay for the opsonophagocytic capacity of pneumococcal antibodies have been developed as laboratory tests to assess the functional capacity of antibodies. The best functional measure, however, appears to be the assay for opsonic capacity, which is the host's primary mechanism of protecting itself against pneumococcal infections. Because of its importance, the opsonophagocytosis assay (OPA) methodology has been greatly improved in the last decade and can now be performed for a large number of samples with ease and precision using only a small sample volume. In this chapter, we will review OPA methods for pneumococcal antibodies and the experience with OPA in clinical trials, stressing the need for further standardization of OPA.

### IMPORTANCE OF PNEUMOCOCCAL OPSONOPHAGOCYTOSIS

Although both innate and adaptive immunity synergize in host defense, vaccines enhance primarily the host's adaptive immunity to a specific pathogen and provide the host with immune protection against that pathogen.

By understanding how vaccine-induced adaptive immunity synergizes with innate immunity to enhance host protection, one can design the best laboratory test that correlates with protective immunity. In the case of pneumococcal conjugate vaccines, studies suggest that the vaccine-induced antibodies primarily help phagocytes ingest and kill pneumococci. This mechanism of host protection is described below.

### Mechanisms of Bacterial Opsonophagocytosis

Since Metchnikoff observed phagocytosis and named the phagocytes macrophages about 100 years ago, several types of cells, including dendritic cells and granulocytes, have been recognized as professional phagocytes. Phagocytosis has been found to be involved in several critical and fundamental biological functions, such as removing apoptotic or senescent host cells and ingesting and killing pathogens such as pneumococci. Consequently, cellular processes involved in phagocytosis have been extensively investigated (65).

Phagocytosis begins when phagocytes encounter their targets and become activated by recognizing the targets' pathogen-associated molecular pattern molecules. Subsequently, phagocytes establish direct physical contacts with their targets by binding to the molecules naturally present on the pathogens with phagocyte receptors (e.g., macrophage mannose receptor). Alternatively, phagocytes may establish contacts with target pathogens by binding to the host's opsonins, which have bound to the pathogens. A host has many molecules that can act as opsonins (e.g., surfactant protein A and C-reactive protein), but antibodies and the complement system are the best recognized opsonins. The phagocytic process involving opsonins is called opsonophagocytosis.

When pneumococci invade the host, pneumococci become decorated with appropriate antibodies and become opsonized. The antibodies immobilized on the bacteria can bind to one of several receptors for the Fc portions of immunoglobulin G (IgG) antibodies (Fc $\gamma$ Rs) on the host phagocytes, activate them, induce pseudopodium formation, and initiate the phagocytic process (8). CD64 (Fc $\gamma$ RI) is the high-affinity receptor for antibodies of the IgG1, IgG3, and IgG4 subclasses (70). CD32 (Fc $\gamma$ RII) is the low-affinity receptor for IgG1, IgG2, and IgG3 antibodies (70). CD32 is encoded by two alleles: the H131 allele encodes histidine at residue 131, and the R131 allele encodes arginine at the same location. The R131 form has a low affinity for IgG2 antibodies (70; chapter 6). The HL-60 cell line that is used as phagocytes in the OPA is homozygous for the R131 allele (15). FcR-mediated opsonization may have some in vivo relevance (60), but the deletion of all FcRs has

no effect on the ability of anti-capsular antibodies to passively protect against pneumococcal challenge (59).

The immobilized antibodies further opsonize bacteria by activating complement proteins and coating the bacteria with C3b, iC3b, and C3d, which correspond to the third complement component, C3, at three different stages of degradation (chapter 7). Of these, iC3b strongly binds to CR3 (CD11b/CD18) and is the most powerful opsonin. Upon recognizing iC3b, phagocytes take up bacteria without inducing pseudopodium formation and proinflammatory mediator production (8). The affinity of CR3 for iC3b increases when the phagocytes are activated (6), and complement-mediated phagocytosis occurs most effectively with activated phagocytes. C3b and C3d also bind, respectively, to CD35 (CR1) on granulocytes and CR2 on B cells, but their role in phagocytosis seems to be minor (chapter 7). After phagocytes ingest the bacteria, the resulting phagosomes containing the bacteria are fused with granules containing enzymes in granulocytes or with lysosomes in macrophages. This fusion is followed by a prominent burst of oxidation, activation of phagocytic enzymes, and killing of the bacteria. Although some bacteria are killed slowly within phagocytes (16), the entire process of recognition, ingestion, and killing of bacteria is very rapid (15 to 20 min) for many bacteria (17). Consequently, *in vitro* OPA for pneumococci requires relatively short incubation periods.

### Opsonophagocytosis Is the Primary Defense Mechanism against Pneumococci

Many observations suggest that *in vivo* defense against pneumococcal infections depends on opsonophagocytosis involving phagocytes, early complement components, and antibodies. The need for neutrophils in defense against pneumococci has been clearly shown for neutropenic patients receiving chemotherapy (25) and animals made neutropenic (9, 80). Also, the need for antibodies to pneumococcal capsular polysaccharide (PS) is shown by patients with Wiskott-Aldrich syndrome or Bruton's agammaglobulinemia. These patients are unable to make antibodies to pneumococcal capsular PS and are thus susceptible to pneumococcal infections but become resistant to pneumococcal infections after gamma globulin treatments. In addition, the need for C3 and CR3 in defense against pneumococcal infections has been clearly established (48, 52, 76; chapter 7). Studies of C1q deficiencies showed that the classical pathway is the main complement activation pathway required for the host protection, and the lectin pathway may influence C3 deposition only slightly in some cases (20). Complement-mediated bacteriolysis is probably

not important in pneumococcal infections since deficiencies in the lytic cascade involving components C5 through C9 are associated with meningococcal but not pneumococcal infections.

The *in vivo* importance of opsonophagocytosis is further supported by the fact that several pneumococcal virulence molecules enhance opsonization avoidance. Pathogenic pneumococci have carbohydrate capsules which do not fix complement and thus act as a shield that covers the inner structures that attract host opsonins. The thickness of the capsule can be associated with resistance to opsonization (27). Many pathogenic pneumococci also express molecules like PspA and PspC (CbpA) that are associated with the deactivation of complement (20, 51, 52; chapter 7). Considered together, all of these observations indicate that the antibody's ability to enhance opsonophagocytosis should be a good measure of pneumococcal vaccine-induced immunity.

### PS VACCINATION ELICITS OPSONOPHAGOCYTIC ANTIBODIES

Given that opsonophagocytosis is essential in host defense against pneumococci, pneumococcal vaccines are designed to induce opsonic antibodies. Since the carbohydrate capsule of pneumococci is well known to elicit opsonic antibody, inducing antibodies against that capsule is the basis for all pneumococcal vaccines clinically used to date. We describe below two different types of clinically available vaccines and the various laboratory measures that were used to estimate their protective efficacy.

#### Currently Available Pneumococcal PS Vaccines Elicit Anti-Capsular PS Antibodies in Adults and Older Children

Capsular PS, if sufficient in size, can stimulate adult B cells largely independent of T-cell help and can induce adults to produce anti-capsular PS antibodies (chapter 6). The anti-capsular antibodies are mostly of the IgG2 subclass, although IgG1, IgM, and IgA antibodies are regularly produced in small amounts (35, 43, 72). The early PS vaccine contained 14 serotypes, but the current PS vaccine contains 23 different capsular PSs (53). Although the PS vaccine can elicit opsonic antibodies and is effective among adults, its efficacy is low among older adults (63), and it is not immunogenic in young children (less than 2 years old), a major target population for pneumococcal vaccines.

#### Currently Available PCVs Elicit Anti-Capsular Antibodies in Infants and Young Children

To produce a pneumococcal vaccine that is effective among young children, pneumococcal PSs were conjugated to protein carrier molecules to form a PS-protein conjugate vaccine. This pneumococcal conjugate vaccine (PCV) becomes T cell dependent, induces B-cell memory, and can elicit anti-PS antibodies in young children. PCVs elicit more IgG1 antibodies than IgG2 antibodies in young children (35, 72). IgG1 antibodies bind Fc receptors better and fix complement better than IgG2 antibodies. PCV7-CRM elicits opsonic antibodies and has been found to be highly effective. The prevalence of invasive pneumococcal infections (IPDs) has decreased dramatically since the conjugate vaccine was introduced to clinical use in 2000 (74). Studies have found that pneumococcal antibody levels greater than 0.18 to 0.35 µg/ml are associated with protection in young children (24, 75). Antibody levels measured by enzyme-linked immunosorbent assay (ELISA) are therefore widely used as a measure of vaccine-induced protection in young children.

PCVs also induce immune memory, which may permit the host to achieve protective levels of antibodies in response to pathogen exposure. Some researchers have therefore suggested that the level of immune memory induced with conjugate vaccines should be used as a measure of vaccine-induced protection. However, protection against meningococci and haemophili appears to correlate better with antibody levels at the time of exposure than with immune memory (26). Consequently, immune memory may not be sufficient as the measure of PCV-induced protection.

#### Concentrations of Anti-Capsular Antibodies Estimated with ELISA Can Be Inadequate in Predicting Protection

Although ELISA is adequate as a surrogate measure of immune protection for young children, ELISA alone appears to be inadequate in many situations for several reasons (32). The first reason is the nonspecificity of the ELISA that was used before 2000. Before the introduction of the third-generation ELISA (73), the old pneumococcal antibody ELISA detected antibodies cross-reacting with contaminants in the capsular PSs that were used for antibody measurements (10, 11, 73, 79). Since cross-reactive antibodies are more abundant in adult sera than in child sera, ELISA is less useful for studying vaccines in adults than in children.

Second, even with the improved ELISA, antibodies are sometimes detected even though no functional antibody activity is detected by either animal protection

studies or other functional antibody assays. Examples of this phenomenon have been reported to occur in splenectomy patients (64), patients with human immunodeficiency virus (HIV) infection (13), bone marrow transplant patients (44), and the elderly (57). The elderly may produce pneumococcal antibodies with sufficient avidity to bind capsular PS adsorbed on ELISA plates but with insufficient avidity to induce opsonophagocytosis. Furthermore, different conjugate vaccines may elicit antibodies with different opsonic potencies (78).

Third, capsular PS epitopes become altered when the PS is immobilized on the ELISA plate. Consequently, ELISA may quantify the antibodies binding to the altered epitope. For instance, a monoclonal antibody was found that opsonized pneumococci in a serotype-specific manner and bound free capsular PS in solution but did not bind the PS immobilized on ELISA plates (66). Such an epitope modification may occur when PS is chemically conjugated to the small plastic particles used for the flow cytometric bead array method (Luminex assay) (37, 45). The Luminex assay is functionally equivalent to multiplex ELISA and has much more analytical throughput than the conventional ELISA method. This potential epitope modification should be carefully investigated before replacing the standard pneumococcal antibody ELISA method with the flow-cytometric bead array method.

## LABORATORY ESTIMATES OF OPSONOPHAGOCYTOSIS

OPA is the most desirable surrogate assay for measuring pneumococcal vaccine-induced immunity. However, unlike the OPA useful in basic science research, an OPA useful in vaccine development must be robust, efficient, and standardized enough to be capable of producing precise results for multiple serotypes for many samples with only small amounts of sera. For the last 10 years, investigations of OPA methods have been under way to develop an OPA with just those capabilities. As a result of those efforts, OPA methods are rapidly evolving. Because this is such a dynamic field, we have used a historical approach to describe the various OPA methods.

### Opsonophagocytic Killing Process Can Be Replicated In Vitro

Many investigators have replicated opsonophagocytosis in vitro to study factors important in the biological process. The classical in vitro system is to expose target bacteria to antibodies, complement, and phagocytes and then to enumerate the number of surviving bacteria.

However, this classical approach is very tedious to perform, primarily due to the counting of colonies. Consequently, many researchers have developed various alternative OPA methods requiring no colony counting, including a radiolabeled-bacterium uptake assay (41, 69), a fluorescent-bacterium uptake assay (23, 39, 69), chemiluminescence (5), and an oxidative-burst generation assay (62). While these assays have been used with various degrees of success, they all indicated that opsonophagocytosis can be replicated in vitro.

### A Killing-type OPA Was Developed as a Reference Assay

Recognizing the special needs for OPA, investigators at the Centers for Disease Control and Prevention (CDC) in collaboration with colleagues at Emory University and the University of Rochester described a single-serotype OPA developed specifically in 1997 to evaluate pneumococcal vaccines (56). The CDC OPA employed the classical killing assay but incorporated several important innovations: the assay used a cell line (HL-60) as phagocytes, frozen aliquots of the target pneumococci, and microcolonies of pneumococci as assay readouts. The counting of microcolonies was critical in handling a large number of samples because it dramatically reduced the biological wastes by allowing one to place reaction mixtures from many reaction wells in a single petri dish. The use of a cell line permitted the standardization of phagocytes. These investigators found that, for their OPA method, human complement can be replaced with baby rabbit complement, a commercially available material. In addition, the CDC made their target bacteria widely available and published a detailed protocol enabling others to duplicate their OPA procedure. Since no other pneumococcal antibody OPA has been so extensively evaluated and standardized, this OPA method was widely adopted by various investigators for clinical studies and has served as the reference method until now (55).

In addition, the CDC OPA served as the basis for the assays developed by two pneumococcal vaccine manufacturers: Wyeth and GlaxoSmithKline (GSK) (18, 21). While these two widely used OPAs are both single-serotype killing assays using HL-60 cells and rabbit complement, they have several differences from the CDC OPA. For instance, the assay was modified at Wyeth to add complement early in the opsonization reaction when the reaction volume is only half of its final level. This modification results in the transient exposure of target bacteria to a twofold-higher complement concentration. In the GSK assay, opsonization and phagocytosis phases were merged. Also, both assays use differ-

ent target bacteria than the CDC OPA. The significance of these differences has not yet been determined.

While the development of the single-serotype killing OPA was an important milestone, the assay had several limitations. First, many investigators in Europe had difficulty using HL-60 cells as phagocytes. Further investigation later showed that HL-60 cell lines provided by European cell banks are not useful as phagocytes, whereas HL-60 cells from the United States are (15). This example further illustrates the need to standardize the assay. Second, this OPA did not employ a standard serum to normalize the results. Consequently, when the CDC investigators led the first attempt to standardize OPA among five different laboratories, the interlaboratory variation in OPA results was still significant: 75% of the titers were within one dilution of the median titer, and 88% of the titers were within two dilutions of the median titer (54). Lastly, the most serious limitation of the method was that microcolonies were visualized under the dissection microscope and colony counting was too tedious to be used routinely.

Several groups of investigators overcame the difficulties of colony counting in different ways. One approach was to use chromogenic (33) or fluorogenic (2) dyes, which produce optical signals proportional to the number of bacteria in the reaction wells. While this approach worked, it was cumbersome, and direct counting of bacterial colonies was preferable. Ultimately, several groups independently found ways to count microcolonies rapidly. Two groups succeeded in adapting counters developed for spot ELISA to count OPA microcolonies (19, 34). Another group found that a tetrazolium dye, triphenyl tetrazolium chloride (TTC), added to agar plates can stain pneumococcal colonies red (28, 58) and make the microcolonies discernible to conventional bacterial colony counters (28). In fact, the TTC-stained microcolonies are so distinct that digital camera images of the TTC-stained microcolonies can be emailed for colony counting (49). This approach should make automated colony counting widely available. In summary, by 2005, colony counting had become as fast and easy as reading the optical densities of ELISA plates.

### Development of a Flow Cytometer-Based Uptake OPA

Because colony counting was so difficult, several groups adapted flow cytometers to determine the number of fluorescent bacteria that had been ingested by phagocytes (23, 39). This uptake assay obviated the need to count colonies and could be adapted to handle a large number of samples. Also, the uptake assay could use either killed bacteria or antigen-coated latex particles

and could analyze serum samples containing antibiotics (38). Studies found that the uptake assay results correlated with those of the classical killing assay, although the uptake assays were not as sensitive as the killing assay (69). Both the killing and uptake assays measure the process of opsonophagocytosis; however, the uptake assays use nonviable targets, and therefore, they do not measure the proportion of killed bacteria. Furthermore, counting ingested bacteria with a flow cytometer is significantly slower than automated microcolony counting.

### Development of Multiplex OPA Methodologies

Typically, PCVs contain capsular PSs from 7 to 13 different serotypes, and conjugate vaccine evaluations require performing OPAs on a large number of serotypes for each serum sample. There is a need for methods that can perform many assays with a small volume of serum since one cannot obtain much serum from young children. To meet this need, two different multiplex OPAs have been developed.

One approach is to measure phagocytosis using a multiplex flow cytometry assay. In this uptake assay, different bacteria are tagged with different fluorochromes and a mixture of the different bacteria is allowed to be ingested by phagocytes in the presence of antibody and complement. The number of each type of target bacteria in the phagocytes can then be determined by multicolor flow cytometry (38). In some cases, fluorescent target bacteria are replaced with fluorescent latex particles coated with capsular PS (38). One multiplex assay using four different types of fluorescent latex particles has been developed for seven serotypes (4, 6B, 9V, 14, 18C, 19F, and 23F). The results correlated with those obtained with the reference killing assay, with  $r$  values ranging between 0.68 and 0.92 (38). Compared with automated colony counting, this assay is relatively slow at enumerating ingested target particles (7, 38). Also, one must assume that PS-coated latex particles faithfully mimic the phagocytosis and PS epitope expression of natural bacteria. However, this assay has the advantages of not requiring bacterial culture and of being able to be performed with samples containing antibiotics.

Another approach is based on the killing-type OPA using a mixture of antibiotic-resistant pneumococci as target bacteria (3, 7, 28, 40). For this assay, different target bacteria are prepared to be resistant to different antibiotics. A mixture of target bacteria is then mixed with phagocytes, antibodies, and complement. The number of surviving bacteria of each serotype is determined by plating the reaction mixture onto agar plates

containing antibiotics for the selection of the organisms that are resistant. This multiplex OPA method is very similar to the classical killing assay, its biological relevance is easy to accept, and it requires no special equipment. Consequently, many laboratories can readily adopt the assay method. Indeed, this approach is robust enough that one can perform assays with multiple serotypes, from two (40) to seven (3). Also, this approach can be applied to an OPA using a dye whose fluorescence is proportional to the number of bacteria (2). The use of the dye can eliminate the need to count bacterial colonies. The multiplex killing method was favored in the 2005 OPA meeting in Atlanta (55).

Recently, a well-characterized four-serotype multiplex, killing-type OPA was described for use with 13 serotypes (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F) (7). The assay procedure is very similar to the CDC OPA (56), but the assay conditions were further optimized and the assay performance was validated. Consequently, the multiplex assay results are highly correlated with the single-serotype OPA results ( $r > 0.97$ ) and are relatively precise. The four-serotype multiplex OPA reduces the required amount of serum significantly enough to be useful for studying infants. Also, the detailed assay protocol and critical reagents are readily available online. This four-serotype multiplex OPA may be useful as a reference assay in the future.

## EXPERIENCE WITH OPA IN PNEUMOCOCCAL VACCINE TRIALS

Although OPA results are the best biomarker of protective immunity, OPA has not been relevant to vaccine studies because it was technically difficult to perform. With the methodological improvements described above, OPA has now become a practical tool for assessing vaccine efficacy. Consequently, it is important to review all the data available to date to see if OPA provides theoretical as well as real advantages in vaccine evaluations. Clinical studies have measured opsonophagocytic antibody activity in young children and adults. Because of the past methodological difficulties, these studies tended to be small in size. They are nevertheless informative and are thus described below.

### Pneumococcal Conjugate Vaccine Trials among Infants with OPA Estimates

Table 1 summarizes the results of recent studies that used both OPA and ELISA to investigate the efficacy of pneumococcal vaccines in healthy young children. The studies employed both killing- and uptake-type OPAs, but they used rabbit complement, HL-60 cells as phagocytes, and bacteria as phagocytic targets. These studies

examined a relatively small number of samples (<100 per group), except for a GSK study, which was described in a recent review (55). The GSK study examined more than 1,000 sera and clearly indicated the feasibility of performing OPA on a large scale.

The total OPA titer tends to increase with repeated immunizations with a PCV, but the functional capacity of induced antibodies does not always increase. Antibody functional capacity is expressed here as a ratio of the ELISA result to the OPA titer, and low numbers indicate superior antibody function. However, the functional capacity has sometimes been expressed as a ratio of the OPA titer to ELISA result. While both expressions are correct, the two expressions can be confusing, and readers should be careful in reading reports on antibody functionality. Table 1 also shows that the correlations between ELISA and OPA results are not ideal but are high enough to make ELISA useful as a surrogate assay for studying pneumococcal vaccines in normal infants.

Nevertheless, several examples described below support the contention that OPA is an important addition to ELISA in assessing vaccine efficacy. One example is with antibodies to the 19F serotype. The functionality of anti-19F antibodies, which is indicated by bold numbers in Table 1, is at least 10-fold less than the median antibody functionality in most cases (Table 1). The only exceptions are the results of studies with the seven-valent PCV conjugated to an outer membrane protein complex (PCV7-OMPC). This observation was made with both killing and uptake types of OPA methods. Since the efficacy of PCV7-CRM is the lowest for 19F among the seven serotypes, OPA results appear to be more predictive of efficacy than ELISA results for the 19F serotype.

Another example is shown with HIV-infected children. PCVs are less effective in HIV-infected children than in healthy children (30). Yet PCVs elicit equivalent levels of pneumococcal antibodies in healthy and HIV-infected children as determined by ELISA (29, 36). When OPA titers were determined, HIV-infected children had lower OPA titers than non-HIV-infected children in response to PCV immunization (36).

In addition to providing more accurate results for HIV-infected children, OPA may also be useful in predicting cross protection induced with PCVs. When sera from young children immunized with the five-valent PCV (PCV5-CRM) were studied with ELISA and OPA, it was found that levels of antibody to the 19A serotype were relatively high but that OPA titers for 19A were very low (78). Clinical experience with PCV7-CRM, which is very similar to the PCV5-CRM, showed that PCV7-CRM is not effective against 19A (42). These

**Table 1** Pneumococcal conjugate vaccine trials with OPA estimates conducted in infant populations

Vaccine and vaccination times <sup>a</sup>	Group size <sup>b</sup>	Population location	OPA titers (range of GMTs)	Functional capacity (range in ng/ml) <sup>c</sup>	OPA type (no. of serotypes studied)	Correlation to IgG ELISA result (range of <i>r</i> values)	Reference
PCV11-D-T (6, 10, 14)	47	Philippines	12.8–277	16–119	Killing (5)	0.53–0.74	50
PCV11-D-T (6, 10, 14, 36)	45	Philippines	125–3,051	4–54	Killing (5)	0.73–0.79	50
PCV11-D-T (8, 16, 24)	28	Finland and Israel	34–837	2–27	Killing (5)	0.51–0.65	77
PCV11-D-T (8, 16, 24, 144)	28	Finland and Israel	148–1,730	2–42	Killing (5)	0.51–0.78	77
PCV9-CRM (6, 10, 14)	56	South Africa	Not given	2–76	Uptake (3)	0.41–0.56	36
PCV7-CRM (8, 12, 16)	140	Germany	Not given	2–216	Killing (7)	Not given	18
PCV7-CRM (8, 16, 24)	55	Finland	26–143	10–97	Killing (3)	0.7–0.84	12
PCV7-CRM (8, 16, 24, 144)	55	Finland	78–606	13–59	Killing (3)	0.63–0.89	12
PCV7-OMPC (8, 16, 24)	55	Finland	<8–51	9–92	Killing (3)	0.60–0.71	12
PCV7-OMPC (8, 16, 24, 144)	55	Finland	17–91	13–96	Killing (3)	0.56–0.90	12
PCV11-PD (12, 16, 20)	106	Czech Republic and Slovakia	42–2,619	Not given <sup>d</sup>	Killing (11)	Not given	61
PCV11-PD (12, 16, 20, 144)	148	Czech Republic and Slovakia	108–4,640	Not given <sup>d</sup>	Killing (11)	Not given	61

<sup>a</sup>Numbers in parentheses indicate vaccination times in weeks. Serum samples were obtained about 4 weeks after the last vaccination. PCV11-D-T, 11-valent PCV conjugated to diphtheria and tetanus toxoids; PCV11-PD, 11-valent PCV conjugated to protein D.

<sup>b</sup>The number of persons in the largest group is shown here. Some groups in the study had slightly fewer members.

<sup>c</sup>Functional capacity indicates the ELISA-determined concentration divided by the opsonization titer, except for the German study with PCV7-CRM, for which it means the ELISA-determined concentration giving an opsonization titer of 8. Functional capacity for serotype 19F is indicated in bold.

<sup>d</sup>Serotypes with lower OPA titers had negative or low vaccine efficacy (i.e., serotypes 3, 18C, and 19F).

examples show that OPA can be more informative than ELISA in certain specific situations.

### Pneumococcal Vaccine Trials for Older Children or Adults with OPA Estimates

The 23-valent pneumococcal PS vaccine (PPSV23) is immunogenic among adults and children older than 2 years of age, but it has low efficacy among immunocompromised and very old adults. Consequently, PCVs, which are designed primarily for the pediatric population, are being investigated for older adults as well as adults or children with risk factors such as HIV infection (13, 67) and a history of bone marrow transplant (44) and splenectomy (64). To assess how OPA can be useful in these evaluations, we have summarized several immunogenicity trials with older children and adults employing OPA and presented the summary in Table 2. The OPAs employed by the studies listed in Table 2 used rabbit complement, HL-60 cells as phagocytes, and bacteria as phagocytic targets, except for the OPA in the study by Tarragó et al. (67), which used human granulocytes and human complement.

Anttila et al. reported significant levels of correlation (*r*, 0.7 for pre- and 0.9 for postvaccination samples) for

sera from healthy adults (*n* = 42) immunized with any of four different vaccines, similar to those found for older infants (1). But most studies listed in Table 2 show less correlation between ELISA and OPA results for adults than the studies listed in Table 1 show for young children. In some adult populations (e.g., unimmunized persons, HIV-positive persons, and older adults), the correlation is extremely low (Table 2). This finding suggests that ELISA and OPA measurements should be considered as independent analytical measures for adult populations. This independence may reflect the poor specificity of pneumococcal antibody ELISA for adults. Most unimmunized adults have antibody levels higher than 0.3 µg/ml (even after absorption with 22F PS), and yet they are in groups at high risk for IPD (13, 57, 71). Thus, currently available information on minimum levels of antibody for protection would not be as useful in vaccine studies of adults as it was in studies of young children.

Limited information does support the contention that OPA results may be more reflective of immune protection than ELISA results. In elderly adults (>65 years old), PPSV23 is not as effective as it is in young adults (63). Yet, ELISA-based studies showed that elderly adults can produce as much antibody as young adults

**Table 2** Pneumococcal vaccine studies with OPA estimates conducted with older children and adults

Group	Vaccine(s) and vaccination times <sup>a</sup>	Group size	Target population	OPA titers (range of GMTs)	OPA type (no. of serotypes studied)	Correlation to ELISA IgG results (range of <i>r</i> values)	Reference(s)
1	PPSV23	46	Elderly adults	22–76.6	Killing (5)	0.3–0.62	57
2	No vaccine	46	Elderly adults	5.2–9.3		Not given	
3	PPSV23	12	Young adults	71.5–352.1		0.61–0.86	
4	No vaccine	12	Young adults	7.5–25.3		Not given	
5	PCV7-CRM, PCV7-CRM (0, 8)	15	HIV <sup>+</sup> adults	38.5–169	Killing (5)	0.25–0.74	13, 14
6	PCV7-CRM, PPSV23 (0, 8)	18		18.8–56.6		0.25–0.74	
7	PPSV23	16		19–83		0.25–0.74	
8	No vaccine	18		11.8–48.9		Not given	
9	No vaccine	56	HIV <sup>+</sup> children	3.5–31	Uptake (3)	0.22–0.54	67
10	PCV7-CRM	56		13.5–161.5		0.07–0.67	
11	PCV7-CRM, PCV7-CRM (0, 8)	56		9.4–67.6		0.41–0.75	
12	PCV7-CRM, PCV7-CRM, PPSV23 (0, 8, 16)	11	Children with sickle-cell disease	290–1,810	Uptake (7)	0.41–0.70	71
13	PPSV23	12		6–215			
14	PCV7-CRM	30	Renal transplant recipients	64–238.9	Uptake (7)	0.18–0.6	31
15	PPSV23	30		28.4–203.3			
16	No vaccine	60		12.4–111.4			
17	PCV7-CRM at 0.2-fold dose <sup>b</sup>	44	Elderly adults ages 70 to 79 with prior PPSV23 immunization	69–733	Killing (7)	Not given	22
18	PCV7-CRM at 1-fold dose <sup>b</sup>	44		228–4,572			
19	PCV7-CRM at 2-fold dose <sup>b</sup>	44		402–3,947			
20	PCV7-CRM at 4-fold dose <sup>b</sup>	44		344–4,699			
21	PPSV23	44		59–1,049			
22	No vaccine	205		17–249			

<sup>a</sup>Numbers in parentheses indicate vaccination times in weeks. Serum samples were obtained about 4 weeks after the last vaccination in most cases except for groups 10 and 11. Group 10 was bled 8 weeks later, and group 11 was bled 20 weeks later.

<sup>b</sup>In this study, variable doses were given, and the doses are shown relative to the regular pediatric dose of 0.5 ml.

(57). However, elderly adults produce antibodies that are less opsonic than those produced by young adults (57). Also, a recent study found that PCV7-CRM elicits antibodies with superior functional capacity compared

to those elicited by PPSV23 in elderly adults (22). Similarly, OPA (but not ELISA) showed that HIV-infected adults produce less-opsonic antibodies than healthy adults (13). Opsonization depends on antibody avidity

as well as on antibody concentration (57, 66, 68). These discrepancies between ELISA and OPA results may be due to the low avidity of the antibodies made by elderly adults (57). These findings support the usefulness of OPA in assessing PCV efficacy among adults.

While an opsonization titer of 8 may be sufficient to protect young children against IPD, many unimmunized adults have titers that range from <8 to >100, suggesting that in adults the minimum OPA titers associated with protection are higher than 8 and may also be serotype dependent (Table 2). A titer of 64 has been suggested in the past to be the protective titer for adults vaccinated with a pneumococcal PS vaccine alone (57). However, establishing the protective level will require additional investigations using standardized OPA and involving many individuals and many different pneumococcal serotypes. One should therefore consider that the protective opsonization titer for adults has not yet been defined and would be different than that for young children.

### Lessons Learned from Using OPA in Vaccine Trials

Experience in the last decade has shown that even the labor-intensive CDC OPA can be used in large-scale trials. Perhaps the most important finding from that experience is that OPA results correlate with clinical outcomes better than ELISA results do. This finding clearly indicates the need to use OPA more in the future than it has been used previously, especially in evaluating vaccines for adults. Thus, we now need an efficient OPA method that has high-throughput capability and requires less serum than the current methods.

In the absence of follow-up efforts to maintain the CDC assay as the standard assay, various laboratories have modified it. Consequently, it is difficult to compare OPA results from different clinical studies. Therefore, another critical and urgent issue is the need for a standardized OPA that is based on a large number of participating laboratories and on shared reference materials, standard serum, and a sustained follow-up standardization effort.

### FUTURE DIRECTIONS OF PNEUMOCOCCAL OPA

In view of increased needs for OPAs and improved OPA methodology, it is now important to standardize OPA by incorporating the recent improvements. One needs to define the protective levels by using the new assay. Also, this standardization should open new applications for

OPA in the evaluation of other pneumococcal vaccines. These future activities for OPA are discussed below.

### Standardization of OPA and Its Requirements

To standardize OPA, one needs a reference OPA that is based on an indisputable analytical principle and that uses universally available assay components. The reference OPA should also be well characterized as a result of the extensive evaluation of assay parameters (e.g., the limit of detection, limit of quantitation, precision, linearity, specificity, and accuracy). This characterization would make the OPA robust, help in replicating the assay in distant locations, and make it become compliant with good laboratory practice (GLP) requirements. GLP compliance is essential for vaccine manufacturers. (The GLP requirements are described on the websites listed in reference 55.) Furthermore, the reference OPA should be technologically current. For instance, the reference assay should use automated colony counting, which has replaced the most cumbersome step in the classical OPA. Also, the reference assay should adopt a multiplex format since new pneumococcal vaccines contain many serotypes and testing for this large number of serotypes in young children must be done with small amounts of serum.

OPA uses complex biological reagents that cannot be completely controlled. Thus, even with a well-characterized reference assay and carefully selected reagents, the assay results would vary significantly in different locations and over time. Therefore, a reference serum with assigned OPA values should be analyzed in each run of the OPA, and the results of test samples should be standardized to the results for the reference serum. The variation in assay results should then be reduced. Such a reference serum is currently being prepared under the leadership of the U.S. Food and Drug Administration.

Opsonic capacities have been commonly expressed as titers. Titers are traditionally determined by diluting serum samples in two-fold serial dilutions and identifying the dilution at which a specific qualitative phenomenon (e.g., agglutination or hemolysis) is observed. Opsonization titers were generally determined as the serum dilution that results in the killing of 50% or more of the target bacteria (56) or in the uptake of 50% or more of the fluorescent targets, although 90% killing was used in some cases (3). By their definition, titers have a systematic bias for results lower than the true values and produce discontinuous results, which are less amenable to statistical analysis.

As the enumeration of colonies has become easier, the exact dilution at which 50% of the bacteria are

killed can be estimated using various interpolation methods. The 50% cutoff is preferred as the end point since the point is more robustly estimated than a cutoff that is near the extremes of the opsonophagocytic curve (55). In addition to avoiding systematic bias, the interpolation also produces results with continuous values, which permit efficient statistical analysis. To distinguish classical discontinuous opsonization titers from the continuous opsonization capacity estimated by interpolation, it has been proposed to adopt a new term (55).

Experience with ELISA showed that standardizing the interpolation method is as important as standardizing assay methods or reagents (46, 47). Thus, the method for estimating the exact dilution at which the 50% point is attained needs additional investigation. Although simple point-to-point interpolation has been used successfully, the four-parameter log-logistic method uses the entire dose-response curve and is emerging as a popular method for data interpolation (18). Also, this calculation method can be used to normalize the results for test samples to those for the reference serum used in each assay run. Other investigators have also used a five-parameter curve fitting method (38). In addition, some calculation programs have additional algorithms to handle atypical titration curves (e.g., curves with a prozone-like phenomenon).

The goal of OPA standardization is not to allow only one type of OPA method but to have OPA results that are comparable. Consequently, one should expect that other assays will coexist because the other assays are either more efficient than the reference assay or have been clinically validated in the past. However, these operational OPAs should be bridged to the reference assay so that the results of different assays can be better compared. A similar functional-equivalence approach was used for the standardization of the ELISA methodology (46, 73). Consequently, OPA standardization would require a sustained and active effort to maintain that standardization.

### Determination of Minimal Level of Protection for Various Populations and Diseases

An opsonophagocytic titer of 8 was initially associated with ELISA concentrations of  $\geq 0.2 \mu\text{g/ml}$  (24). A similar correlation was found in the recent report by Henckaerts et al. (18). However, the minimum levels of antibody required for 50% killing vary widely by serotype (Table 1) (32). Also, the minimum levels of protection may be different for diseases other than IPD, for elderly adults, and for other populations at risk. In addition, OPA has not been standardized yet. Thus, additional studies are needed to better establish the minimal levels of protection in other at-risk populations.

### Use of OPA for Pneumococcal Vaccines Based on Other Antigens

As conjugate vaccines containing a large number of serotypes are difficult and expensive to manufacture, there has been a concerted effort to develop pneumococcal vaccines based on noncapsular PS. Candidate molecules for the noncapsular vaccines include many pneumococcal proteins, such as PspA, pneumolysin, and PsaA, among others. If any of these molecules become a successful pneumococcal vaccine, it would allow us to have a pneumococcal vaccine that is both easy to manufacture and can provide broad protection coverage independent of capsule serotypes.

At present, protein candidates are being investigated for their capacity to elicit antibodies that can opsonize pneumococci. Also, one should consider that the relevant phagocytes for the protein vaccines may be macrophages rather than granulocytes. Nevertheless, opsonophagocytosis plays a central role in protecting the host against pneumococcal infection. In fact, some of the pneumococcal proteins are known to interfere with the deposition of complement, which is critical for opsonization. Thus, OPA should be important and should be useful in assessing the new protein-based vaccines.

### Application of OPA to the Evaluation of Vaccines for Other Pathogens

Opsonization is central for protection by antibodies to gram-positive bacteria such as *Streptococcus agalactiae* and *Streptococcus pyogenes*. Opsonization may be also relevant in protection against diseases caused by gram-negative bacteria such as meningococci (4). However, since OPA has been technically difficult to perform, it has not played an important role in evaluating vaccines against these pathogens. Technical improvements that are critical to improving pneumococcal antibody OPA can readily be applied to measuring the opsonic capacity of antibodies to other pathogens. Furthermore, technical improvements of OPA can be applied to improve serum bactericidal assays, another type of functional assay of antibodies widely used to evaluate antibodies to gram-negative bacteria such as haemophili and meningococci. Therefore, the functional capacity of antibodies would be measured more widely in the future. The widespread use of OPA may result in a paradigm shift in vaccine evaluations—from measuring antibody concentrations to measuring antibody functions.

### CONCLUSION

Opsonophagocytosis is the primary mechanism of protection against pneumococcal infections and is recognized as the best correlate in evaluating the protection

provided by pneumococcal vaccines. Because of technical improvements over the last decade, in vitro opsonophagocytic activity can be determined for many samples and under standardized conditions. OPA can be used in large immunogenicity trials involving diverse populations (55). OPA will likely also become the basis for evaluating the efficacy of protein vaccines. Standardizing OPA would allow for a wider use of this methodology and for an improved correlation between tests for protective immunity and clinical outcomes and could lead to a better definition of protective titers in various populations and for various diseases.

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Helena Käyhty  
Stephen Lockhart  
Lode Schuerman

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# Immunogenicity and Reactogenicity of Pneumococcal Conjugate Vaccines in Infants and Children

Several investigational pneumococcal conjugate vaccines (PCVs) have been evaluated in phase II immunogenicity and reactogenicity studies with infants. The investigational PCVs studied during the last 15 years have differed in the dose of polysaccharides (PSs), number of serotypes and carrier proteins, and the formulation (with or without an adjuvant) and have been studied with various other childhood vaccines and administered according to various schedules (1, 3, 4, 6, 12, 13, 15, 22–26, 32, 34, 41, 42, 45–47, 52, 55, 58–60, 62, 67, 71, 72, 74–78, 82, 84–86, 88, 95–97). The immunogenicities of the PCVs have been determined both in phase II studies and in phase III studies in parallel with efficacy trials (9, 27, 28, 30, 36, 43, 50, 51, 61, 63, 68, 80, 82, 83, 87, 91).

In contrast to pneumococcal PS vaccines (PPSVs), PCVs are immunogenic in young infants and children. The immunological basis for the enhanced immunogenicity is described in detail in chapter 6. Antibodies to capsular PS are believed to be the main mediators of protection after vaccination with pneumococcal vaccines, and accordingly, the determination of the concen-

trations of serotype-specific antibodies has been used for assessing the immunogenicities of PCVs. Early immunogenicity studies used a radioimmunoassay, but more recently, different modifications of a standardized enzyme-linked immunosorbent assay (ELISA) have been used (chapter 14). To increase the specificity of the assay, the present ELISA protocol includes adsorption with both pneumococcal cell wall PS and serotype 22F PS (19).

Opsonophagocytosis mediated by antibody and complement is the most important host defense mechanism, and accordingly, the demonstration of the killing of pneumococci in an opsonophagocytic assay (OPA) (chapter 15) is considered to be an important secondary end point for evaluating the PCVs (93).

The measurement of affinity and its maturation can provide useful additional information about the immune response. High-avidity antibodies can have greater functional capacity than low-avidity antibodies (7, 90), and the increase in avidity is regarded as a marker of the development of immunological memory (38). The avidity of antibodies can be measured by a modified ELISA

using a chaotropic agent to dissociate low-affinity antibodies (chapter 14).

PCVs prevent mucosal infections (acute otitis media [AOM] and colonization), and thus, some groups have also made efforts to characterize the mucosal immune response after vaccination in the hope of finding serological correlates of mucosal protection. Such studies have addressed immunoglobulin G (IgG) and secretory IgA antibody concentrations in salivary fluid (16, 53–59) or the demonstration of circulating antibody-secreting cells on their way back to the effector sites in the mucosa (53, 54).

## IMMUNOGENICITY OF PCVS IN EFFICACY TRIALS

### IPD

Invasive pneumococcal disease (IPD) has been an end point in four efficacy trials (chapter 21). The first one was conducted in Northern California (the Northern California Kaiser Permanente [NCKP] trial) (8, 9). Subsequent trials were conducted among American Indian, South African, and Gambian infants (20, 44, 64). The vaccine efficacy against IPD caused by the vaccine serotypes varied between 76.8 and 97.4%, being lowest among American Indian, Gambian, and human immunodeficiency virus (HIV)-positive South African infants.

Serotype-specific serum antibody concentrations in the trial populations were tested with a standard ELISA without 22F adsorption. After the third dose of the vaccine, the geometric mean concentrations (GMCs) of antibodies tended to be lowest among the Californian infants (Fig. 1). Serological data for non-HIV-infected and HIV-infected infants have been reported for a small number of South African vaccinees (50). The GMCs in the two groups were not significantly different, but the level of functional activity seemed to be lower among HIV-positive infants. This finding is in concordance with the apparently lower level of vaccine efficacy in HIV-infected children (44, 49). The NCKP trial yielded the highest point estimate of efficacy, while the estimates were lowest in the studies conducted in The Gambia and among American Indians. However, this variation was not reflected in GMCs of antibodies after the third dose (Fig. 1). The booster dose given in the second year of life may have contributed to the high efficacy among the NCKP infants but was not sufficient to provide a similarly high degree of efficacy among the American Indian infants. Thus, the apparent differences in the efficacy (chapter 21) seem to be dependent on the epidemiology and infection pressure. The average esti-

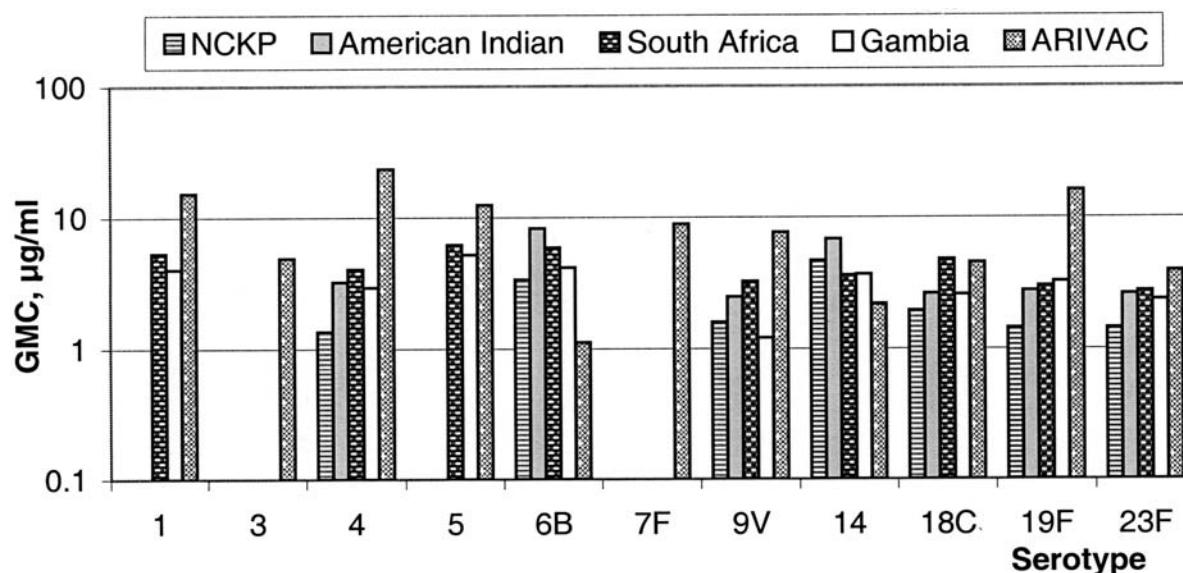
mated antibody concentration thresholds predicting protection (chapter 23) were calculated in the above-listed studies to be 0.2 µg/ml for the NCKP infants, 0.68 µg/ml for the South African infants, and 1.0 µg/ml for the American Indian infants (38, 83). Based on a meta-analysis of the data from these three efficacy trials, WHO experts agreed on the use of the threshold of 0.35 µg/ml in noninferiority evaluations of new PCVs or PCV formulations compared to PCVs for which efficacy against IPD has been demonstrated (83, 93). None of the trials included a sufficient number of IPD cases to provide reliable serotype-specific thresholds.

### Pneumonia

The efficacy of a 7-valent PCV conjugated to cross-reactive material related to diphtheria toxin (PCV7-CRM), a 9-valent PCV conjugated to CRM (PCV9-CRM), or an 11-valent PCV conjugated to diphtheria and tetanus toxoids (PCV11-D-T) against community-acquired pneumonia with radiological confirmation has been evaluated in four trials (chapter 22): those done among infants from Northern California (11), South Africa (44), The Gambia (20), and the Philippines (46a). The immunogenicity profiles are similar (Fig. 1), and the apparent differences in the efficacy (chapter 22) may be more dependent on the infection pressure than the immunogenicities of the PCVs.

### AOM

Three trials (30, 43, 68) have looked at the efficacy of PCVs against culture-confirmed AOM in infants, and a fourth trial estimated the efficacy of PCV7-CRM against recurrent culture-confirmed AOM among older children (92) (chapter 20). Two trials were conducted in parallel among infants in Finland (the Finland otitis media [FinOM] vaccine trials) with PCV7-CRM and a seven-valent PCV conjugated to the meningococcal outer protein complex (PCV7-OMPc) (30, 43). The third infant trial (the pneumococcal otitis efficacy trial [POET]) was conducted in Czech Republic and Slovakia (68) and used a novel, investigational 11-valent PCV in which the *Haemophilus influenzae*-derived protein D (PD) was used as the carrier protein (PCV11-PD). The observed levels of efficacy of these vaccines against pneumococcal AOM caused by vaccine serotypes were very similar (56 to 57.6%). It has to be noted, however, that the case definitions were different in the POET and the FinOM vaccine trials and that, thus, the efficacy data are not directly comparable. Further, the antibody concentrations in the FinOM trials were tested with an ELISA without a 22F PS adsorption step, and those in



**Figure 1** GMCs of antibodies to vaccine serotypes reported in connection with the PCV efficacy trials with invasive pneumococcal disease and/or radiologically confirmed pneumonia as end points (36, 61, 63, 83, 87).

the POET were tested with an ELISA with 22F PS adsorption, which should be kept in mind when comparing the results.

The mean concentrations were somewhat lower than those reported for the IPD efficacy trials (Table 1). Significant serotype-specific protection against serotypes 6B, 14, and 23F could be detected in all three studies (chapter 21). For serotype 19F, the point estimates were lower and significant protection by PCV7-OMPC and PCV11-PD could be demonstrated. For serotype 6B,

PCV7-CRM induced the highest antibody concentrations, but the efficacy was 80 to 90% in all three trials, suggesting that even the lower concentrations evoked by PCV7-OMPC or PCV11-PD were sufficient to provide good protection. Similarly, PCV7-CRM induced the highest response against serotype 23F, while the point estimates of efficacy varied between 52 and 72.3%.

The booster dose given in the second year of life in all three trials evoked a good antibody response showing that the primary series had primed the infants. This

**Table 1** GMCs of antibodies to pneumococcal PSs of vaccine serotypes in samples from infants in efficacy trials with an end point of culture-confirmed AOM caused by the vaccine serotypes

Trial <sup>a</sup> (reference)	Sample set (n) <sup>b</sup>	GMC (µg/ml) of antibody to serotype:										
		1	3	4	5	6B	7F	9V	14	18C	19F	23F
FinOM trial of PCV7-CRM (unpublished data) <sup>d</sup>	Post 3 (376)	NA <sup>c</sup>	NA	2.55	NA	2.68	NA	2.85	7.58	4.05	3.63	2.83
	Post 4 (470)	NA	NA	3.53	NA	10.45	NA	4.2	9.31	5.98	4.82	6.13
FinOM trial of PCV7-OMPC (39)	Post 3 (376)	NA	NA	3.45	NA	0.35	NA	1.79	3.23	1.02	3.19	0.67
	Post 4 (374)	NA	NA	6.50	NA	2.34	NA	4.06	5.96	3.45	8.65	2.41
POET (67)	Post 3 (140–143)	1.58	3.78	2.16	1.92	0.62	2.34	1.60	3.00	1.49	2.60	0.9
	Post 4 (118–143)	2.55	2.83	2.50	3.11	2.17	4.66	3.63	6.48	2.71	4.88	3.55

<sup>a</sup>The FinOM trials used ELISA without 22F adsorption, while the POET study used 22F adsorption.

<sup>b</sup>Post 3, samples taken after the third dose, post 4, samples collected after the fourth dose; n, number of subjects.

<sup>c</sup>NA, not applicable.

<sup>d</sup>The samples corresponding to the results given here differ from those described in published reports (27, 30).

result was clearly demonstrated for PCV11-PD in an immunogenicity study that showed significantly higher mean antibody concentrations after the booster dose at 12 to 15 months of age than after the first dose of PCV11-PD at the same age of 12 to 15 months (55).

The FinOM data showed that there was an inverse association between the antibody concentration and the risk of AOM (39). The mean antibody concentration predicting protection after vaccination varied between serotypes so that a lower concentration of antibodies was needed to protect against serotype 6B AOM than against AOM caused by other studied serotypes. In the POET, the low number of breakthrough cases did not allow similar analysis, but also in that study, the mean concentration of antibody and the opsonophagocytic activity against serotype 19F were lower in post-primary series samples from subjects with breakthrough cases than in samples from those who did not develop AOM caused by serotype 19F (80).

In all three studies, the functional activity of anti-pneumococcal antibodies was also measured (Table 2). Because of the methodological differences, the POET and FinOM data cannot be compared directly. When each serotype is considered separately, the antibody concentration and the functional activity seem to correlate well. However, the OPA results show differences among serotypes (Table 2), and OPA results may be even better associated with protection, at least for PCV7-CRM and PCV11-PD (28, 35, 80), than the antibody concentration. In all three studies, less antibody was needed for killing 50% of serotype 6B strain bacteria than for killing 50% of serotype 19F strain bacteria, which is in concordance with the efficacy of the three PCVs against these serotypes.

## IMMUNOGENICITIES OF DIFFERENT PCVS IN THE SAME POPULATION

Several investigational 4- to 11-valent PCVs have been tested in separate studies in Finland, each including a fairly small number of infants (1, 3, 4, 24, 27, 41, 42, 55, 58, 59, 71). The same schedule of three doses in infancy and a booster dose in the second year of life and the same ELISA methodology without 22F adsorption (42) have been used throughout the studies. Figure 2 shows the GMCs of antibodies against serotypes contained in 7- to 11-valent PCVs after the administration of the third and the fourth (booster) doses to the Finnish infants (24, 27, 55, 58, 71). After the third dose (Fig. 2A), the GMCs were similar for serotypes 4, 9V, and 19F while for the other serotypes there was some variation; PCV7-CRM induced the highest antibody concentrations for most of the serotypes. After the booster dose (Fig. 2B), the trend was similar: results for serotypes 6B, 14, 18C, and 23F showed the greatest variation among the PCVs and among the studies.

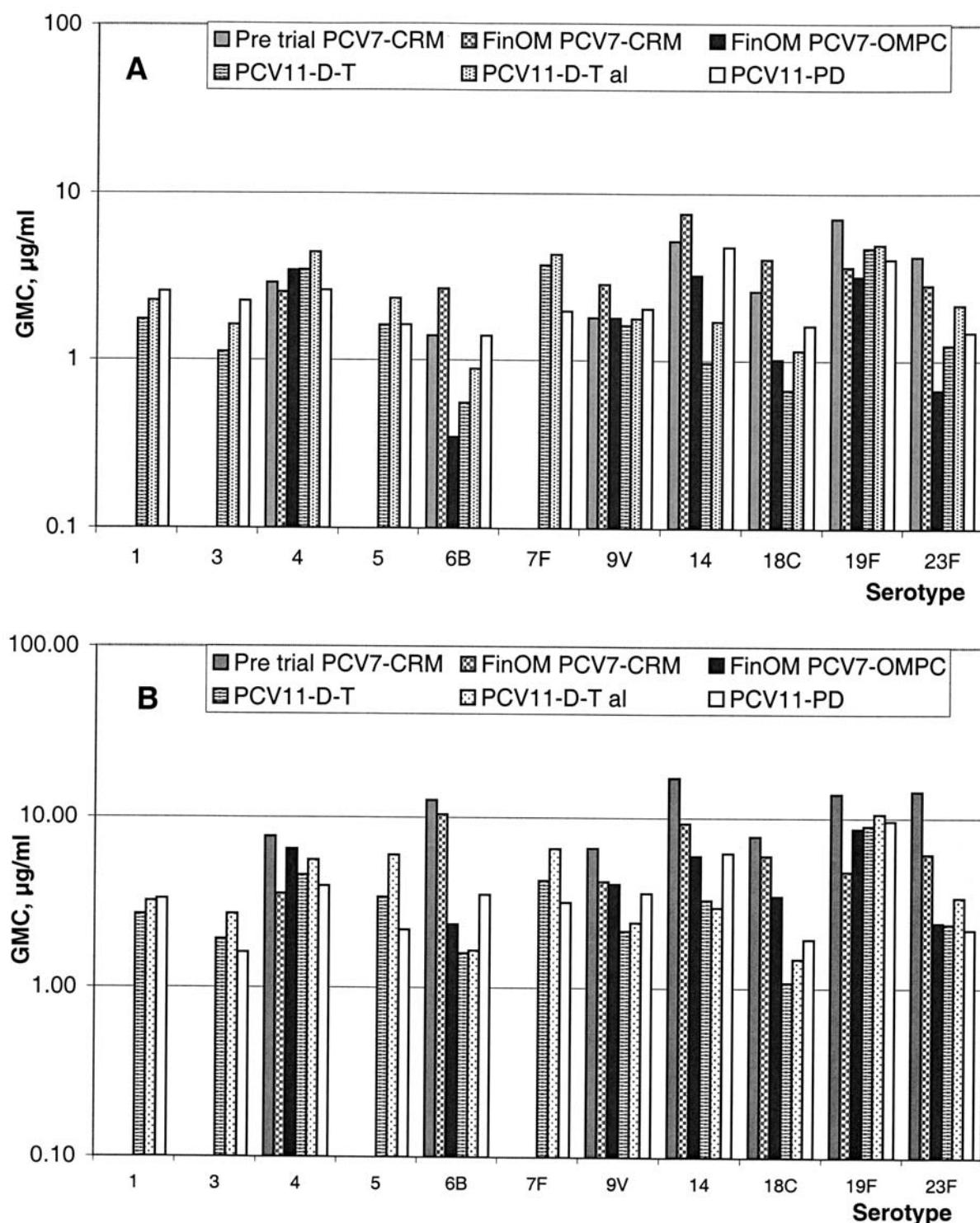
## IMMUNOGENICITIES OF PCVs IN DIFFERENT POPULATIONS

The licensed PCV7-CRM or the similar PCV9-CRM has now been used in several populations. Figure 3 compares antibody concentrations for four vaccine serotypes (6B, 14, 19F, and 23F) as determined in 17 studies among African, American Indian, Californian, German, British, Canadian, Finnish, and Icelandic infants after immunization with three doses of PCV7-CRM or PCV9-CRM in infancy (13, 15, 34, 36, 45, 51, 58, 61, 63, 76–79, 83, 88). When comparing results from different

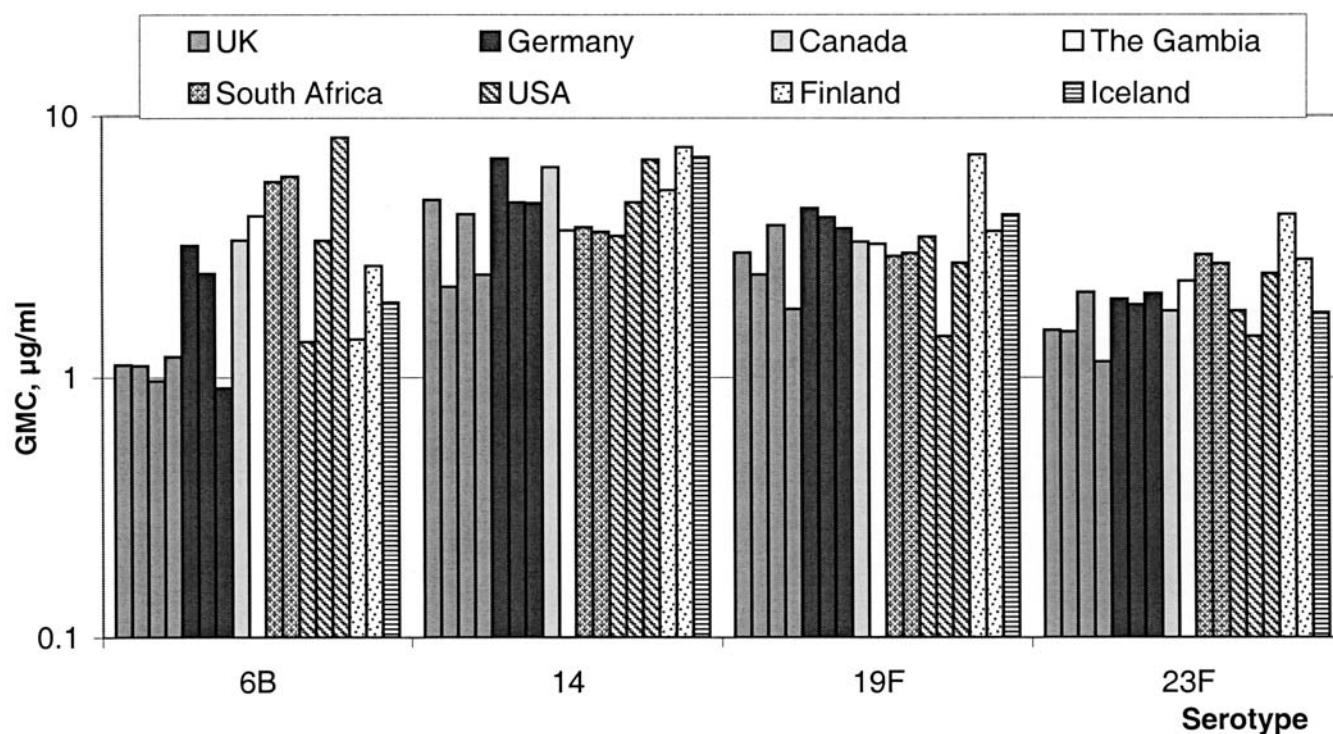
**Table 2** Geometric means titers (GMT) of opsonophagocytic antibodies against pneumocci of the indicated serotypes in samples from infants in FinOM trials and POET with an end point of AOM caused by the vaccine serotypes<sup>a</sup>

Trial, vaccine, and no. of subjects (references)	Serotype	% Efficacy (95% CI)	Post-third dose	GMT ( $\mu$ g/ml) Post-fourth dose
FinOM, PCV7-CRM, 53–55 (27, 28, 30)	6B	84 (62–93)	143	606
	19F	25 (−14–51)	26	78
	23F	59 (35–75)	39	173
FinOM, PCV7-OMPC, 44–52 (28, 39)	6B	79 (58–89)	15	91
	19F	37 (1–59)	51	84
	23F	52 (28–68)	<8	17
POET, PCV11-PD, 106–148 (67, 79)	6B	87.6 (58.4–93.6)	567.5	1,045.6
	14	95.5 (66–99.4)	1,262.2	1,872.4
	19F	44.4 (8.3–66.3)	106.5	406.6
	23F	72.3 (24.8–89.8)	1,722.5	2,925.0

<sup>a</sup>OPA data received from the FinOM and POET studies cannot be compared because of the methodological differences.



**Figure 2** Immunogenicities of 7- to 11-valent PCVs in Finnish infants after a primary series at 2, 4, and 6 months (A) and after a booster dose in the second year (B). GMCs are given for PCV7-CRM used previously (58) and in the FinOM vaccine trial (unpublished data), for PCV7-OMPC used in the FinOM vaccine trial (43), for PCV11-D-T with and without an aluminum adjuvant (24, 71), and for PCV11-PD (55).



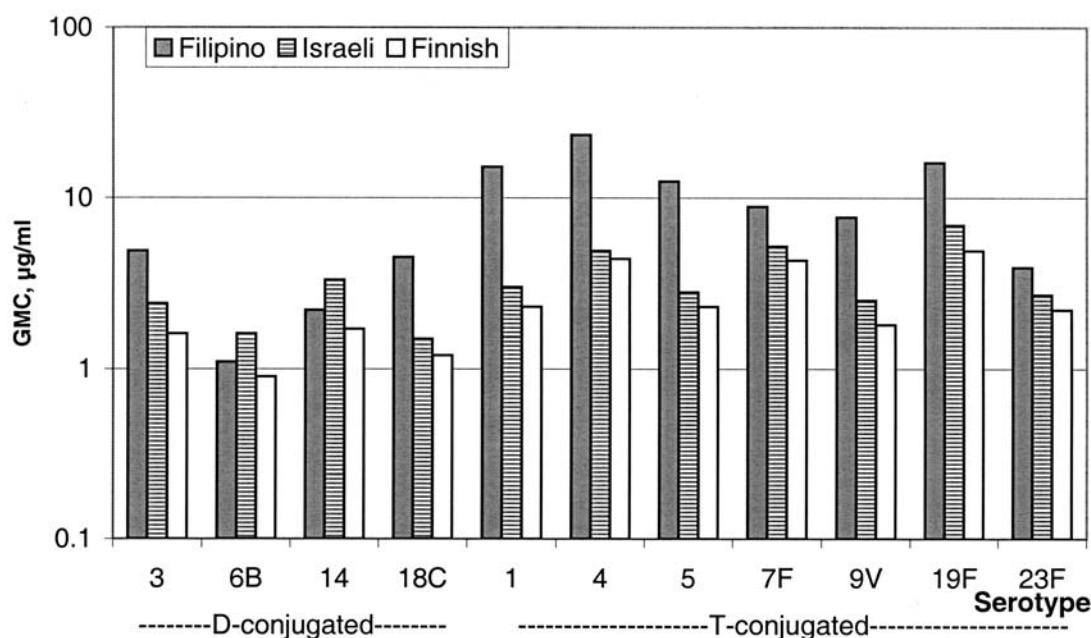
**Figure 3** GMCs of antibodies after three primary doses of PCV7-CRM or PCV9-CRM in studies in the United Kingdom (13, 15, 34, 76), Germany (45, 78, 88), Canada (77), The Gambia (61), South Africa (36, 51), the United States (63, 75, 83), Finland (unpublished; reference 58), and Iceland (unpublished; reference 84). The PCV was given at 2, 3, and 4 months in the United Kingdom, Germany and The Gambia; at 2, 4, and 6 months in Canada, the United States and Finland; at 3, 4, and 5 months in Iceland; and at 6, 10, and 14 weeks in South Africa.

studies, one has to remember that the ELISAs have been performed in different laboratories and the vaccines have been given with different schedules (at 2, 3, and 4 months in the United Kingdom, Germany, and The Gambia; 3, 4, and 5 months in Iceland; 2, 4, and 6 months in the United States, Canada, and Finland; or 6, 10, and 14 weeks in South Africa) and with different concomitant vaccines. Results for serotype 6B seem to show the greatest variation among populations.

The investigational mixed-carrier aluminum adjuvant-containing PCV11-D-T was given to Filipino, Israeli, and Finnish infants in two separate studies (71). In all three countries, the first three doses of PCV11-D-T were given concomitantly with whole-cell pertussis pathogen (wP) containing combination vaccines. The antibody concentrations were determined at the National Public Health Institute, Helsinki, Finland, with the same methodology without 22F adsorption. After the third dose, given at 14 weeks in the Philippines and at 6 months in Israel and Finland, the Filipino infants showed higher concentrations of antibodies to most of

the 11 serotypes and the difference was most notable for serotypes conjugated to tetanus toxoid (TT) (Fig. 4). The difference remained for the TT-conjugated serotypes when the mean concentrations were compared after the booster dose, given at 9 months in the Philippines and at 12 months in Israel and Finland. Possible reasons for the higher-level responses among Filipino infants may be priming through tetanus vaccination of mothers in the Philippines, natural boosting by earlier carriage acquisition in the Philippines than in Finland or Israel, genetic factors, antibodies elicited by cross-reacting antigens, and the vaccination schedule. Of interest is that in a comparison of different *H. influenzae* type b (Hib) conjugate vaccines in Filipino infants, the one with a TT carrier was the most immunogenic (14).

Serological data for the novel 11PCV-PD are available from the immunogenicity studies in Finland (55) (where the vaccine was administered at 2, 4, 6, and 12 to 15 months simultaneously with a diphtheria-tetanus-acellular pertussis protein [DTaP]-inactivated poliovirus [IPV]-Hib vaccine), the Philippines (33) (where the vac-



**Figure 4** GMCs of antibodies after three primary doses of PCV11 with serotypes conjugated to diphtheria toxoid or TT in samples from children in the Philippines (immunized at 6, 10, and 14 weeks) and Israel and Finland (immunized at 2, 4, and 6 months) (71). Serotypes 3, 6B, 14, and 18C were conjugated to diphtheria toxoid (D), and serotypes 1, 4, 5, 7F, 9V, 19F, and 23F were conjugated to tetanus protein (T).

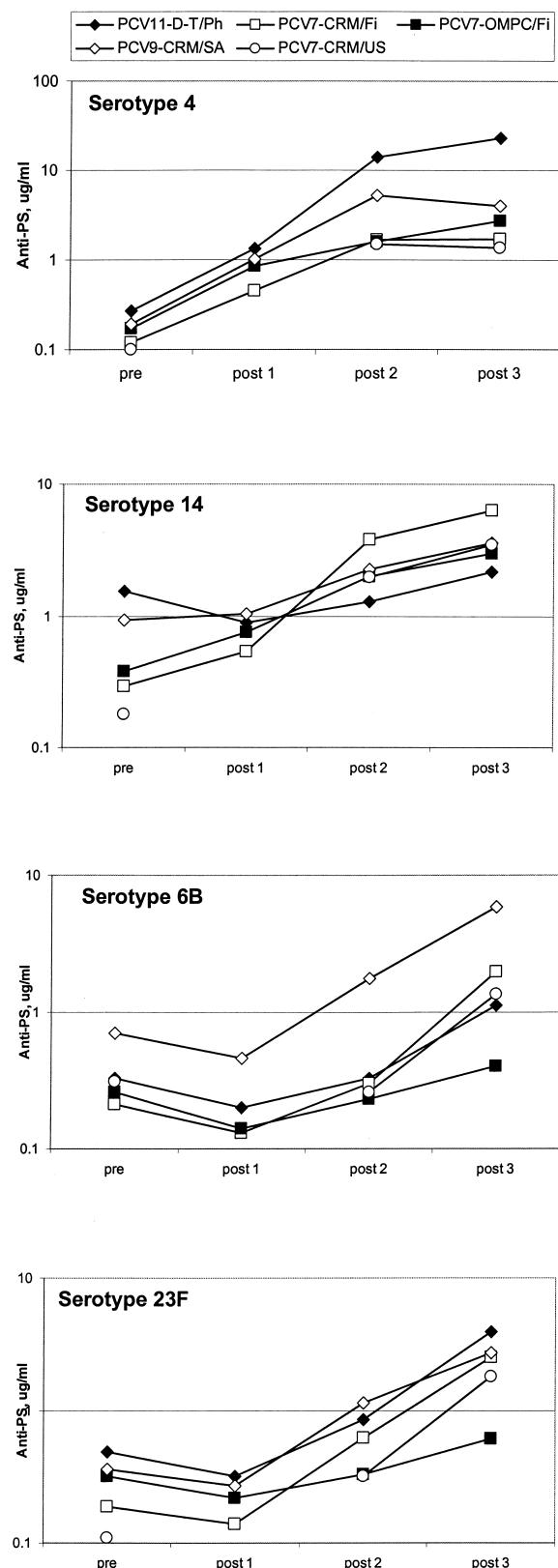
cine was administered at 6, 10, and 14 weeks simultaneously with a diphtheria-tetanus-wP [DTwP]-hepatitis B virus [HBV]-Hib vaccine), and from the phase III study in the Czech Republic and Slovakia (68) (where the vaccine was administered at 3, 4, 5, and 12 to 15 months simultaneously with a DTaP-HBV-IPV-Hib vaccine). The antibody concentrations were determined either at GlaxoSmithKline Biologicals or at the National Public Health Institute. The mean concentrations after the third dose were very similar in the three populations, as were those after the fourth dose given in Finland and the Czech Republic and Slovakia (55, 68).

### NUMBER OF DOSES OF PCVs IN PRIMARY SERIES

The efficacy studies have used three-dose priming with boosting in the second year of life (typically at 2, 4, 6, and 11 to 15 or 11 to 18 months [the 3+1 schedule]) or a three-dose schedule (the local Expanded Program on Immunisation schedule of 6, 10, and 14 weeks or 2, 3, and 4 months) (9, 20, 30, 43, 44, 46a, 64). A number of countries have, however, used different schedules for routine infant immunization, and many use the PCV with a schedule consisting of two primary doses in in-

fancy (at approximately 3 and 5 months of age) followed by a booster dose at 11 to 18 months (the 2+1 schedule). In addition, to be able to adopt the PCVs in resource-poor countries, immunization series of fewer than three doses would offer a possibility to lower the costs of immunization. Immunogenicity data for estimating the alternative schedules come mainly from three different sources: studies on the kinetics of the antibody response during the course of PCV vaccination in infancy, studies comparing the different schedules directly in randomized trials, and studies that use data from a separate study as a reference.

Studies on the kinetics of antibodies in samples from children from Finland, South Africa, the Philippines, and the United States (27, 36, 72, 75) show very similar trends in spite of the use of different PCVs and different schedules (Fig. 5). Typically, serotypes 6B and 23F induce antibody increases only after the second or third dose, while for serotypes 4 and 14, increases can be found already after the first dose. Anderson et al. and Ekström et al. have reported the kinetics during a course of three doses of PCV7-OMPc administered to U.S. and Finnish children. The kinetics are very similar, even if the mean antibody concentrations are not comparable due to differences in the assays (6, 27).



Leach et al. compared groups that received PCV5-CRM at 2, 3, and 4 or at 2 and 4 months of age (46). Higher antibody concentrations were noted in the group that had received three doses than in the group receiving two doses, but due to the small numbers of vaccinees, the difference was significant only for serotype 14. The situation was similar for subjects at 9 months of age. In a Finnish study, PCV4-OMPC was given to subjects at 4 and 6 months or at 2, 4, and 6 months, and the three-dose schedule induced higher responses than the two-dose schedule for all four serotypes (42). An Icelandic study randomized infants to receive PCV9-CRM given in combination with a group C meningococcal conjugate vaccine (MCV-C) either at 3 and 5 months or at 3, 4, and 5 months, with a booster dose at 12 months. The mean antibody concentrations after the primary courses were similar for the two groups (Table 3), with the exception of the mean concentrations of antibodies to serotypes 6B and 23F (unpublished data and reference 84), which were higher after three doses. After a booster dose of either PCV9-CRM or PPSV, no differences were noted.

Goldblatt et al. compared data from two studies that had a schedule of either 2, 3, 4, and 12 months or 2, 4, and 12 months (34). They found very similar mean antibody concentrations in infants at 5 months of age (Table 3). In addition, the avidity maturation (studied for serotypes 6B, 14, and 19F) and the booster responses at 12 months were similar, suggesting that similar to the three-dose schedule, the two-dose schedule had induced the development of immunological memory. Espesito et al. and Käyhty et al. reported antibody concentrations for the 2+1 schedule (3, 5, and 11 or 12 months) of PCV7-CRM for Italian and Swedish infants, respectively (32, 41). Compared to historical controls, i.e., results from studies that have used the same vaccine with the 3+1 schedule (9, 27, 30), the GMCs for serotypes 6B and 23F remained low after the primary course, but no clear-cut differences were noted for other serotypes (Table 3). After the booster at 11 or 12 months, the GMCs were similar to those found after boosting in previous studies using three doses in infancy. In general,

**Figure 5** Kinetics of antibody responses to the adjuvant-containing PCV11-D-T in Filipino infants (Ph) (72), to PCV7-CRM and PCV7-OMPC in Finnish infants (Fi) (27), to PCV9-CRM in South African infants (SA) (36), and to PCV7-CRM in U.S. infants (75). The GMCs are given for serotypes 4, 6B, 14, and 23F before vaccination (pre) and after the first, second, and third doses (post 1, post 2, and post 3, respectively). The course of vaccination took place at 6, 10, and 14 weeks in the Philippines and at 2, 4, and 6 months in Finland and the United States.

**Table 3** GMCs of antibodies to serotype 4, 6B, 14, and 23F pneumococcal PSs after primary series and booster doses in studies that have compared, directly or indirectly, schedules including two or three primary doses in early infancy with PCV boosting at 12 months of age

Vaccine	Country(ies) (reference [52])	Schedule according to ages (mos) of vaccines (no. of vaccines)	GMC (μg/ml) of antibody to serotype:							
			4	6B	14	23F				
		Post-primary series	Post-booster dose	Post-primary series	Post-booster dose	Post-primary series	Post-booster dose			
PCV9-CRM	Iceland [83] <sup>a</sup>	3-5-12 (108) 3-4-5-12 (110)	2.34 2.97	3.87 4.30	0.69 1.94	9.42 14.01	4.69 6.95	8.75 10.15	0.91 1.77	2.83 4.42
PCV9-CRM	United Kingdom (34)	2-4-12 (82) 2-3-4-12 (73)	2.05 1.82	5.55 5.19	1.01 1.12	9.68 11.11	3.20 4.78	13.88 18.48	1.15 1.52	6.11 6.40
PCV7-CRM	Sweden and Finland (30, 40)	3-5-12 (99) 2-4-6-12 (57)	4.43 1.70	9.43 2.56	0.30 2.00	4.92 9.05	3.37 6.28	11.67 10.82	0.88 2.51	4.59 6.25

<sup>a</sup>Some data presented here are from unpublished results.

the findings of comparative studies described here suggest that the 2+1 dose schedule induces satisfactory immune responses with the possible exception of responses to serotypes 6B and 23F. The data on the impact of PCV vaccination on respiratory-tract infections in Italy also speak for good effectiveness of this schedule (31).

A Filipino study showed similar antibody concentrations at 9 months of age in children immunized with three doses of PCV11-D-T at 6, 10, and 14 weeks of age and those immunized with a single dose at 18 weeks of age (47). The relative avidity was tested for four serotypes and was lower in the one-dose group for serotypes 5, 6B, and 23F but not for serotype 19F (40). Several studies comparing alternative schedules of PCV7-CRM dosing are ongoing. The data on antibody concentrations, the development of memory, and the functional activity of antibodies from those studies will be available in the near future.

## RESPONSE TO A BOOSTER DOSE OF PCV OR PPSV

The need for booster doses of conjugate vaccines in industrialized countries has become evident from the experience with Hib and MCV-C in the United Kingdom; the three-dose schedule carried out in early infancy without a booster dose did not provide satisfactory long-lasting protection (73, 89). The South African follow-up study suggests that HIV-infected children would benefit from a booster immunization while non-HIV-infected children may have persistent protection due to natural boosting via pneumococcal colonization or cross-reacting antigens (49). The inclusion of a booster dose is believed to have a significant effect on the reduction of colonization and hence indirect protection, since the antibody concentrations in booster dose-immunized children are highest in the second and third years of life, when colonization by and transmission of pneumococci are frequent (chapter 19).

In general, independently from the PCV formulation or the primary immunization schedule, the booster dose is able to induce antibody concentrations higher than or similar to those induced by the primary immunization. In many cases, the PPSV boosting induces higher mean antibody concentrations than the PCV boosting (1, 4, 28, 34, 37, 43, 55, 58, 84, 85, 97), suggesting that a schedule including a PCV in infancy and boosting with a PPSV could be used as a cheaper alternative schedule, although the feasibility of such a program has been questioned. There are also concerns about hyporesponsiveness after the vaccination with a PPSV in early childhood, though

this effect has been better documented for group C meningococcal PS than for pneumococcal PS (48). The use of a comparison of antibody responses after a PPSV booster dose in previously unimmunized and PCV-immunized children has been suggested for studying the development of B-cell memory during the primary course (38). The determination of booster responses to PPSVs has been used, e.g., for testing the persistence of memory (34, 79) or the induction of memory by different schedules (34), different PCV doses (4), and different vaccine combinations (15) and for comparing responses of preterm and full-term infants (76).

### PERSISTENCE OF ANTIBODIES AFTER VACCINATION WITH PCVs

The long-term persistence of antibodies has been monitored for PCV9-CRM in South Africa (37, 49), for PCV7-CRM in Finland (5), and for PCV11-PD in the Czech Republic (79). In addition, phase II studies with the investigational PCV4 or PCV8 formulations included follow-up until the age of 2 to 3 years (1, 4, 59). After 6.2 years of follow-up in South Africa, protection persisted among the non-HIV-infected children (77.8%) but declined from 65 to 38.8% (95% confidence interval [CI], 7.8 to 65.2%) among HIV-infected children. Consistent with this difference, the proportions of HIV-infected children with IgG antibody levels of  $\geq 0.35$   $\mu\text{g/ml}$  were similar for the placebo and PCV groups for most of the serotypes, while significantly higher proportions of non-HIV-infected PCV recipients than of non-HIV-infected children in the placebo group had such

levels (49). Huebner et al. report (Table 4) the antibody concentrations in a cohort of South African vaccinees up to 18 months of age (37). Their conclusion is that even without a booster dose, the antibodies remain significantly higher in the PCV9-CRM recipients up until the age of 18 months than in the placebo group.

The persistence of antibodies in children at the age of 4 to 5 years in the PCV7-CRM and control (HBV vaccine) groups was evaluated in the FinOM vaccine trials. The antibody concentrations had declined, but less so for the frequently carried serotypes 6B, 14, 19F, and 23F (5) than for the other vaccine serotypes (Table 4). A subset of the sera has recently been reanalyzed with an ELISA with 22F adsorption, and the conclusion is very similar (unpublished data). The follow-up of a subset of children that previously participated in the POET study showed a similar trend (Table 4). The PCV11-PD group maintained significantly higher antibody concentrations up to their fourth year of life for all other pneumococcal vaccine serotypes, except for serotype 3, and the difference was most clear cut for the most frequently carried serotypes 6B, 14, and 19F and the cross-reactive serotype 6A (79).

### NEW FORMULATIONS OF PCVs

Experimental PCVs currently in clinical trials with infants include the mixed-carrier PCV10-PD-D-T, which has nontypeable *H. influenzae* PD, diphtheria toxoid, and TT as carriers and includes serotypes 1, 5, and 7F in addition to the PCV7 serotypes (17), and PCV13-CRM, which has increased valency compared to the licensed

**Table 4** Persistence of antibodies after immunization of South African, Czech, and Finnish infants with PCVs<sup>a</sup>

Country (reference)	Age of subjects	Vaccine (no. of subjects in group)	GMC ( $\mu\text{g/ml}$ ) of antibody to serotype:										
			1	3	4	5	6B	7F	9V	14	18C	19F	23F
South Africa (37)	18 mos	PCV9-CRM (211–214)	0.27	NA	0.19	0.58	1.07	NA	0.39	1.1	0.22	0.83	0.24
		Control (211–217)	0.11	NA	0.07	0.36	0.13	NA	0.28	0.17	0.11	0.40	0.05
Czech Republic (79)	3.6 yrs	PCV11-PD (50)	0.12	0.34	0.13	0.20	1.59	0.31	0.23	2.05	0.16	3.59	0.92
		Control (49)	0.04	0.20	0.05	0.07	0.13	0.04	0.06	0.17	0.04	0.33	0.08
Finland (5)	4–5 yrs	PCV7-CRM (382)	NA	NA	0.51	NA	3.33	NA	0.95	1.94	0.76	4.66	6.19
		Control (341)	NA	NA	0.33	NA	0.59	NA	0.58	0.58	0.34	1.38	0.36

<sup>a</sup>Shown are the GMCs of antibody to each vaccine serotype in samples from children at the indicated ages who had received a PCV or a control vaccine in infancy. NA, not applicable.

PCV7-CRM, containing the additional serotypes 1, 3, 5, 6A, 7F, and 19A (18). PCV10-PD-D-T induced IgG antibody concentrations and functional immune responses comparable to the responses to PCV11-PD, for which efficacy against AOM has been demonstrated (unpublished data). PCV13-CRM induces IgG and functional antibody responses to the seven original vaccine serotypes similar to those induced by PCV7-CRM. PCV13-CRM is also immunogenic for the six new serotypes and is as well tolerated as PCV7-CRM (unpublished data).

## EFFECTS OF CARRIER-MEDIATED INTERACTIONS

Most conjugate vaccines use protein carriers that are identical to protein antigens used in other pediatric vaccines, such as TT and diphtheria toxoid, or are immunologically related, such as CRM<sub>197</sub>, which is cross-reactive with diphtheria toxoid. The mechanisms of carrier-mediated interactions are discussed in chapter 6.

### Increased Antibody Response to the Carrier Protein

In a number of PCV7-CRM trials, the response to a primary series of doses of a diphtheria-tetanus-pertussis combination vaccine (DTP) alone has been compared to the response to DTP coadministered with PCV7-CRM. The GMCs of antibodies against diphtheria toxoid were generally higher in the group given PCV7-CRM in studies with both wP (15)- and acellular pertussis protein (aP)-containing combinations (15, 45, 78, 88).

In a study in Canada, the sequential use of DTP at 2, 4, and 6 months with PCV7-CRM at 3, 5, and 7 months increased the GMC of anti-diphtheria toxoid compared to the concomitant use of PCV7-CRM and DTP at 2, 4, and 6 months (77). Although this result could be interpreted as a reduced response with concomitant administration, it should also be remembered that the sequentially dosed subjects effectively received six doses of diphtheria toxoid or a cross-reactive antigen, CRM<sub>197</sub>. A U.S. study reported very similar data (67).

Studies investigating PCV7-CRM administered alone to infants have not been performed. PCV11-D-T given to infants at 18 weeks of age was able to boost the diphtheria toxoid and TT responses of Filipino infants who had received a DTwP vaccine at 6, 10, and 14 weeks (65). Similarly, the vaccination of toddlers with PCV11-D-T (66) was able to boost responses to diphtheria toxoid and TT.

Nontypeable *H. influenzae* PD has been used as a carrier protein in PCV11-PD, tested in POET, where it was shown to induce an immune response that provided

protection against nasopharyngeal carriage and AOM episodes caused by *H. influenzae* (68).

### Increased Antibody Response to Other Conjugate Vaccines Using the Same Carrier Protein

When PCV7-CRM was administered at the same time as a CRM<sub>197</sub>-based Hib conjugate vaccine (polyribosyl ribitol phosphate [PRP]-CRM), the mean concentration of antibody to Hib PS was doubled compared to that in subjects not receiving concomitant PCV7-CRM (15). A similar effect was observed when a CRM<sub>197</sub>-based MCV-C was administered with PRP-CRM (29). However, in a U.S. study, PCV7-CRM was given at the same times as or 2 weeks after DTaP-HBV-IPV and PRP-CRM vaccines, and no significant differences in anti-Hib PS responses were observed (67).

### Reduced Antibody Response to Other Conjugate Vaccines and Related Protein Antigens

In a Finnish dose-ranging study of a four-valent PCV conjugated to TT (PCV4-T), infants also received PRP-T and DTwP combination vaccines including TT (4, 21). After the three primary vaccinations, both anti-Hib PS and anti-TT GMCs were inversely related to the PCV4-T dose (21). A similar Israeli, placebo-controlled study of a single dose level of PCV4-T also showed reduced anti-Hib PS responses in the subjects given concomitant PCV4-T (21, 25). Although PCV7-CRM in general showed evidence of enhancement of the response to concomitantly administered related antigens (see above), there is also some evidence of carrier-mediated suppression with CRM (13, 82).

### Interactions between PCVs and Immunologically Unrelated Vaccines

A number of studies have examined the immunogenicities of commonly used vaccines administered with and without PCVs. These include studies with DTaP combinations (trivalent up to hexavalent combinations, also including HBV, PRP-T, and IPV) (45, 55, 69, 77, 78, 88), DTwP combinations (74), HBV vaccines (45, 77, 82, 88), an oral polio vaccine (82), an inactivated polio vaccine and PRP-T (10, 74, 77, 78, 88), measles-mumps-rubella vaccine (10), and a monovalent attenuated human rotavirus vaccine (81). Further studies have been conducted with PCV9-CRM (36, 61), PCV9-CRM-MCV-C-CRM (13), and PCV11-PD (68). Although minor differences between groups are sometimes seen for particular antigens in vaccines given together with PCVs, in general these differences are variable between studies and unlikely to be of clinical significance.

Although there is a substantial amount of information from randomized trials on the impact of PCV7-

CRM on concomitant vaccines, there is less information on the impact of the concomitant vaccines on antibody responses to PCVs. This is because it is not possible to randomize children to groups not receiving the “standard of care.” However, it is clear (see sections above) that in all trials, a large number of children have achieved a protective antibody response regardless of the concomitant standard vaccines that they received. To that extent, concomitant standard vaccines clearly have no significant deleterious impact on the immunogenicities of PCVs.

Four trials have, however, included infants given different concomitant vaccines. In three trials, differences in concomitant vaccines did not have any effect (67, 77, 82). An Israeli study compared two groups, one receiving investigational PCV11-D-T concomitantly with DTwP-IPV-PRP-T vaccine and the other receiving it with a DTaP-IPV-PRP-T vaccine. The response after the third and fourth doses to TT-based conjugates was significantly lower among infants who had received an aP-containing combination vaccine (23).

## NEED FOR ALUMINUM ADJUVANT

In a trial in the United Kingdom, PCV9-CRM-MCV-C-CRM reconstituted with aluminum phosphate was compared to PCV9-CRM-MCV-C-CRM reconstituted with saline (13). The adjuvant-containing formulation was significantly more immunogenic than the non-adjuvant-containing formulation, as indicated by GMCs, for four of the nine pneumococcal types after the three-dose primary series and for seven of the nine types after a booster dose. There was a trend towards more local reactogenicity with the adjuvant-containing formulation but no difference in systemic reactogenicity, fever, or adverse events. These data support the use of an aluminum adjuvant for the optimal efficacy of PCV-CRM.

In a study of PCV11-D-T, a formulation with aluminum hydroxide induced higher GMCs of antibodies, but differences were not statistically significant. In general, there were no significant differences in the avidities of antibodies (71, 95). The OPA-determined activity was higher for serotype 6B with the adjuvant-containing formulation but showed no difference for the other four serotypes examined (96).

## REACTOGENICITY

Local reactions due to PCV7-CRM have generally been less frequent and less severe than those due to DTwP vaccines (15, 23, 74, 75, 82), whereas the frequency and severity of such reactions have been similar to those of

local reactions due to combination vaccines including aP (19, 45, 67, 77, 78, 88). Giving PCV7-CRM together with combination vaccines containing wP appears to increase slightly the rate of fever measured rectally of  $>38^{\circ}\text{C}$  (74, 75), as does administering the vaccine with combinations containing aP (9, 45, 67, 78, 88, 97). However, fever exceeding  $39^{\circ}\text{C}$  was unusual across all trials. The frequency and severity of local reactogenicity, fever, and other systemic symptoms associated with PCV11-PD, PCV10-PD-D-T, and PCV13-CRM are similar to those of such symptoms associated with PCV7-CRM (unpublished data, reference 18).

A Filipino study compared the reactions at the sites of injection of DTwP-PRP-T and adjuvant-containing PCV11-D-T, given concomitantly. The rate of local reactions at the PCV injection site was equal to or less than that at the DTwP-PRP-T site (70). The rate of fever ( $>38^{\circ}\text{C}$ ) was 40% after the first dose and around 20% after the second and third doses. A similar study done among Israeli and Finnish infants reported somewhat higher rates than the Filipino study for any local reactions and fever for the adjuvant- and no-adjuvant-containing PCV11-D-T sites after the second and third doses (24, 94). In general, the DTwP-PRP-T-IPV site showed higher reaction rates. Fever ( $>38^{\circ}\text{C}$ ) was found in 36 to 56% of subjects, depending on the dose and the formulation. The adjuvant-lacking formulation induced lower rates of local reactions than the adjuvant-containing formulation.

In studies conducted in Finland (55) and the Czech Republic and Slovakia (POET) (68), local reactions (pain, redness, and swelling at the site of injection) were more common after vaccination with PCV11-PD than after vaccination with the control hepatitis B and hepatitis A vaccines, respectively. Fever (rectal temperature of  $\geq38^{\circ}\text{C}$ ) was reported after the primary doses of PCV11-PD for 36.5% of subjects in Finland and for 26.8% of subjects in the POET, compared to 16.7 and 17.3% in the respective control groups. After the booster dose, fever of  $\geq38^{\circ}\text{C}$  was reported for 41.2% of the subjects in Finland and for 13.2% of the subjects in the POET. High-grade fever ( $>39^{\circ}\text{C}$ ) was recorded rarely. The reactogenicity profile is not different from that reported for PCV7-CRM administered with DTaP-IPV-Hib or DTaP-HBV-IPV-Hib vaccines (55, 69, 88).

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Lisa A. Jackson  
George R. Siber

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## Immunogenicity and Safety in Adults

Older adults and persons with chronic illness are at elevated risk of invasive pneumococcal infection, and in many countries the 23-valent pneumococcal polysaccharide vaccine (PPSV) is recommended for those high-risk groups. Clinical trials documenting the effectiveness of the PPSV in reducing the risk of pneumococcal pneumonia in young adult South African gold miners provided data supporting the licensure of the PPSV for adults in the United States in 1977 (3, 32). Since that time, postlicensure observational studies have indicated that the PPSV reduces the risk of pneumococcal bacteremia in immunocompetent older adults (6, 14, 29).

The public health benefit of the 23-valent PPSV for the targeted populations of seniors and adults with chronic illness is limited, however, because this vaccine does not appear to be effective in adults that are immunocompromised due to factors such as malignancy or the receipt of immunosuppressive medications (6, 14, 29), who are at greatly elevated risk for pneumococcal bacteremia compared to the general population (9, 19), and vaccine effectiveness in immunocompetent adults declines with time (29). Revaccination with the PPSV is not routinely recommended for seniors because of con-

cerns regarding immune tolerance to repeated doses of polysaccharide vaccine.

Taking these factors into account, Fry and colleagues modeled the impact of a strategy of one-time vaccination with the PPSV at age 65 on the burden of invasive pneumococcal disease in seniors in the United States. They estimated that an ongoing program that achieved 90% PPSV coverage in 65-year-olds would prevent only 21% of all cases of invasive pneumococcal disease in seniors (9). The public health benefit of the PPSV for adults is also limited by the apparent lack of impact of vaccination on the risk of all-cause pneumonia (10, 14).

There is, therefore, a need for better interventions to prevent pneumococcal infections in adults. The success of pneumococcal polysaccharide-protein conjugate vaccines in children (4, 5) suggests the possibility that these vaccines may also offer advantages over the PPSV for adults. During the past decade, a number of studies have evaluated the immunogenicity and safety of various pneumococcal conjugate vaccine (PCV) formulations in immunocompromised (see chapter 18) and immunocompetent adults. This chapter summarizes the findings from evaluations of PCVs in immunocompetent adults.

## VACCINE FORMULATIONS EVALUATED FOR IMMUNOCOMPETENT ADULTS

In the past two decades, 15 PCV formulations have been evaluated for immunocompetent adults (Table 1). These vaccine formulations vary in terms of important characteristics such as the valency, the serotypes included, the carrier protein, the saccharide size, the saccharide content, and the inclusion of adjuvants. For example, the saccharide content per serotype ranges from a high of 2.5 µg to a low of 1 µg, with a trend toward lower saccharide contents in more recently developed vaccine formulations. These differences mean that the results of evaluations of one PCV formulation cannot necessarily be extrapolated to other formulations. Of the 15 formulations evaluated, only one, the seven-valent PCV conjugated to a mutant, nontoxic diphtheria toxoid, CRM (PCV7-CRM [Prevnar]), has been licensed, and this formulation is currently approved for use in children under 7 years of age in the United States and a number of other countries.

## IMMUNOGENICITY

CVs have consistently been shown to induce an immunoglobulin G (IgG) response (1, 2, 15–17, 23–26, 27a, 28, 30, 35–37), as well as IgA and IgM responses (16, 24, 25, 36), in immunocompetent adults. Two early studies evaluated the immune response to monovalent PCV formulations by using radioimmunoassays (8, 27), but since that time, all clinical studies of PCVs in immunocompetent adults have assessed the IgG antibody response by using enzyme-linked immunosorbent assays (ELISAs) and, in some cases, additional immunologic assays, such as the opsonophagocytic assay (OPA).

### IgG ELISA Antibody Response to PCV Compared with that to PPSV

To assess whether PCVs may offer an advantage over PPSVs in adults, it is of interest to know whether PCVs induce higher serotype-specific IgG antibody levels than PPSVs. Thirteen studies with published results have compared the IgG immune response, as measured by ELISA, to PCV with the immune response to 23-valent PPSV in immunocompetent adults. Table 2 summarizes the results of the 11 studies that evaluated a single-dose formulation of an investigational PCV compared to the standard PPSV, and Table 3 summarizes the results of the two dose-ranging studies of the licensed PCV7-CRM in elderly participants.

### Single-Dose Studies

Ten of the 11 studies summarized in Table 2 used a second-generation ELISA protocol that included single absorption with cell wall polysaccharide to remove non-capsule-specific antibodies; the most recent study used a third-generation ELISA protocol that also included absorption with 22F polysaccharide. The largest of the 11 studies, that by Ahmed and colleagues, was conducted primarily to evaluate the PCV in human immunodeficiency virus-infected persons but also evaluated 99 healthy human immunodeficiency virus-uninfected adults, who were randomly assigned to receive an investigational five-valent oligosaccharide CRM vaccine or a PPSV (1). Among those healthy adults, postvaccination geometric mean concentrations (GMCs) of antibodies to serotypes 6B, 18C, and 23F were significantly higher in the PCV group than the PPSV group, but those of antibodies to serotypes 14 and 19F did not differ between the two groups.

The other 10 studies were relatively small (four had 10 or fewer participants per study group, and none had more than 25 participants per group) and were thus not adequately powered to identify moderate differences in postvaccination antibody levels between the PCV and PPSV groups. Two of those 10 studies reported a significantly higher antibody response in the PCV group than in the PPSV group for any serotype. In the first, Shelly and colleagues evaluated 50 healthy nonelderly adults and 50 seniors who were assigned to receive either the PCV or the PPSV (30). Among the younger adults, the postvaccination GMC for one of the two serotypes assessed was significantly higher in the PCV group than in the PPSV group, but among the elderly adults, there were no significant differences between the conjugate and polysaccharide vaccine groups. Interestingly, in this study the response to the CRM conjugate vaccine appeared to vary according to the prevaccination diphtheria antibody level and tended to be greater in persons with higher prevaccination diphtheria antibody levels (31). The older adults tended to have lower diphtheria antibody levels than the younger subjects, suggesting that lower carrier immunity may have contributed to the lower immunogenicity of the conjugate vaccine in the older study group. In the second study, Scott et al. evaluated 30 adults randomized to receive a 13-valent PCV or PPSV (27a). Significantly higher GMCs were found in the PCV group for 3 of the 12 serotypes common between the two vaccines.

In addition to the published reports of the studies described above, an additional phase 1 trial of a 13-valent CRM<sub>197</sub> PCV in healthy adults has been reported in abstract form (28). In that study, 30 Japanese adults 20 to

**Table 1** Characteristics of 15 polysaccharide-protein conjugate vaccine formulations evaluated in published clinical studies that included immunocompetent adults

Manufacturer	Valency	Serotypes	Carrier protein <sup>a</sup>	Saccharide type	Saccharide content (µg/serotype/0.5-ml vol)	Adjuvant	Reference(s)
NIH	1	6A	TT	Polysaccharide	50	None	Schneerson et al., 1986 (27)
	1	6B	TT	Polysaccharide	12	None	Jonsson et al., 2002 (15) Vidarsson et al., 1998 (36)
Eli Lilly	1	12F	DT	Polysaccharide	2.5	None	Fattom et al., 1990 (8)
Lederle	1	12F	DT	Polysaccharide	2.5	None	Fattom et al., 1990 (8)
University of Rochester	1	Multiple formulations	DT	Multiple formulations	Multiple	None	Anderson and Betts, 1989 (2)
Connaught	4	6B, 14, 19F, 23F	DT	Polysaccharide	10	None	Nieminen et al., 1998 (24)
Pasteur-Merieux	4	6B, 14, 19F, 23F	TT	Polysaccharide	10	None	Nieminen et al., 1998 (24)
Merck	4	6B, 14, 19F, 23F	OMPC	Polysaccharide	2	None	Kroon et al., 2000 (17)
	4	6B, 14, 19F, 23F	OMPC	Polysaccharide	1	None	Nieminen et al., 1998 (25)
Lederle-Praxis	5	6B, 14, 19C, 19F, 23F	CRM <sub>197</sub>	Oligosaccharide	10	Aluminum phosphate	Ahmed et al., 1996 (1); Shelly et al., 1997 (30); Powers et al., 1996 (26); Lottenbach et al., 1999 (21); Soininen et al., 1999 (34)
Wyeth	7	4, 6B, 9V, 14, 18C, 19F, 23F	CRM <sub>197</sub>	Polysaccharide	2 for all serotypes except 6B; 4 for 6B	Aluminum phosphate	Kamboj et al., 2003 (16); Jackson et al., 2007 (12); de Roux et al., 2007 (7)
Merck	7	4, 6B, 9V, 14, 18C, 19F, 23F	OMPC	Polysaccharide	1 for all serotypes except 6B; 2.5 for 6B	None	Storek et al., 1997 (35); Molrine et al., 1995 (23)
	7	4, 6B, 9V, 14, 18C, 19F, 23F	OMPC	Polysaccharide	1 for serotypes 4, 14, and 18C; 1.5 for 9V; 2 for 19F; 3.5 for 6B	None	Lottenbach et al., 1999 (21)
Aventis Pasteur	11	1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F	Mixed: DT or TT	Polysaccharide	1 for serotypes 1, 4, 5, 7F, 9V, 19F, and 23F; 3 for serotypes 3, 14, and 18C; 10 for 6B	Formulations with and without aluminum phosphate were evaluated	Wuorimaa et al., 2001 (37)
Wyeth	13	1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F	CRM <sub>197</sub>	Polysaccharide	2 for all serotypes except 6B; 4 for 6B	Aluminum phosphate	Scott et al., 2007 (27a) Scott et al., 2006 (28)

<sup>a</sup>TT, tetanus toxoid; DT, diphtheria toxoid.

**Table 2** Published results of clinical trials excluding dose-ranging studies comparing the ELISA-measured IgG response to PCV with the response to PPSV in immunocompetent adults

Study	PCV <sup>a</sup>	Range of subject ages (yrs)	No. of subjects/vaccine group (vaccine)	Measure of postvaccination response	Result (GMC [ $\mu$ g/ml] or % of subjects) for serotype:							Summary
					4	6B	9V	14	18C	19F	23F	
Molrine et al., 1995(23)	7-valent PCV with OMPC	Not stated	20 (PCV) 19 (PPSV)	GMC of Abs	7.1 9.4	5.7 8.6	4.3 7.1	10.3 9.9	5.8 8.4	5.7 8.4	5.6 6.4	No difference between groups
Ahmed et al., 1996 (1)	5-valent PCV with CRM <sub>197</sub>	18–65	49 (PCV) 50 (PPSV)	GMC <sup>b</sup> of Abs	9 <sup>c</sup> 4.5		23 15	20 <sup>c</sup> 6	6 5	129 <sup>c</sup> 4		Significantly greater response to PCV for 3 of the 5 serotypes
Powers et al., 1996 (26)	5-valent PCV with CRM <sub>197</sub>	50–85	23 (PCV) 23 (PPSV)	% of subjects $\geq$ 2-fold increase in Ab levels	70 61		70 70	96 87	39 70	83 70		No difference between groups
Shelly et al., 1997 (30)	5-valent PCV with CRM <sub>197</sub>	18–45	25 (PCV) 25 (PPSV)	GMC of Abs	10.9 <sup>c</sup> 3.7		17.3 12.6					In nonelderly adults, significantly greater response to PCV for 1 of the 2 serotypes evaluated; in seniors, no difference between groups
			60–78		3.2 4.4		8.8 18.2					
Storek et al., 1997 (35)	7-valent PCV with OMPC	18–50	10 (PCV) 19 (PPSV)	Median value	22.9 17.4	5.7 3.4	22.0 15.2	11.4 9.3	32.7 9.7	11.5 9.4	16.7 12.1	No difference between groups
Nieminen et al., 1998 (25)	4-valent PCV with OMPC	23–56	10 (PCV) 8 (PPSV)	GMC of Abs	2.5 0.9		2.6 3.9		4.2 14.8	2.1 3.5		No difference between groups
Kroon et al., 2000 (17)	4-valent PCV with OMPC	26–47	9 (PCV) 10 (PPSV) <sup>d</sup>	GMC <sup>b</sup> of Abs	1.6 5		9 5		4 12	2.5 4		No difference between groups
Wuorimaa et al., 2001 (37)	11-valent PCV with mixed DT and TT	22–35	10 (PCV) –alum 10 (PCV) +alum 10 (PPSV)	GMC of Abs	2.7 2.4 1.9	7.1 15.5 3.7	3.5 1.6	4.4 25.3	8.1 7.6	11.9 8.9	5.2 4.3	No difference between PCV and PPSV groups
Jonsson et al., 2002 (15)	Serotype 6B PCV with TT	55–75 (subjects had COPD) <sup>e</sup>	10 (PCV) 9 (PPSV)	GMC of Abs	4.7 5.7							No difference between groups
Kamboj et al., 2003 (16)	7-valent PCV with CRM <sub>197</sub>	22–35	15 (PCV) 9 (PPSV)	GMC of Abs	5.2 7.2	14.8 11.7	5.6 9.3	11.9 12.8	17.9 10.6	16.0 15.1	17.5 6.1	No difference between groups
Scott et al., 2007 (27a)	13-valent PCV with CRM <sub>197</sub>	18–49	15 (PCV) 15 (PPSV)	GMC of Abs	2.2 2.3	24.5 <sup>c</sup> 7.3	5.1 3.6	12.9 11.4	17.8 <sup>c</sup> 2.9	14.4 4.3	17.4 <sup>c</sup> 2.5	Significantly greater response to PCV for 3 serotypes

<sup>a</sup>TT, tetanus toxoid; DT, diphtheria toxoid; Ab, antibody.

<sup>b</sup>Values are approximate and were estimated based on a graphical presentation of the data in the original publication.

<sup>c</sup>Value for PCV group was significantly different ( $P < 0.05$ ) from value for PPSV group.

<sup>d</sup>Historical control group.

<sup>e</sup>COPD, subjects had chronic obstructive pulmonary disease.

Table 3 Results of dose-ranging studies of PCV7-CRM (Prevnar) comparing the ELISA-measured IgG responses to various PCV dosages with the response to PPSV in immunocompetent elderly adults

Study(ies)	Range of subject ages (yrs)	Prior PPSV exposure	Vaccine and dose <sup>a</sup>	No. of subjects	Postvaccination GMC (µg/ml) of antibodies specific to serotype:					Summary	
					4	6B	9V	14	18C		
de Roux et al., 2007, and Lode et al., 2004 (7, 20)	≥70	None	None (baseline)	413	0.2	1.0	0.9	1.9	1.1	1.2	1.0 Dose response to PCV.
			PCV, 1×	110	3.1 <sup>b</sup>	8.0 <sup>b</sup>	9.8 <sup>b</sup>	17.1 <sup>b</sup>	13.0 <sup>b</sup>	5.5	12.4 <sup>b</sup> Significantly greater response to PCV at 1× than to PPSV for 6 of 7 serotypes
			PCV, 2×	107	5.0 <sup>b</sup>	12.9	8.6 <sup>b</sup>	20.6 <sup>b</sup>	14.7 <sup>b</sup>	15.5	10.3 <sup>b</sup>
			PCV, 4×	107	6.1 <sup>b</sup>	14.9	12.2 <sup>b</sup>	26.2 <sup>b</sup>	23.4 <sup>b</sup>	8.1 <sup>b</sup>	14.2 <sup>b</sup>
			PPSV	109	1.4	4.4	3.6	8.5	6.8	4.4	3.8
Jackson et al., 2007 (12)	70–79	One prior PPSV <sup>c</sup>	PCV, 0.2×	44	1.1	1.7	2.6	4.6	4.2	3.4	2.2 Dose response to PCV.
			PCV, 1×	44	1.5	2.6	3.2	8.8	5.0	3.4	3.4 Significantly greater response to PCV at 2× than to PPSV for 4 of 7 serotypes
			PCV, 2×	44	2.8 <sup>b</sup>	3.8	5.3 <sup>b</sup>	7.3	8.8 <sup>b</sup>	6.0	5.9 <sup>b</sup>
			PCV, 4×	44	2.8 <sup>b</sup>	4.7	6.2 <sup>b</sup>	8.9	8.7 <sup>b</sup>	5.6	7.2 <sup>b</sup>
			PPSV	44	1.1	2.7	2.1	7.0	4.5	4.3	2.3

<sup>a</sup>All studies evaluated a dose of 23-valent PPSV of 0.5 ml (standard dose), 1×, standard pediatric dose of PCV7-CRM with 2 µg of each polysaccharide except 6B (4 µg); 0.2×, one-fifth the standard pediatric dose of PCV7-CRM with 0.4 µg of each polysaccharide except 6B (0.8 µg); 2×, twice the standard pediatric dose of PCV7-CRM with 4 µg of each polysaccharide except 6B (8 µg); 4×, four times standard pediatric dose of PCV7-CRM with 8 µg of each polysaccharide except 6B (16 µg).

<sup>b</sup>GMC differed significantly ( $P < 0.05$ ) from that for PPSV group.

<sup>c</sup>Participants received one prior PPSV on or after their 65th birthday and at least 5 years prior to enrollment.

50 years of age living in the United States were randomized to receive 13-valent PCV or PPSV subcutaneously. This trial was undertaken in anticipation of later trials to be conducted in Japan, where subcutaneous administration is the standard route.

In this last study, the immune response to the 13-valent-vaccine antigens was robust and postvaccination GMCs in the 13-valent-PCV group tended to be higher than those in the PPSV group.

### Dose-Ranging Studies

The results of two large dose-ranging studies of PCV7-CRM (Prevnar) in seniors have been recently reported (Table 3) (7, 12, 20). Both of those studies used a third-generation ELISA protocol that included double absorption with cell wall polysaccharide and type 22F polysaccharide to remove non-capsule-specific antibodies. The study by Jackson and colleagues included elderly adults 70 to 79 years of age who had received one dose of the 23-valent PPSV on or after their 65th birthday and at least 5 years prior to enrollment in the study (12). At enrollment, participants received either one of four volumes (0.1, 0.5, 1, and 2 ml) of the PCV7-CRM (licensed dose, 0.5 ml) or the licensed 0.5-ml dose of 23-valent PPSV. There were no significant differences in GMCs of antibodies between participants given the standard 0.5-ml pediatric dose of PCV7-CRM and the group given PPSV. However, the group given a 1-ml volume of PCV7-CRM had significantly higher GMCs of antibodies to four of the seven vaccine serotypes than the PPSV group, which is consistent with a dose response. There was little additional further increase in GMCs in the group that received 2 ml of PCV7-CRM.

The study by de Roux and colleagues assessed PPSV-naïve seniors 70 years of age and older (7, 20). At enrollment, participants received one of three PCV dose formulations or the licensed 0.5-ml dose of the 23-valent PPSV. Among the three PCV groups, the levels of the antigens from the seven serotypes in the PCV7-CRM were varied by reconstituting an investigational lyophilized nine-valent CRM PCV (PCV9-CRM) with the liquid PCV7-CRM (Prevnar). Participants assigned to receive PCV received either 0.5 ml of PCV7-CRM alone (standard-dose group), 0.5 ml of PCV7-CRM plus one dose of PCV9-CRM (a twofold vaccine dose and a standard AlPO<sub>4</sub> dose), or 1 ml of PCV7-CRM plus two doses of PCV9-CRM (a fourfold vaccine dose and a twofold AlPO<sub>4</sub> dose). As in the study by Jackson and colleagues, in this study a dose response to PCV was observed. Unlike the results of the study of previously vaccinated seniors, however, in this study of unimmunized

**Table 4** Results of dose-ranging studies of PCV7-CRM (Prevnar) comparing the OPA-measured responses to various PCV dosages with the response to PPSV in immunocompetent elderly adults

Study(ies)	Prior PPSV exposure	Vaccine and dose <sup>a</sup>	No. of subjects	Postvaccination OPA-measured GMT of antibodies specific to serotype <sup>b</sup> :					Summary	
				4	6B	9V	14	18C		
de Roux et al., 2007, and Lode et al., 2004 (7, 20)	None	None (baseline) <sup>d</sup>	413	14	62	144	96	44	15	47
	PCV, 1×	110	1,457*	1,351	2,915*	2,397*	1,318*	182	1,309*	Significantly greater response to PCV at 1× than to PPSV for 4 of 7 serotypes
	PCV, 2×	107	1,505*	2,536	3,511*	2,547*	1,711*	148	1,206*	Significantly greater response to PCV at 1× than to PPSV for 4 of 7 serotypes
	PCV, 4×	107	2,022*	3,914*	5,239*	3,433*	2,172*	359	1,930*	Significantly greater response to PCV at 1× than to PPSV for 4 of 7 serotypes
	PPSV	109	663	809	985	1,011	465	203	302	
										Dose response to PCV.
Jackson et al., 2007 (12)	One prior PPSV <sup>c</sup>	PCV, 0.2×	44	199	52	74	677	388	148	566
		PCV, 1×	44	1,701	419	306*	1,262	681	220	4,501*
		PCV, 2×	44	3,765*	889*	1,259*	966	2,299*	396	2,298*
		PCV, 4×	44	3,063	625*	720*	1,371	1,697*	338	4,624*
		PPSV	44	983	119	46	637	525	330	456
										Values are pooled prevaccination levels.

<sup>a</sup>All studies evaluated a 23-valent PPSV dose of 0.5 ml (standard dose). 1×, standard pediatric dose of PCV7-CRM with 2 µg of each polysaccharide except 6B (4 µg); 0.2×, one-fifth the standard pediatric dose of PCV7-CRM with 0.4 µg of each polysaccharide except 6B (0.8 µg); 2×, twice the standard pediatric dose of PCV7-CRM with 4 µg of each polysaccharide except 6B (8 µg); 4×, four times the standard pediatric dose of PCV7-CRM with 8 µg of each polysaccharide except 6B (16 µg).

<sup>b</sup>Asterisks indicate GMTs significantly higher ( $P < 0.05$ ) than those for PPSV group.

<sup>c</sup>Participants received one prior PPSV on or after their 65th birthday and at least 5 years prior to enrollment.

seniors, the GMCs were significantly higher even in the standard-PCV-dose group than those in the PPSV group for six of the seven vaccine serotypes. Also, the twofold and fourfold doses induced progressively higher levels of antibodies to most serotypes than the standard dose.

### Functional Immune Response Assessed by OPAs

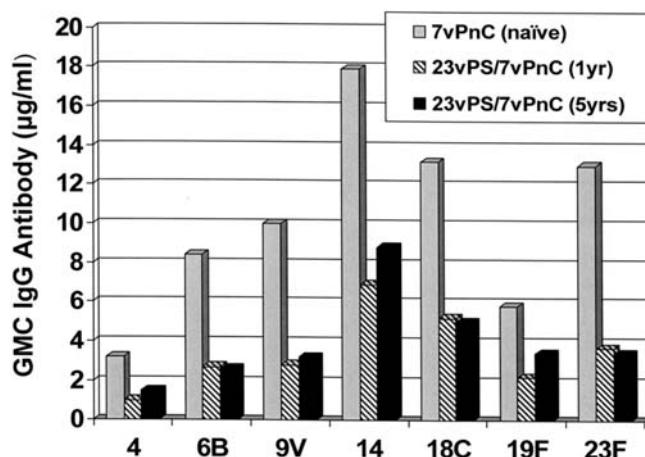
The two dose-ranging studies of PCV7-CRM also assessed the OPA-measured responses to the various doses of PCV and to the PPSV given at enrollment. In both studies, PCV7-CRM and PPSV induced a functional antibody response, and OPA-determined geometric mean titers (GMTs) tended to be higher in the higher-dose PCV groups (Table 4). In general, the patterns of OPA response were similar to the patterns of response measured by IgG ELISA. However, the OPA GMTs from the two studies cannot be compared directly because the OPA has not been standardized among different laboratories.

Similarly, in the published phase 1 trial of a 13-valent PCV-CRM vaccine, OPA GMTs tended to be higher following PCV compared to PPSV (27a).

### Hyporesponsiveness to PCV Induced by Prior PPSV Vaccination

In the study by de Roux and colleagues, participants randomized to receive PPSV at enrollment were given 0.5 ml of PCV7-CRM 1 year later. This method allowed a comparison of the responses to PCV7-CRM between the pneumococcal vaccine-naïve adults given PCV7-CRM at enrollment and the study participants given PCV7-CRM 1 year after PPSV. The ELISA-determined IgG GMCs following the administration of PCV7-CRM were substantially lower in the group that received PCV7-CRM 1 year after PPSV than in the group that received PCV7-CRM at enrollment (Fig. 1). The differences in the post-PCV7-CRM GMCs between the PPSV-immunized and PPSV-naïve groups were statistically significant for all seven serotypes. These results indicate that the administration of PPSV induced hyporesponsiveness to PCV given 1 year later.

In the study by Jackson and colleagues, various dosages of PCV7-CRM were administered at enrollment to seniors who had previously received PPSV at least 5 years prior to enrollment. In that study, the GMCs in response to the standard 0.5-ml dose of PCV7-CRM were similar to the ELISA-measured GMCs determined in the de Roux et al. study for the group given PCV7-CRM 1 year after PPSV (Fig. 1). The two studies were conducted among different populations and used different



**Figure 1** ELISA-measured GMC of IgG antibodies following administration of a 0.5-ml dose of PCV7-CRM (7vPnC) to three groups of elderly adults defined by their previous exposure to PPSV (23vPS). The first group consisted of 110 pneumococcal-vaccine-naïve adults enrolled in the study by de Roux (7), the second group consisted of 78 participants in the de Roux study who received the PCV 1 year after the PPSV, and the third group consisted of 44 adults in the study by Jackson et al. who received the PCV 5 or more years after the PPSV (12). The GMCs following the PCV dose tended to be lower in the groups previously exposed to PPSV than in the pneumococcal-vaccine-naïve group.

laboratories for the ELISA, which have been standardized to give similar results in different laboratories. The GMCs are strikingly similar, suggesting that the administration of PPSV may induce hyporesponsiveness to PCV which persists for at least 5 years.

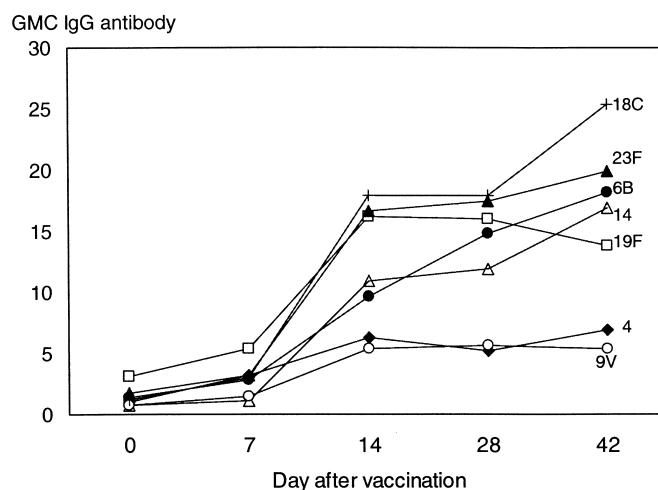
### Variation in Antibody Response by Serotype

The magnitude of the serotype-specific IgG response, as well as the IgA and IgM responses, to PCVs varies by serotype (16) and by vaccine formulation. Although there is variation in the relative ranking of the magnitudes of the responses to various serotypes across studies, in general, the IgG response to serotype 14 tends to be relatively strong while the response to serotype 4 tends to be relatively weak. The reason for these differences is not known, but they do not seem to be due to serotype-specific alterations of the carrier protein due to the conjugation processes for different capsular polysaccharide conjugates, as one study demonstrated that all of the individual components of the seven-valent CRM vaccine induce vigorous CD4<sup>+</sup>-T-cell proliferative responses, that do not vary significantly by serotype (16).

### Kinetics of Antibody Response

One study measured IgG, IgA, and IgM antibody levels by ELISA at intervals of 7, 14, 28, and 42 days after vaccination with PCV7-CRM in 15 pneumococcal vaccine-naïve healthy participants (Fig. 2) (16). In this study, the greatest increase in IgG antibody levels occurred between days 7 and 14. For some serotypes, there was little additional increase after day 14, while for others, there was a continual increase in antibody levels in subsequent samples. For IgM and IgA antibodies, the greatest increase also took place between days 7 and 14, although those antibody levels then tended to decline over subsequent time periods (data not shown). Another study of a mixed diphtheria and tetanus toxoid conjugate vaccine found the highest IgG GMCs on day 14, with no additional increase observed at day 28 (37), but responses were not assessed before 14 days postvaccination in this study.

A study of responses to another CRM conjugate, *Haemophilus influenzae* type b oligosaccharide-CRM<sub>197</sub> (HibTITER), in adults showed similar antibody kinetics, with significant increases by day 7 but the peak responses at day 14 (22). Interestingly, in that study, toddlers and infants achieved peak responses to the vaccine earlier, at day 7. A recent study of responses to a meningococcal group C CRM conjugate vaccine (Meningitec) in adolescents (mean age, 14 years) also showed a rapid peak by day 7 (33). Since delayed antibody responses may be associated with greater risk of invasive infection, the possibility that adults, and particularly the elderly,



**Figure 2** ELISA-measured IgG response to PCV7-CRM at days 7, 14, 28, and 42 in 15 healthy young adults. Adapted from the *Journal of Infectious Diseases* (16) with permission from the publisher.

have slower responses than children deserves further evaluation.

### IgG Subclass Response

In adults, PCVs appear to induce primarily an IgG2 response, as is also seen with PPSV vaccination, which contrasts with the predominantly IgG1 response to conjugate vaccines usually found in children (21, 30, 37). One study has, however, reported that a *Neisseria meningitidis* outer membrane protein complex (OMPC) PCV (PCV-OMPC) induced a greater IgG1 antibody response than PPSV in adults (34).

### Immune Response to a Second Compared with a First Dose of Conjugate Vaccine

Two studies have assessed the response to a second dose of PCV (Table 5).

Kroon et al. evaluated the administration of an OMPC PCV that was “field formulated” by putting together 2 µg each of 6B and 14 polysaccharides in one syringe and 2 µg each of 19F and 23F polysaccharides in another, which were then injected separately (17). In that study, the administration of a second dose of those formulations 30 days after the first resulted in little additional increase in levels of antibodies to serotypes 6B, 19F, and 23F and a modest increase in levels of antibodies to serotype 14 in the nine healthy adults evaluated.

In the dose-ranging study of PCV7-CRM in PPSV-naïve seniors by de Roux and colleagues, 43 participants who received 0.5 ml of PCV7-CRM at enrollment received a second dose of 0.5 ml of PCV7-CRM 1 year later. Antibody levels declined during the 12-month interval between the first and second vaccination but then rose in response to the second PCV7-CRM vaccination. The postvaccination ELISA-measured GMCs and OPA-measured GMTs were generally similar after the first and second doses (Table 5). For types 19F and 23F, OPA titers were significantly higher after the second PCV dose, but for type 18C they were lower in a paired analysis.

### Response to Polysaccharide Vaccine Challenge

Five studies of PCV in immunocompetent adults have assessed the immune response to PPSV given after PCV in order to determine whether exposure to PCV induces an enhanced response to PPSV (Table 6 and Fig. 3). If such a “booster” response were identified, this could be considered evidence supporting an effect of PCV vaccination in priming B cells for a memory immunologic response to subsequent exposure to capsular polysaccharide antigen. It has been suggested that the immune response to polysaccharide vaccines serves as a surro-

gate for the host’s ability to respond to natural exposures.

For the purposes of this chapter, we will define a booster response simply as one involving significantly higher antibody levels in PCV-primed individuals than in unprimed individuals following the administration of the standard licensed dose of PPSV.

Only two studies may have showed a booster response according to this definition. The larger study of vaccine-naïve elderly adults by de Roux et al. showed that the ELISA-determined IgG GMCs after PPSV were higher for six of seven types in PCV-primed individuals than in unprimed individuals, but these differences were not statistically significant. However, OPAs of all samples were also performed. The GMTs following the administration of PPSV 1 year after that of PCV were consistently higher than the GMTs after the administration of PPSV at enrollment, and these differences were statistically significant for serotypes 4, 9V, 14, and 23F. Booster antibody responses assessed as increases in ELISA-measured concentrations post-PPSV administration relative to prevaccination concentrations were lower in the PCV-exposed group, presumably because these subjects still had residual antibody levels from their priming dose of PCV a year earlier.

In a small group of nine nonelderly adults, Kroon observed higher GMCs following a dose of PPSV given 8 months after two doses of a four-valent PCV-OMPC than in unprimed historical controls, but the difference was significant for only one type (14) (Table 6). The increases in antibody levels were similar in the two groups because pre-booster dose antibody levels were higher in the PCV-primed group.

In the dose-ranging study by Jackson and colleagues, all participants received a low-dose (0.1-ml) PPSV challenge 1 year after enrollment (Table 6 shows only the response to the low-dose challenge among participants who received 0.5 ml of PCV7-CRM at enrollment). In that study population of elderly adults who had received PPSV 5 or more years prior to enrollment, the administration of PCV7-CRM did not prime for an enhanced response to the low-dose PPSV challenge. Booster responses according to our definition cannot be assessed in the Jackson study because only one-fifth the standard dose of PPSV was used for the challenge.

The other two studies failed to show enhanced antibody responses to PPSV after conjugate vaccination in adults, but the numbers of subjects were small and the intervals between immunizations were short (2 to 6 months). In the study by Shelly et al., participants who received PPSV 2 months after a single dose of PCV had little response to PPSV (Table 6) (30). Similarly, in the

**Table 5** Antibody response in immunocompetent adults as measured by ELISA and OPA following first and second doses of PCV

Study	Range of subject ages (yrs)	PCV formulation	Interval between PCV doses	No. of samples	Samples tested and method or calculation	GMC (ELISA; µg/ml) or GMT (OPA) of antibodies to or ratio (95% CI) of results for serotype:						
						4	6B	9V	14	18C	19F	23F
Kroon et al., 2000 (17)	26–47	4-valent PCV with OMPC	30 days	9	Post-dose 1, <sup>a</sup> ELISA		1.6		9		4	2.5
					Post-dose 2, <sup>a</sup> ELISA		1.8		13		6	3.0
de Roux et al., 2007 (7)	≥70	PCV7-CRM	1 yr	43 <sup>b</sup>	Post-dose 1, ELISA	3.3	9.5	8.4	18.0	12.4	4.1	12.6
					Post-dose 2, ELISA	3.1	10.0	5.7	16.5	8.6	5.4	15.2
				86	Ratio (and 95% CI) of ELISA results for post-dose 2 and post-dose 1 samples determined	1.0 (0.7–1.3)	1.1 (0.7–1.6)	0.7 (0.5–0.8)	0.9 (0.7–1.3)	0.7 (0.6–0.9)	1.3 (0.9–1.9)	1.2 (0.9–1.7)
					43	Post-dose 1, OPA	1,350	1,560	2,690	3,110	1,510	139
				43	Post-dose 2, OPA	1,390	2,180	2,570	2,830	900	264	4,100
					86	Ratio (and 95% CI) of OPA results for post-dose 2 and post-dose 1 samples determined	1.0 (0.6–1.6)	1.4 (0.9–2.3)	1.0 (0.6–1.5)	0.6 (0.6–1.4)	1.9 (0.4–0.9)	2.7 (1.2–3.1)

<sup>a</sup>Values are approximate and were estimated based on a graphical presentation of the data in the original publication.

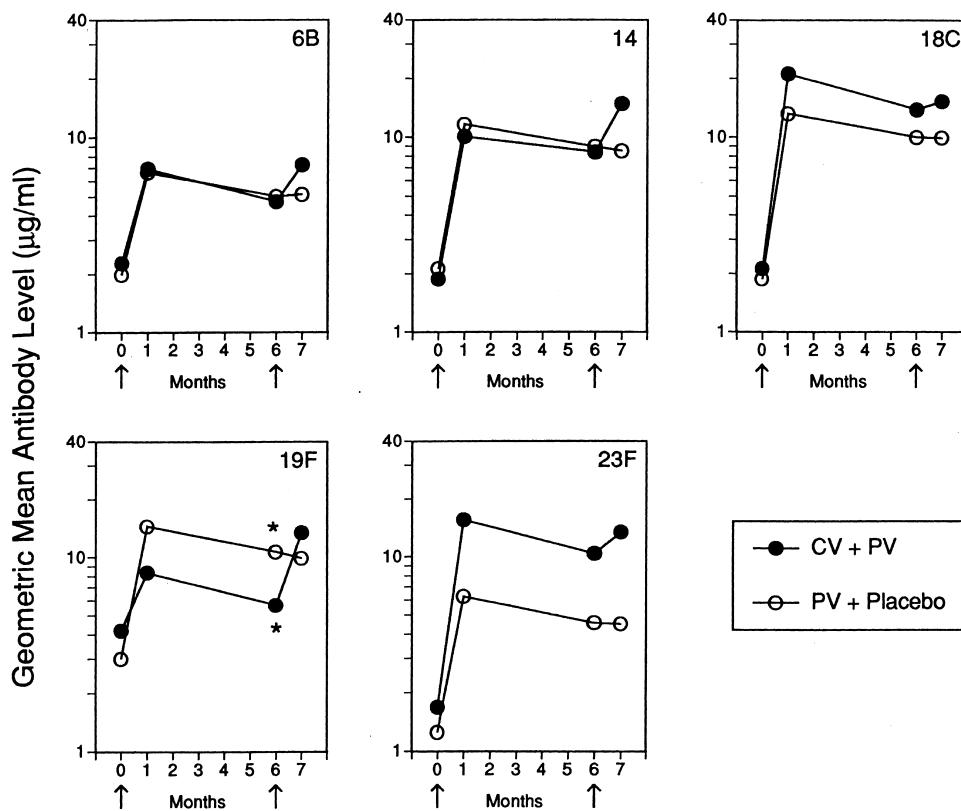
<sup>b</sup>Samples from 43 patients who completed both dose 1 and dose 2 were subjected to a matched analysis.

**Table 6** ELISA-measured IgG response to PPSV challenge given at various intervals after PCV compared with the response to PPSV alone

Study	Range of subject ages (yrs)	PCV	Interval between PCV and PPSV	Measure of vaccine response	Treatment regimen for group	Specimen tested	Result (GMC [ $\mu$ g/ml], % of subjects, or increase [n-fold]) for serotype:							Summary
							4	6B	9V	14	18C	19F	23F	
de Roux et al., 2007 (7)	$\geq 70$	7-valent PCV with CRM <sup>a</sup>	1 yr	GMC of antibodies	PPSV after PCV	Pre-PPSV	0.7	2.0	3.9	7.8	5.0	2.8	6.1	Pre- and post-PPSV GMCs tended to be higher in the group given PPSV 1 yr after PCV than in the group given PPSV alone. Geometric mean increases in post- vs pre-PPSV levels were higher in the PPSV-alone group than in the group given PPSV after PCV
					PPSV	Post-PPSV	1.5	5.0	6.1	15.0	7.1	8.0	7.5	
					alone	Pre-PPSV	0.2	1.1	0.9	1.7	1.2	1.2	0.9	
					PPSV	Post-PPSV	1.5	4.5	3.5	8.6	6.8	4.6	3.7	
				Geometric mean increase (n-fold) in antibody levels post-PPSV compared to pre-PPSV levels	PPSV after PCV		2.3	2.5	1.6	1.9	1.4	2.9	1.2	
					PPSV alone		8.3	4.2	4.0	5.1	5.6	3.7	4.0	
Jackson et al., 2007 (12)	70–79	7-valent PCV with CRM <sup>a</sup>	1 yr	GMC of antibodies	PPSV after PCV	Pre-PPSV	0.8	1.2	1.8	6.5	3.3	2.0	1.5	Little response to a reduced (0.1-ml) dose of PPSV given 1 yr after PCV
					PPSV	Post-PPSV	1.1	1.7	2.2	7.7	4.0	3.0	1.9	
					PPSV alone	Pre-PPSV	0.5	1.4	1.2	4.3	2.5	2.9	1.2	
					PPSV	Post-PPSV	1.1	2.7	2.1	7.0	4.5	4.3	2.3	
				% of subjects with $\geq 2$ -fold increase in antibody levels	PPSV after PCV		14	14	7	7	2	14	5	
					PPSV alone		37	27	22	25	40	15	32	

Shelly et al., 1997 (30)	18–45	5-valent PCV with CRM	2 mo	% of subjects with ≥2-fold increase in antibody levels	PPSV after PCV	0	4		Little response to PPSV given 2 mos after PCV
	60–78			Mean in- crease ( <i>n</i> - fold) in antibody levels	PPSV after PCV	1.2	1.2		
				% of subjects with ≥2-fold increase in antibody levels	PPSV after PCV	0	4		
Kroon et al., 2000 (17)	<50	4-valent PCV with OMPC	8 mo	Mean in- crease ( <i>n</i> - fold) in antibody levels	PPSV after PCV	3.9	3.3	3.8	2.6
				GMC of antibodies	PPSV alone	3.7	4.5	3.7	4.2
					PPSV after PCV	1.4	8	6	4
					Post-PPSV	6	27*	23	11
					PPSV alone	1.2	2	3	1
					Pre-PPSV	5	5	12	4
					Post-PPSV				

\*Standard 0.5-ml pediatric dose of PCV7-CRM.



**Figure 3** Serum IgG antibody responses to capsular types 6B, 14, 18C, 19F, and 23F in older adults after immunization with an investigational 5-valent oligosaccharide CRM PCV (CV) or the 23-valent PPSV (PV). Arrows indicate times of first and second vaccine injections. Closed circles, subjects immunized with the PCV at month 0 and the PPSV at month 6; open circles, subjects immunized with the PPSV at month 0 and a placebo at month 6; asterisks, significant difference in GMC between vaccine groups ( $P < 0.05$ ). Reprinted from the *Journal of Infectious Diseases* (26) with permission from the publisher.

study by Powers et al. (26), the ELISA-measured IgG GMCs in the study group given PPSV 6 months after PCV ( $n = 23$ ) were similar to the GMCs in the group given PPSV at enrollment (Fig. 3).

Further studies of elderly patients using larger numbers of subjects and intervals of 12 months or longer between PCV vaccination and subsequent polysaccharide challenge are needed to verify whether PCV can indeed enhance the response to subsequent PPSV. PPSV-naïve patients may be more likely to demonstrate such enhanced responses than persons previously immunized with PPSV.

However, it should be noted that it is uncertain whether the methods that have been used to assess the response to a polysaccharide challenge, such as the dose of polysaccharide administered and the interval between PCV vaccination and polysaccharide challenge, are optimal for the identification of a memory response.

In addition, perhaps most importantly, it is not clear that the administration of a parenteral polysaccharide challenge is an appropriate method for demonstrating the presence or absence of immune memory in adults. Unlike young children, even unvaccinated adults have been exposed to capsular polysaccharide antigen through carriage and tend to have relatively high baseline levels of serotype-specific antibody. Adults are also not naïve relative to the carrier proteins (tetanus toxoid, diphtheria toxoid, CRM, and OMPC) used for PCV.

Our understanding of the immune responses to PCV and PPSV would be facilitated by the development and/or the application of standardized quantitative assays for the detection of polysaccharide-specific memory B cells and carrier-specific T cells and further analysis of the affinity, opsonic function, and clonotypic diversity of the induced antibody responses in the elderly. The application of these techniques may allow us

**Table 7** Prevalence of fever and local signs and symptoms in elderly subjects following administration of PCV7-CRM<sup>a</sup> compared to those following administration of 23-PPSV

Study	History of pneumococcal vaccination	Study vaccine	% Prevalence of:			
			Fever (temp, $\geq 38^{\circ}\text{C}$ )	Pain in vaccinated arm	Redness in vaccinated arm	Swelling in vaccinated arm
Jackson et al. (13)	One prior PPSV vaccination	PCV	0	34	16	9
		PPSV	2	68**	34	45**
de Roux et al. (7)	No prior PPSV vaccination	PCV	1	38	34	20
		PPSV	3	25	23	17

<sup>a</sup>Standard pediatric dose and volume including 2 µg of each capsular polysaccharide except 6B (4 µg). \*\*,  $P < 0.05$  for comparison of PCV and PPSV groups.

to understand the basis of hyporesponsiveness induced by repeated polysaccharide exposures, whether conjugate vaccines preserve responsiveness or reverse hyporesponsiveness, and differences in immune responses to different serotypes and to different vaccine formulations.

## SAFETY

PCVs are well tolerated in immunocompetent adults (13, 17, 27a, 30, 37). As with PPSVs, fever is rare following PCV vaccination in adults. Local reactions are the most commonly reported adverse events but are usually mild and are self-limited. In previously pneumococcal vaccine-naïve adults, local reactions following the administration of PCV are comparable to or somewhat more frequent than those following the administration of PPSV (26, 30, 37). In adults, the frequency of local reactions is higher after a second dose of PPSV than after a first dose (11), and in the dose-ranging study of seniors who had received PPSV prior to study enrollment, local reactions tend to occur more frequently in the group given PPSV at enrollment than in the group given PCV at enrollment (Table 7) (13).

## SUMMARY

The experience with PCV in adults as described in published reports is limited, and conclusions about the immunogenicity and safety of PCVs must be viewed as preliminary and subject to confirmation in larger, well-controlled studies.

The studies suggest that

1. In PPSV-naïve elderly adults, the licensed pediatric dose of PCV7-CRM induces higher levels of antibodies, as measured by ELISA and OPA, to most serotypes than PPSV (7, 20).

2. In contrast, adults who have received a prior dose of PPSV show little difference in immune responses to the licensed pediatric dose of PCV7-CRM and a second dose of PPSV, but a twofold dose of PCV7-CRM induces higher antibody responses than PPSV (12).
3. Prior PPSV immunization significantly reduces the response to PCV as measured by ELISA and OPA (7).
4. Prior exposure to PCV does not suppress the response to a second dose of PCV (7), which may allow for repeated dosages of PCV.

If conclusions 3 and 4 are confirmed in larger studies, and if PCVs ultimately are approved for adult use, then PCV should be used prior to PPSV for optimal antibody responses.

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Neil French  
Sharon Nachman  
Stephen I. Pelton

18

## Immunogenicity in High-Risk and Immunocompromised Children and Adults

The list of immunocompromising and other conditions that put individuals at high risk for pneumococcal disease is long and applies to a diverse population of adults and children with a spectrum of disease processes and immune defects that predispose to pneumococcal disease (Table 1). The nature of these immune defects is often poorly defined, and they are superimposed upon the normal maturing and aging processes of the immune and physiological systems. They do, however, define important target groups for pneumococcal disease protection.

As many as a quarter of children with invasive pneumococcal disease (IPD) in the developed world may have a significant underlying medical condition (74). The figures for the developing world are unknown. Moreover, these children with immunocompromising conditions suffer from IPD more frequently and with greater mortality than healthy children. In adults, immunocompromise and high-risk conditions account for nearly two-thirds of the total invasive pneumococcal disease burden in the United States (51). In the develop-

ing world, human immunodeficiency virus (HIV) is a major contributor to adult pneumococcal disease, while the contributions of diabetes, chronic cardiopulmonary disease, and the other high-risk conditions are unknown but likely to be significant and increasing.

The 23-valent pneumococcal polysaccharide vaccine (PPSV23) is recommended for use in most of these conditions, but the efficacy of this vaccine in these groups is inconclusive. Pneumococcal conjugate vaccines (PCVs) offer an alternative approach to protection. With the exception of HIV-infected children (43), there are no clinical efficacy data for PCV in these high-risk groups, and appropriately conducted studies of efficacy are needed. The assessment of vaccine efficacy for many of the less frequent immunocompromising conditions in conventional randomized controlled trials with clinical end points is impractical, and thus, immunogenicity studies combined with case control or post-marketing surveillance studies are the most practical way to assess vaccine effectiveness.

Neil French, Infectious Disease Epidemiology Unit, London School of Hygiene and Tropical Medicine, and Karonga Prevention Study, Box 46, Chilumba, Malawi. Sharon Nachman, Department of Pediatrics, SUNY Health Science Center at Stony Brook, Stony Brook, NY 11794-8111. Stephen I. Pelton, Pediatrics and Epidemiology, Boston University Schools of Medicine and Public Health, Maxwell Finland Laboratory for Infectious Diseases, Boston, MA 02118.

**Table 1** Conditions that predispose to pneumococcal infection and mechanisms of susceptibility

Condition	Predisposes through:					Comment	Evaluated in PCV immunogenicity studies
	Defective antibody	Reduced mucosal clearance	Anatomical defects	Complement deficiency	Phagocyte dysfunction		
HIV infection	X	X				Debility interferes with respiratory secretion clearance in late stages	Yes
SCD	X			X	X		Yes
Alcoholism		X			X		No
Transplantation	X				X	Broad disruption of humoral, cellular, and phagocyte immune function	Yes
Chronic heart disease		X					No
Chronic chest disease		X	X		X	Condition includes tuberculosis, asthma, bronchiectasis, and COPD	No
Diabetes					X		No
Chronic renal disease	X			X			Yes
Liver cirrhosis			X		X		No
Malignant disease	X	X	X				
Immunosuppressive therapy	X				X	Similar to transplantation but complicated by underlying disease, e.g., SLE <sup>a</sup>	No
Congenital immunodeficiency(ies)	X				X		No
Belonging to indigenous aboriginal population						Ill-defined reasons for susceptibility	Yes
Cerebrospinal fluid leak			X				No
Cochlear implantation			X				Yes
Asplenia (functional and anatomic)	X			X			Yes

<sup>a</sup>SLE, systemic lupus erythematosus.

## MEASURING IMMUNOGENICITY

Demonstrable immunogenicity to PCV is a requirement for clinical efficacy. However, it is unclear what surrogate *in vitro* measures are best correlated with protection in high-risk populations. The enzyme immunoassay is a robust tool for measuring immunogenicity post-PCV in healthy children (chapter 14). However, it lacks specificity in older adults (14) and in HIV-infected adults (19, 25). Modifications to the assay by the inclusion of a 22F capsular polysaccharide adsorption step improve specificity but have not as yet improved the

predictive value of this assay in adults. Similarly qualitative measures of antipneumococcal immunity are believed to be more specific as predictors of protection (chapter 15). However, a lack of sensitivity may limit their value.

Concentrations of serotype-specific anti-capsular polysaccharide immunoglobulin G (IgG; anti-PPS), measured using a standardized enzyme immunoassay, which have protective predictive value in healthy infants have been defined (78). Whether these same levels of antibody can be applied to immunocompromised children is

uncertain, and there is good evidence that these levels are not predictive in adult groups (25, 63).

HIV-infected children are the only immunocompromised group for which we have randomized clinical trial data confirming efficacy (43). Serological measures from this group parallel the clinical findings (53), although higher concentrations of antibody are necessary to achieve comparable functional activity in the HIV-infected children than in uninfected children (54). This provides some confidence that serological responses in HIV-infected children and probably other immunocompromised pediatric groups are proportional to, if not directly predictive of, clinical efficacy.

## HIV INFECTION

HIV-infected adults and children are between 20 and 100 times more likely to suffer IPD than their age-matched uninfected counterparts. In Africa, rates of pneumococcal disease in HIV-infected children are 40-fold higher than those seen in the developed world. HIV has massively increased the burden of pneumococcal disease, and HIV is estimated to be the attributable cause of at least half of all serious pneumococcal infections and pneumonia cases in the adult population in this region (22, 42). Highly active antiretroviral therapy (HAART) has been associated with a 60% decrease in IPD in the United States, but the risk of disease in HIV-infected individuals remains in the order of 30 times greater than that in HIV-uninfected persons (32). Moreover, relative to other HIV-associated infections, bacterial pneumonia and infection with *Streptococcus pneumoniae* has increased in importance as the other classical opportunistic infections have declined. The high rates of IPD have led to the evaluation of vaccines for the prevention of IPD and pneumococcal pneumonia in HIV-infected adults and children.

The only prospective randomized trial of PPSV23 failed to show benefits of vaccination in a cohort of HIV-infected Ugandan adults; indeed, rates of pneumonia and pneumococcal disease increased in the vaccine recipients (26). In a U.S. retrospective case-control study evaluating the effectiveness of PPSV23 against IPD among HIV-infected adults (11), the effectiveness was 49% overall (95% confidence interval, 12 to 70%), although in a stratified analysis, there was no benefit in African-Americans, with an effectiveness of 24% (95% confidence interval, -50 to 61%). Several immunogenicity studies with PPSV23 have been conducted, and all tend to show significant responses to vaccination but reduced responses compared to those in HIV-uninfected controls (13, 23, 33, 46, 88). The use of antiretroviral

therapy (ART) has not been demonstrated to change the quantitative response to PPSV23 (72, 84).

In HIV-infected children in the pre-HAART era, PPSV23 was demonstrated to have poor immunogenicity (7). In children on HAART, PPSV23 produced relatively modest increases in serotype-specific anti-PPS that correlated with CD4 percentages (85). No clinical efficacy studies of PPSV23 in HIV-infected children have been reported.

## PCV and HIV-Infected Children

Several studies evaluating PCV in HIV-infected infants and older children have been published (Table 2), including the only clinical efficacy trial of the nine-valent PCV conjugated to CRM, a nontoxic mutant diphtheria toxin (PCV9-CRM), in South Africa (43). Early studies with PCV5-CRM given as a single dose to children over 2 years old were disappointing (39). However, when the vaccine was given on a three-dose schedule to infants under 2 years old, good overall responses were achieved and were indistinguishable from those of healthy control children (40). The first trial evaluating PCV7-CRM, in a population of HIV-infected infants who were all on HAART, demonstrated that PCV7-CRM, followed by the PPSV23, was well tolerated, was not associated with adverse reactions, and was immunogenic (64). In the largest U.S. study (PACTG 1024), two doses of PCV7-CRM were given (2 months apart), followed by a single dose of PPSV23 after a further 8-week interval, to 263 HIV-infected youth aged 2 to 19 years (1). The vaccines, given on this schedule, were safe and immunogenic. Anti-PPS levels for four conjugate vaccine serotypes (6B, 14, 19F, and 23F) at 24 weeks after vaccination with PPSV23 were greater than  $\geq 0.5 \mu\text{g/ml}$  for 75% of the participants and  $\geq 1.0 \mu\text{g/ml}$  for 50%. Week 24 antibody concentrations varied directly with CD4 percentages but not with nadir CD4 percentages before HAART, gender, race, or previous PPSV23 (except for serotype 6B). There were no viral load thresholds predictive of significantly reduced responses, but lower viral load at entry predicted higher week 24 antibody concentrations.

A large-scale efficacy study of a PCV9-CRM was performed in South Africa (43). For HIV-infected infants ( $n = 2,577$ ), the short-term serotype-specific efficacy was 65%, compared to 85% efficacy for uninfected children ( $n = 37,258$ ). Not surprisingly, immunogenicity in the HIV-infected group differed from that in the group of age-matched healthy South African controls. Those with more advanced immune suppression had lower anti-PPS levels than the HIV-infected children with less immune suppression and significantly lower levels than

**Table 2** Summary of immunogenicity studies of PCV in high-risk children<sup>a</sup>

Population and yr (reference)	Location	Trial type	Age group(s)	Regimen(s) and group(s)	Findings regarding anti-PPS response	Comments
<b>HIV infected children</b>						
1996 (39)	USA	OL + R	>2 yrs	PCV5-CRM + PPSV23; PPSV23 alone; HIV positive, <i>n</i> = 30; HIV negative, <i>n</i> = 30	GMC of <1 µg/ml for five serotypes (6B,14,18C,19F, and 23F) tested in HIV-positive children; no response to PPSV23 booster dose	No effect of CD4, but numbers small; ART use unclear but no HAART
1997 (40)	USA	OL	<2 yr	Three doses of PCV7-CRM, <i>n</i> = 18; HIV-negative control group, <i>n</i> = 33	88% of all measurements were >1 µg/ml after three doses in HIV-infected children; no difference from uninfected children	Tendency for less advanced HIV-infected children to respond better/quicker
2003 (64)	USA	DB + R	<6 mos	Three doses of PCV7-CRM + PCV7-CRM at 15 mos + PPSV23 at 24 mos, <i>n</i> = 30; PCB, <i>n</i> = 15	GMC of >1 µg/ml for all serotypes after three doses; 67 to 90% had >1 µg/ml after three doses	ART recipients with variable regimens; majority on HAART; minimal symptoms
2005 (81)	Greece	OL	1.5–12 yrs	Two doses of PCV7-CRM + PCV7-CRM at 12 mos in HIV-positive group, <i>n</i> = 14; two doses of PCV7-CRM in HIV-negative group, <i>n</i> = 21	29% response rate after first PCV in HIV-infected group; no significant response to second PCV; no response to PCV booster dose	11 of 14 infected children on HAART; adequate response defined as >2-fold increase in anti-PPS concentration for >2 of 4 serotypes measured
2005 (86)	Spain	OL	3–18 yrs	Two doses of PCV7-CRM in HIV-positive group, <i>n</i> = 56	Early waning of anti-PPS GMC	All on HAART and PPSV23 in past 12 mos
2005 (54)	South Africa	DB + R	<3 mos	Three doses of PCV9-CRM (HIV positive, <i>n</i> = 30; HIV negative, <i>n</i> = 63)	63–93% of HIV-infected children achieved >0.35 µg/ml after three doses; response lower in symptomatic children; 46–78% developed measurable killing	No ART use in children; all stages of disease represented; study nested in clinical efficacy trial
2006 (1)	USA	OL		Two doses of PCV7-CRM + PPSV23, <i>n</i> = 225	62–88% had ≥1 µg/ml post-PPSV23; immunogenicity best in those with highest CD4, undetectable viremia in plasma, and longer duration of HAART at baseline	CD4 level at vaccination more predictive of response than nadir CD4 cell count
<b>SCD patients</b>						
1998 (90)	USA	OL + R	>4 yrs and adults	Two doses of PCV7-CRM + PPSV23, <i>n</i> = 23; PPSV23, <i>n</i> = 23	Responses to PCV7 serotypes better in PCV arm; GMC of >1 µg/ml for all serotypes after PPSV23	Response to 6B increased by second dose of PCV7-CRM but not for other serotypes
2000 (67)	USA	OL	<2 mos and 2–12 mos	Three doses of PCV7-CRM + PPSV23; one dose of PCV7-CRM + PPSV23; SCD patients, <i>n</i> = 47; controls, <i>n</i> = 14	56–100% had >1 µg/ml after three PCV doses in first regimen; 31–71% had >1.0 µg/ml after one PCV dose in second regimen; significant responses after PPSV23 indistinguishable by SCD status	38 of 47 completed full vaccine schedule; PPV23 booster at 24 mos

2000 (66)	USA	OL	<2 mos and 2–12 mos	Three doses of PCV7-CRM + PPSV23; one dose of PCV7-CRM + PPSV23; SCD patients, <i>n</i> = 38; controls, <i>n</i> = 10	31–35% of SCD infants had measurable killing after PPSV23, similar to controls; serotypes 6B and 14 assessed	Radiometric opsonic assay; control numbers small; PPSV23 booster at 24 mos
<b>PPSV23 nonresponders</b>						
1998 (80)	USA	OL	2–13 yrs	PPSV23 + PPSV23, <i>n</i> = 11; PPSV23 + PCV7-CRM, <i>n</i> = 17 (nonresponders); PPSV23, <i>n</i> = 67	Levels $\geq 1.3 \mu\text{g}$ for between 35% (6B) and 88% (14) of subjects; significant increases in anti-PPS for all seven PCV7 serotypes	Participants were clinic attendees for recurrent respiratory infections; groups were defined by initial response to PPSV23; nonresponders were given PCV
2000 (91)	Germany	OL	3–18 yrs	Two doses of PCV7-CRM; nonresponders, <i>n</i> = 22; controls, <i>n</i> = 21	25–70% had $>1.0 \mu\text{g/ml}$ after second PCV; two-thirds of subjects achieved an OPA titer of $>1:64$ for 23F after second PCV	Various immunoglobulin deficiencies in nonresponders
2005 (73)	Germany	OL + R	2–14 yrs	Two doses of PCV7-CRM + PPSV23, <i>n</i> = 16; PPSV23 + PPSV23, <i>n</i> = 17	Measurements at 7 and 28 days post-PPSV23 booster; GMC of $\geq 1.0 \mu\text{g/ml}$ for all PCV7 types measured; higher levels in PCV recipients	All children had recurrent airway infections and history of nonresponse to at least two doses of PPSV23; PPSV23 booster after 1 yr
<b>Hematopoietic transplant recipients</b>						
2007 (69)	UK	OL + R	1–18 yrs	Auto and AlloHCT, first vaccine at 15 mos; two doses of PCV7-CRM + PPSV23, <i>n</i> = 8; PPSV23 + PPSV23, <i>n</i> = 7	GMC of $>1.0 \mu\text{g/ml}$ for all PCV serotypes after two doses; 85% had $\geq 0.35 \mu\text{g/ml}$ for all seven serotypes in PCV arm vs 17% in PPSV arm; numbers too small for postbooster comparison	All had underlying malignant disease; vaccines given in sequence with other infant vaccines; PPSV23 given at 24 mos
2007 (56)	Germany	OL	1–17 yrs	AlloHCT, <i>n</i> = 53; three doses of PCV7-CRM 6–9 mos posttransplant	GMC of $\geq 0.5 \mu\text{g/ml}$ for all serotypes after second PCV; complete protection in 56% and 77% after second and third PCV, respectively	Serologic response defined by anti-PPS concentration of $\geq 0.5 \mu\text{g/ml}$ or $>2$ -fold increase
<b>Solid organ transplant recipients</b>						
2005 (52)	USA	OL	2–18 yrs	Two doses of PCV7-CRM + PPSV23; transplant recipients, <i>n</i> = 25; healthy controls, <i>n</i> = 23	GMC of $>1 \mu\text{g/ml}$ for all serotypes after first PCV7-CRM; no significant increases after further vaccination	Heart transplant recipients generated lower responses than liver recipients; no effect from immunosuppressive regime used
<b>ROM patients</b>						
1999 (8)	USA	OL + R	18–72 mos	PCV7-CRM or PPSV23; otitis-prone children, <i>n</i> = 64; otitis-free children, <i>n</i> = 48	GMC of $>1 \mu\text{g/ml}$ for all four serotypes vs one for PPSV23	Four serotypes assessed (6B, 14, 19F, and 23F)
2003 (89)	The Netherlands	DB + R	1–2 yrs and $>2$ yrs	Two doses of PCV7-CRM + PPSV23, <i>n</i> = 24; PCV7-CRM + PPSV23, <i>n</i> = 24; controls ( <i>n</i> = 48) received a hepatitis vaccine	GMC of $>1 \mu\text{g/ml}$ for four of seven serotypes after PCV and six of seven after PPSV23	Nested immunogenicity substudy of clinical efficacy trial

<sup>a</sup>OL, open label; DB, double blind; R, randomized; PCB, placebo; ART, any antiretroviral therapy; HAART, highly active ART; anti-PPS, anti-pneumococcal capsular polysaccharide IgG; autoHCT, autologous hematologic stem cell transplant; alloHCT, allogeneic hematologic stem cell transplant; USA, United States; UK, United Kingdom; OPA, opsonophagocytic activity.

healthy controls (54). HIV-infected vaccine recipients were also less likely to have functional antibody to all studied serotypes (54), and the data suggested that greater amounts of antibody were necessary in HIV-infected children to achieve opsonophagocytic activity comparable to that in healthy controls. Several years after the primary series, participants were offered a booster dose of PCV7-CRM and evaluated for long-term efficacy of the vaccine against IPD as well as long-term immunogenicity (53). Among HIV-uninfected children, the estimated efficacy rate was stable (77% at follow-up, compared to 83% seen initially), while the estimated efficacy rates in the HIV-infected cohort dropped from 65% initially to 39% at follow-up. The long-term immunogenicity study (mean follow-up time was 4.8 years) showed that vaccine serotype-specific anti-PPS concentrations of  $>0.35 \mu\text{g/ml}$  were present in the majority of HIV-uninfected healthy children while the proportion of HIV-infected children, with geometric mean concentrations (GMC) of  $>0.35 \mu\text{g/ml}$  varied by serotype (4, 45%; 6B, 77%; 9V, 36%; 14, 81%; 18C, 19%; 19F, 81%; and 23F, 39%). HIV-infected children demonstrated a more rapid decline in antibody concentrations, suggesting a likely need for a booster dose. The latter finding has also been confirmed in symptomatic HIV-infected children receiving HAART (81).

Current recommendations are that all HIV-infected infants and children should be vaccinated with a three-dose primary series and a booster dose of PCV. The South African experience suggests that immunization will result in a significant reduction in IPD and pneumococcal pneumonia even in HIV-infected children who are not receiving HAART.

### PCV and HIV-Infected Adults

Studies with PCVs have so far been restricted to four published reports and a small number of conference abstracts (Table 3) (3, 18, 45, 57). The first study used PCV5-CRM and showed immunogenicity similar to that of PPSV23 in HIV-infected adults, despite superior immunogenicity of PCV compared to that of PPSV23 in HIV-uninfected adults. In the second study, two doses of an experimental four-valent PCV conjugated to *Neisseria meningitidis* outer membrane protein complex (OMPC; PCV4-OMPC), followed by a PPSV23 booster dose, showed superior immunogenicity compared to PPSV23 for serotypes 14, 19F, and 23F but not for 6B. The relatively small amount of 6B capsular polysaccharide used in the vaccine may in part explain the poor response to this serotype. Responses were greater in those subjects with CD4 cell counts of  $\geq 200 \text{ cells}/\mu\text{l}$ .

The two more recent published studies have both used PCV7-CRM. In the U.S. study (18), immunogenic-

ity was assessed in a population with diverse exposure to ART. PCV7-CRM recipients had higher postvaccine anti-PPS levels and geometric mean opsonophagocytic titers for the five serotypes studied (4, 6B, 9V, 14, and 23F) than PPSV23 recipients. Although there was minor serotype variation, a second dose of PCV7-CRM did not add significantly to immunogenicity. Subgroup analyses did not show any association between CD4 cell count, HIV viral load, ART use, and response to vaccination.

In the Ugandan study (57), PCV7-CRM was given to a cohort of survivors from an earlier PPSV23 clinical efficacy trial. None of the study participants were receiving ART, and half of the group had received PPSV23 in the past 5 years. PCV7-CRM induced significant responses to all seven serotypes. There was a clear relationship to CD4 cell count, and for four of the seven serotypes, a second dose of PCV7-CRM induced further increases in anti-PPS IgG levels. A comparison of PCV7-CRM response data to historical data on PPSV23 response in the same Ugandan population (25) showed that a single PCV7-CRM produced anti-PPS concentrations for serotypes 19F and 23F similar to those produced by PPSV23.

A more recent but small study from Malawi has shown good immunogenicity of PCV7-CRM in HIV-infected adults not taking HAART (24). Postvaccination anti-PPS concentrations and geometric mean killing titers were similar to those in PCV7-CRM-vaccinated HIV-uninfected controls up to 6 months postvaccination (37). Combined in this study was an assessment of anti-PPS responses in saliva and in bronchoalveolar lavage fluid. Significant increases in salivary IgA (24) and in IgG in bronchoalveolar lavage fluid (27) were measured in the HIV-infected group, similar to the response in the HIV-uninfected group. Increases in alveolar anti-PPS levels have been measured previously in this population after pneumococcal infection (29) but not after PPV23 vaccination (S. Gordon, personal communication). This result suggests different immunological handling of PCV compared to PPSV in this group.

Immunogenicity studies to date with PCVs in HIV-infected adults confirm these vaccines to be safe and of greater immunogenicity than PPSV23. In most reports, these differences are modest and their clinical implications are uncertain. Clinical efficacy trials are required, although likely to be problematical where PCV is in routine use in infants. Studies of IPD during the pre-HAART era demonstrated that the serotypes causing disease in children and, to a lesser extent, in adults were primarily those found in the PCV7 (55). More recent studies demonstrate that disease due to nonvaccine serotypes may be a problem in Africa (28), and serotype replacement disease is increasing in importance in HIV-

infected adults where infant PCV administration is in use (20). These observations suggest that a second-generation PCV with increased valency will be valuable for the HIV-infected population if it proves to be clinically efficacious.

### SCD

Children with sickle-cell diseases (SCD) in the United States and Western Europe are particularly susceptible to pneumococcal infection. IPD risk is related to a naïve immune system, the near-universal occurrence of auto-splenectomy, and defective activation of the alternative pathway of complement (9). IPD rates peak in 1-year-old children and were estimated at 36 to 63 per 1,000 persons per year in children between 1 and 2 years of age and at 9.5 to 19 per 1,000 persons per year for children under the age of 10 (2). Three studies of the serotype distribution of invasive isolates in children with SCD identified 71 to 97% as contained within PCV7 (15).

Studies of PPSV in children with SCD have not demonstrated consistent immunogenicity or efficacy. A randomized trial of PPSV14 in 242 Jamaican children with SCD who were less than 3 years of age (34) did not confirm the efficacy of the vaccine against IPD. In U.S. children, PPSV14 has been shown to induce variable and age-dependent anti-PPS responses (68).

The immunogenicity and safety of PCV7-CRM in children and adolescents has been confirmed in three published studies (Table 2) and further work from west Africa (recorded in the Cochrane database). In all, the vaccines were found to be safe and immunogenic (15, 66, 67, 90). The use of PPSV23 as a booster vaccine was associated with substantial increases in anti-PPS in all three study groups, while the PCV-PCV-PPSV regimen was shown to induce greater concentrations of anti-PPS than PPSV alone for all seven serotypes in a study of older children and young adults (90). Based on these immunogenicity results, current recommendations for children with SCD include immunization with PCV7-CRM in infancy, a booster dose at 12 to 15 months, PPSV23 at 24 months of age, and a booster dose of PPSV 3 to 5 years later (5).

### FUNCTIONAL AND ANATOMICAL ASPLENIA

The spleen is critical for initiating antibody production and clearing opsonized bacteria from the circulation. In addition, splenectomy will be performed or functional aplenia will arise as a consequence of other underlying

conditions which of themselves may predispose to pneumococcal infection, such as SCD and hematological malignancies. PPSV23 is recommended for use in groups with these conditions, but vaccine efficacy varies by different underlying pathological conditions. In a retrospective study from the United States in the early 1990s, PPSV23 efficacy was estimated at 77% overall for the prevention of IPD (12). However, serological studies have reported variable outcomes. Responses in splenectomized but otherwise healthy individuals show responses similar to those of controls, but responses in individuals with underlying malignant disease are reduced (21, 31, 70, 76, 79). There is a recognition that some individuals postsplenectomy do not respond to PPSV and remain at high risk for serious disease (44).

PCVs are attractive vaccines for use in splenectomized individuals. As they are T-cell dependent, vaccine responses may take place in germinal centers outside of the spleen and bypass the requirement for a functioning splenic marginal zone. Trials of PCV in groups without functioning spleens have been reported for those recovering from Hodgkin's disease, SCD patients (see other sections), and those who had total or near-total splenectomy for hereditary spherocytosis. In the latter study, PCV7-CRM was immunogenic, with between 2.2 and 14.6% of subjects failing to reach an anti-PPS level of  $\geq 1 \mu\text{g/ml}$  (83). PCV7-CRM has been successfully used to overcome nonresponsiveness to PPSV23 in previously splenectomized individuals (60).

### BONE MARROW TRANSPLANTS

*S. pneumoniae* is an important pathogen in patients undergoing bone marrow transplantation (BMT) or peripheral blood stem cell transplantation, with two patterns of pneumococcal disease recognized (47). Early-onset disease is observed in both allogeneic and autologous BMT and peripheral blood stem cell transplant patients within the first 35 days. Later-onset disease occurs after 100 days, is more frequent in allogeneic than in autologous BMT, and is the most common bacterial infection in such patients. Functional hyposplenism secondary to irradiation, chronic graft-versus-host disease, and decreased IgG2 antibody contribute to the risk of IPD and the poor outcome. Case fatality rates are approximately 20% (17, 47).

Stem cell transplant recipients respond poorly to T-cell-independent polysaccharide antigens in the first 2 years following the transplant, with gradual maturation of the immune response thereafter. Several investigators have reported failure to elicit protective responses to PPSV when administered prior to 2 years after the transplant.

**Table 3** Summary of immunogenicity studies of PCV in adult and adolescent immunocompromised groups

Population and yr (reference[s])	Location	Trial type	Regimen(s) and group(s)	Findings regarding anti-PPS response	Comment
<b>HIV-infected patients</b>					
1996 (3)	USA	OL + R	PCV5-CRM (HIV positive, <i>n</i> = 92; HIV negative, <i>n</i> = 49); PPSV23 (HIV positive, <i>n</i> = 91; HIV negative, <i>n</i> = 50)	GMC of >1.0 µg/ml for all five serotypes; PCV and PPSV23 responses similar in HIV-infected groups; response CD4 dependent	10 µg of polysaccharide per serotype
2000 (45)	The Netherlands	OL	Two doses of PCV4-OMPC + PPSV23 (HIV positive, <i>n</i> = 30; HIV negative <i>n</i> = 9); PPSV23 (historic controls: HIV positive, <i>n</i> = 50; HIV negative, <i>n</i> = 10)	19–100% of HIV-positive subjects had ≥1.0 µg/ml after second PCV; response CD4 dependent and inferior to that of HIV-negative subjects, of which 43–100% had ≥1.0 µg/ml after PPSV23; higher response with PCV priming	2 µg of each polysaccharide; PPSV23 given at 10 mos; 6B, 14, 19F, and 23F studied
2001 (18)	USA	DB + R	PCV7-CRM + PPSV23; two doses of PCV7-CRM; PCB + PPSV23; PCB + PCB; HIV positive, <i>n</i> = 25 (each group)	GMC of >1.0 µg/ml for all serotypes assessed after first PCV; PCV regimens superior to PPSV23 only, with higher final OPA titer; two doses of PCV7-CRM no better than one except for 6B	67 completed study; response CD4 independent; all had CD4 cell count of >200 cells/µl; HAART use by 42%; AIDS illness in 19%
2005 (57)	Uganda	OL	PPSV23 + two doses of PCV7-CRM, <i>n</i> = 54; PCB + two doses of PCV7-CRM, <i>n</i> = 55	GMC of >1.0 µg/ml for all seven PCV serotypes after first PCV; significant increase after second PCV for serotypes 4, 6B, 19F, and 23F; CD4-dependent responses	Past participants in PPSV23 clinical efficacy trial; PCV7-CRM 5 yrs after PPSV23 or PCB; responses unaffected by past PPSV23 or PCB; no ART use
2006 (24, 27, 37)	Malawi	DB + R	Two doses of PCV7-CRM (HIV positive, <i>n</i> = 10; HIV negative, <i>n</i> = 10); two doses of PCB (HIV positive, <i>n</i> = 10; HIV negative, <i>n</i> = 12)	GMC of >1.0 µg/ml for all serotypes after first PCV; no increase after second PCV; similar pattern for OPA response; salivary and bronchoalveolar responses also increased after first PCV; responses statistically indistinguishable by HIV status	Serotypes 6B, 14, 19F, and 23F assessed; no subjects on ART; numbers too small to investigate CD4 effects; whole-cell killing assay used to determine killing titer
<b>Solid organ transplant recipients</b>					
2003 (48, 49)	Canada	DB + R	Renal transplant recipients; one dose of PCV7-CRM, <i>n</i> = 30; one dose of PPSV23, <i>n</i> = 30	GMC of >1 µg/ml all serotypes after first PCV; 17–50% response in PCV recipients; response to PCV tended to be better than that to PPSV23; at 3-yr follow-up, significant declines but no difference by vaccine	Response defined by 2-fold or greater increase and anti-PPS GMC of >1.0 µg/ml at 3-yr follow-up: PCV, <i>n</i> = 23; PPSV23, <i>n</i> = 24

2006 (50)	Canada	OL	Liver transplant recipients; PCV7-CRM + PPSV23, $n = 24$ ; PCB + PPSV23, $n = 26$	GMC of $>1 \mu\text{g/ml}$ for all seven serotypes post-PCV; no significant difference in anti-PPS or OPA titer by vaccine group
Hematopoietic transplant recipients				
2003 (58)	USA	OL + R	AlloHCT; pretransplant donors and patients given PCV7-CRM or PCB; posttransplant subjects given three doses of PCV7-CRM at 3, 6, and 12 mos	Protection against PCV7-CRM serotypes posttransplant in donor PCV vs nil recipients: 67% vs 36% after one dose of PCV posttransplant; 75% vs 64% after three doses of PCV
2005 (6)	USA	OL + R	AutoHCT; pretransplant patients given PCV7-CRM or nil before stem cell harvesting and three doses of PCV7-CRM at 3, 6, and 12 mos after transplant	Protection against PCV7-CRM serotypes posttransplant in preharvesting PCV vs nil recipients: 68 vs 32% after one dose of PCV posttransplant; 87 vs 61% after three doses of PCV; GMC higher at all time points in preharvesting group, significantly so for serotypes 18C, 19F, and 23F after three doses of PCV
2005 (71)	USA	OL + R	AutoHCT; pretransplant patients given PCV7-CRM or nil before stem cell harvesting and two doses of PCV7-CRM at 1 and 3 mos after transplant	Response to PCV in preharvesting vaccine group but not in other group; response waned except in T-cell experimental arm
Splenectomy patients				
2005 (83)	Germany	OL	PCV7-CRM + PPSV23, $n = 39$	GMC of $\geq 1 \mu\text{g/ml}$ between 85.4% (6B) and 97.6% (19F) for the PCV serotypes after PCV dose; no increase in GMC after PPSV23
Hodgkin's disease patients				
1995 (59)	USA	DB + R	One dose of PPSV23, $n = 58$ ; one dose of PCV7-OMPC, $n = 70$ ; all 128 treated for Hodgkin's disease; 20 healthy controls received one dose of PCV7-OMPC	GMC of $>1.0 \mu\text{g/ml}$ for all seven serotypes post-PCV but mean increase very low, between 1.0- and 1.4-fold; GMC lower in patients after PCV7-OMPC than PPSV23 for all seven serotypes
COPD patients				
2002 (35)	Iceland	OL	COPD patients, PCV1-T ( $n = 10$ ) or PPSV23 ( $n = 9$ ); controls, PCV1-T ( $n = 15$ )	GMC postvaccine of $>1.0 \mu\text{g/ml}$ in 80, 77, and 93% of PCV and PPSV recipients and control groups, respectively
				PCV a single valency of 12 $\mu\text{g}$ of 6B polysaccharide conjugated to tetanus toxoid carrier

\*OL, open label; DB, double blind; R, randomized; PCB, placebo; ART, any antiretroviral therapy; HAART, highly active ART; anti-PPS, anti-pneumococcal capsular polysaccharide IgG; autoHCT, autologous hematologic stem cell transplant; alloHCT, allogenic hematologic stem cell transplant; USA, United States; UK, United Kingdom; OPA, opsonophagocytic activity.

PCV immunogenicity has been investigated in several trials with different PCV7-CRM regimens. Reasonable response rates can be expected when PCV is given late after transplant (69). With earlier vaccination, e. g., first vaccination given at 3 or 6 months, protective antibodies to all seven PCV serotypes after three doses occur in between 64 and 75% of patients, but multiple dosing is essential, with poor responses common after the first dose (6, 56, 58). In allogenic transplant recipients, donor immunization with PCV pre-bone marrow harvesting enhances the response of the recipient to PCV posttransplant, particularly to the first dose (58). In autologous stem cell transplant patients, there are similar findings. Individuals who receive a dose of PCV prior to stem cell harvesting have better responses to the first PCV and overall better responses after three doses (6). This approach has been extended further with PCV given preharvesting combined with ex vivo autologous T-cell activation. Responses to PCV given 1 month after transplant were achieved (71).

## SOLID-ORGAN TRANSPLANTS

Solid-organ transplant recipients are at increased risk of pneumococcal disease. The risk varies with the nature of the transplant; however, recipients have a lifelong increase in risk due to the immunosuppressive therapy required for transplant survival, and recurrent disease is common. PPSV23 has been recommended for solid-organ transplant recipients; however, immunogenicity tends to be poor whether the vaccine is given before or after transplantation (10, 38).

The immunogenicity and safety of a PCV-PCV-PPSV regimen has been evaluated in children who have undergone solid-organ transplants. Antibody concentrations regarded as protective ( $>0.5 \mu\text{g/ml}$ ) developed in 49 to 92% of recipients, depending on the serotype, compared to 78 to 100% of healthy controls. Antibody concentrations were comparable among transplant recipients receiving tacrolimus alone or in combination with other agents. Heart transplant patients had lower type-specific antibody responses than liver transplant patients. No serious adverse events were observed (52).

PCV7-CRM has been studied in adult renal and liver transplant recipients (48, 50). In the patients studied, PCV7-CRM was no more immunogenic than PPSV23. In the case of the renal transplant recipients, a cohort at the 3-year follow-up point had similar anti-PPS levels independent of past PCV or PPSV23 receipt (49). Variation in schedules, and in particular the timing of repeat doses of PCV, merits further investigation in these patients.

## CHILDREN WITH RECURRENT OTITIS MEDIA

There is increasing evidence that many otitis media (OM)-prone children demonstrate subtle deficits in response to PPS antigens or the outer membrane protein P6 from *Haemophilus influenzae*. The immunologic abnormality most often identified is partial IgG subclass or IgA deficiency (87). Children with recurrent OM (ROM), but not parents with a history of ROM, frequently lack detectable anti-PPS against capsular types 6A and 19F, in contrast to healthy age-matched controls (36). The findings suggest that some individuals with ROM have delayed immune maturation rather than an intrinsic inability to respond to pneumococcal polysaccharide. Notwithstanding these studies, most children with ROM will not have a quantifiable immunologic abnormality. Furthermore, the response to PPSV in children less than 2 years of age, the time when OM is most common, is inadequate to prevent recurrent disease.

Two studies, one nested within a clinical efficacy trial, have evaluated PCV7-CRM use in children with ROM. In the first study, PCV elicited higher antibody concentrations against all four serotypes studied (6B, 14, 19F, and 23F), and responses were comparable to those in otitis-free children (8). Veenhoven et al. reported that anti-PPS concentrations in children with ROM immunized with PCV and PPSV were  $>1.0 \mu\text{g/ml}$  for all PCV serotypes, with the exception of 6B, which remained  $<1.0 \mu\text{g/ml}$  in children immunized between 12 and 24 months (89). This study was nested within a clinical trial, and although protective antibody concentrations were achieved following immunization, PCV7 failed to reduce the number of episodes of recurrences. However, pneumococcal serotypes accounted for few middle-ear infections in this selected cohort.

## POLYSACCHARIDE NONRESPONDERS

Nonresponse to polysaccharide antigens is relatively common, with as many as 1 in 10 adults failing to mount an effective serological response to at least half of the 10 serotypes measured in studies of PPSV23 (61). The clinical consequences of nonresponsiveness vary from asymptomatic to recurrent pneumonia and upper-respiratory-tract infections in children, adolescents, and adults. The pathophysiological processes which underlie nonresponsiveness are multiple, although nonresponse has been associated with genetic inheritance (61) and defects in immunoglobulin production (14, 87).

PCV offers an alternative approach to induce anti-PPS responses when PPSV23 has failed. Musher et al. reported responses to multiple and complex regimens of

PCV (eight-valent PCV conjugated to diphtheria toxin [PCV8-D], PCV5-CRM, and PCV1-OMPC) in adults who had previously failed to respond to PPSV23 (62). Five of seven subjects eventually mounted significant responses. Zielen et al. reported responses to two doses of PCV7-CRM in children and adolescents who suffered recurrent annual infections (91). After a single dose, only anti-PPS for serotype 19F exceeded 1 µg/ml. After two doses, this level was exceeded for serotypes 4, 9V, 14, and 19F. These findings were followed with a further study confirming good responses at 1 year to a PPSV23 booster dose in a population of PPSV23-nonresponsive children and adolescents. PCV would therefore seem to offer an alternative to PPSV23 for these groups.

## MALIGNANT DISEASE

Individuals with cancer have a relative risk of pneumococcal disease 20 to 50 times that of the general population (51). Their situation is often compounded by other factors which increase pneumococcal disease risk (e.g., smoking, immobility, and chemotherapy) such that the underlying predisposing defects are varied and not related just to immune dysfunction. Immunogenicity studies of PPSV in patients with solid tumors are scarce, but response has been recorded as normal or reduced (65). In hematological malignancies, immunogenicity is poor, although asplenia is also a feature of many of these patients (21, 31, 75, 77). A small randomized trial of a PPSV17 in individuals with lung cancer did not confirm the efficacy of PPSV23 to prevent IPD (41).

There are no studies of PCV in individuals with solid tumors. Molrine et al. reported responses to a single dose of a PCV7-OMPC in patients treated for Hodgkin's disease at least 2 years previously (59). Postvaccine anti-PPS levels were lower in PCV7-OMPC than in the PPSV23 recipients. The quantities of pneumococcal polysaccharide contained in the PCV7-OMPC (1 and 2.5 µg per serotype) may have adversely affected the immunogenicity of this vaccine. Further studies are required with the current licensed PCV products.

## COPD

Chronic obstructive pulmonary disease (COPD) increases the risk of IPD to up to 10 times that of the age-matched population (51). The contribution of *S. pneumoniae* to noninvasive morbidity in this group is also believed to be large. Pneumococcal vaccines would have increased value for patients with this condition if they were able to provide protection against bronchitis and pneumonia, and thus, PCV is an attractive option for this patient group.

Four prospective trials have investigated the role of PPSV23 in COPD patients (30). No significant protection against pneumonia, death, the exacerbation of disease, or IPD has been measured. Immunogenicity studies with PPSV23 are few and have concentrated on the impact of cotreatment with steroids at the time of vaccination (16, 82). In these studies, postvaccination concentrations of anti-PPS were comparable to those in healthy controls.

A single study has reported the response to an experimental serotype 6B one-valent PCV conjugated to tetanus toxoid (PCV1-T; 10 µg of polysaccharide) (35). The postvaccine concentration of anti-PPS for serotype 6B was similar to that produced by PPSV23 and to the response in healthy controls. Opsonophagocytic titers showed variable responses, with only 4 of the 10 COPD PCV recipients showing a rise in titers. Larger studies in this important vaccine target group and the assessment of the mucosal activity of PCV are required.

## SUMMARY

To date there is a modest body of information on the immunogenicity of PCVs in immunocompromised and high-risk groups. Several key areas need further investigation. Although a direct relationship exists between immunogenicity and protection, this needs further evaluation, as the relationship in immunocompromised individuals may not match the findings in healthy children. PCVs induce mucosal responses, and direct measurement of these and/or studies of pneumococcal carriage may be a suitable method of vaccine evaluation. Inadequate information exists on vaccine immunogenicity in many large and important high-risk groups, e.g., diabetics and chronic cardiopulmonary disease patients. The timing of vaccination in children is based on the established childhood vaccine schedules. These will need to be reviewed and modified for immunocompromised infants and children, with more frequent booster doses. In adult groups, no consensus exists on optimal dosing schedules. If efficacy trials are to be pursued in adults, it would be prudent to systematically evaluate immunogenicity with a range of schedules. As additional PCVs with broader serotype coverage become available, understanding their role in immunocompromised populations will become increasingly important.

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# *Efficacy and Safety*

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IV

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Katherine L. O'Brien  
Ron Dagan  
P. Helena Mäkelä

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## Nasopharyngeal Carriage

### INTRODUCTION

The pneumococcus is a normal component of the nasopharyngeal (NP) mucous membrane microflora; it is believed that most episodes of NP colonization cause no symptoms or disease, although recent data indicate that respiratory tract symptoms may occur (108). However, the pneumococcus is one of the most common etiologies of respiratory tract infections like sinusitis, otitis media, and pneumonia, as well as nonrespiratory infections like meningitis, sepsis, and bacteremia. When pneumococcal disease occurs, it is the host's own NP pneumococci that serve as the source of the disease-causing strain. Therefore, the medical importance of asymptomatic pneumococcal NP carriage rests in its role as both the reservoir of bacteria transmitted to others, thereby propagating the asymptomatic colonization state within the community, and the immediate source of disease-causing pneumococci in the host. As a corollary, the prevention of pneumococcal NP colonization and transmission would be expected to effectively prevent pneumococcal disease. Does the pneumococcal conjugate vaccine (PCV) prevent such transmission? What is the

effect of PCV on the asymptomatic carriage state among individuals and within the community as a whole? Accurately interpreting the findings of studies addressing these questions requires an understanding of the determinants of NP carriage and immunity to this state.

### Determinants of Carriage Prevalence

Pneumococcal carriage prevalence is strongly dependent on the ages of individuals and the presence of pneumococcal carriers in the immediate environment. A newborn soon becomes colonized by bacteria from its surroundings, including pneumococci. Typically, pneumococcal carriage prevalence is high in developing countries, with some children becoming colonized within days after birth. Fifty to 90% of children are found to be colonized by several months of age; the cumulative frequency of colonization among children this age is nearly 100%, indicating that almost every child experiences at least one episode of colonization by this time. Once children pass 10 years of age, the prevalence of colonization declines to about 10% (36, 37, 49, 65, 70, 84, 125) and remains at this level for ages up through adulthood.

By contrast, in more affluent populations, the first episode of pneumococcal colonization may be delayed until several months of age, and a steady state of colonization prevalence of 40 to 50% may not be reached until after 2 years of age. These peak rates of colonization are followed by a reduction in NP colonization prevalence around the age of 5 years, which continues down to levels of 10% or less in adults (1, 5, 39–41, 46, 53, 103, 114). Few data on NP colonization among the elderly are available.

The distribution of pneumococcal serotypes also varies with age. In general, pneumococci that are found to colonize children are most often of serogroups 6, 19, and 23. These have previously been termed pediatric serogroups because they are found less commonly among the colonizing types later in life (6, 10, 36, 39, 43, 84, 102, 114, 117). However, this term has also been taken to mean that these serotypes are found to cause disease only among children, which is incorrect. These serogroups share some characteristics, including the observation that they not only are commonly found in the asymptomatic carriage state in children but also are a common cause of invasive pneumococcal disease in this age group. Furthermore, they are poorly immunogenic in infants and children under 2 years of age when encountered in the natural setting. For these reasons, they have been considered to be essential components of PCV. By contrast, both carriage and invasive disease-causing pneumococci isolated from adults show broader serotype distributions than among children.

The implicit role of carriers in transmitting pneumococci to close contacts has been directly assessed in family studies (2, 7, 53, 66, 81, 102, 108). These studies have shown that the colonization of the newborn occurs earliest in families with other children and that the risk of colonization correlates with the number of siblings. Day care centers provide even greater opportunities for close contact and allow specific serotypes to spread in microepidemics throughout the center and on into the families of the children attending the day care (6, 11, 31, 33, 117).

Colonization is likely promoted by viral respiratory infections (7, 27, 102, 114, 118, 123). In a birth cohort study, pneumococcal carriage prevalence in children who were seeking medical care for acute respiratory infection or otitis media symptoms was double that in healthy children of the same age (114). The excess colonization was shown to be due to new carriage acquired within the period the symptoms of the respiratory infection lasted (113); it remains possible that the pneumococcal colonization incidence did not increase but that the detection of existing colonization increased as a re-

sult of greater pneumococcal carriage density secondary to the viral infection. Antibiotics clear or reduce pneumococcal carriage, but this effect is transient and the carriage returns to the original prevalence within a week after the cessation of antimicrobial therapy (12, 39, 64, 71, 114). Furthermore, pneumococci resistant to the antimicrobial agent used are overrepresented among pneumococci carried after the treatment (12, 64).

Pneumococcal carriage is not a continuous state. In fact, once established on the NP mucosa, the pneumococci are cleared within weeks to months, only to have another pneumococcal strain appear within the next few weeks or months. Thus, a child experiences numerous carriage episodes with different pneumococcal strains (i.e., serotypes or clones) (39, 53, 65). The duration of a carriage episode has been explored in several studies and is reported to vary widely (i.e., 5 to 290 days) depending on the serotype and the age of the carrier (2, 10, 15, 16, 25, 38, 46, 84, 109, 110). These studies inferred the duration of colonization by assessing carriage at sequential sampling times, not by directly observing the acquisition and clearance events themselves. There were certainly episodes of acquisition and clearance which were missed entirely by these studies, whose precision of estimates was highly dependent on the sampling frequency. Mathematical modeling of the NP acquisition and clearance process suggests a wide variation in the duration of carriage, with many very short episodes resulting in mean duration times between 15 and 60 days.

It is also well known that more than one pneumococcal serotype or clone can coexist in the nasopharynx of an individual. In some populations, by using methods designed to detect multiple-serotype carriage, up to 30% of carriers have been shown to carry more than one serotype simultaneously (38, 44, 70, 89, 90, 93). However, typically one serotype predominates, with second or third types present as minority strains (39, 52, 108). With the exception of one study (93), studies of PCV impact on carriage have used laboratory methods designed to identify only the dominant serotypes, and thus, the discovery of the true prevalence of multiple-serotype or multiple-clone carriage awaits the development of practical, highly sensitive laboratory methods for the detection of multiple-strain colonization, as was recommended by a WHO working group (8, 92).

### Mechanisms of Mucosal Colonization by Pneumococci

The cellular- and molecular-level events associated with colonization and its clearance are incompletely characterized. The attachment of the pneumococcus to na-

sopharyngeal epithelial cells is a required initiating step. Several potential receptors have been identified by *in vitro* systems (14, 112, 130). We presume the existence of special niches for pneumococcal attachment and the subsequent establishment of colonization, processes in which the newly arriving pneumococcus has to evade the local host defenses and compete with the resident polymicrobial flora, including other strains of pneumococci (68, 72). A precise understanding of the human cellular mechanisms operating at this stage is not available, and it is unclear which findings from experimental animal studies (chapter 5, this volume) are relevant to humans. Nevertheless, it is well known that pneumococcal morphology (*i.e.*, opaque versus transparent) varies; opaque phenotypes are correlated with disease, and transparent phenotypes are correlated with NP colonization (61, 127).

Characteristics of the invading pneumococci certainly play a role in the ability of the organisms to colonize, but there is only partial understanding of what these characteristics are and their roles. Both epidemiologic and experimental animal studies have demonstrated that virulence, or the capacity to cause invasive disease, can vary by pneumococcal serotype or other clonal characteristics (9, 42, 68, 104, 110, 116). Fewer studies have addressed strain variability in colonization parameters, such as the establishment, duration, and transmission of colonization to another host (80, 109). The now-available full-genome sequences of several strains of pneumococci have opened up a powerful means to unravel the determinants and molecular mechanisms of pneumococcal virulence (4, 45, 94, 98). Recent research taking advantage of these data has already shown the involvement of many genes in colonization, pointing at the importance of colonization to the bacterium itself. The polysaccharide (PS) capsule, so important to protect the pneumococcus from phagocytic killing in invasive infections, also plays a role in colonization: unencapsulated mutants are inefficient colonizers in mouse infection experiments. Recent findings suggest that the effector mechanism in this case is not phagocytosis but rather a physicochemical one, so that the capsule prevents the bacteria from being trapped in the mucus and exported by the local ciliary action, one of the most basic defenses of the upper airways (87).

### Role of Acquired Immunity in Colonization

Pneumococcal colonization induces a variety of immune responses in humans, including the production of antibodies directed at both surface proteins (25, 36, 58, 70, 89) and the PS capsule (34, 50, 78, 86, 106, 107, 111, 131). Antibody production against pneumococcal pro-

teins begins in infancy, whereas the production of antibodies directed to capsular PSs does not typically begin until the second or third year of life. The role of this naturally acquired immunity to proteins and PS in modifying colonization is an important area of vaccine development research, as this immunity provides a key strategy for identifying possible vaccine candidates.

Experimental work with mice has raised the notion that immune pathways other than antibodies to the carbohydrate capsule may be important in the natural development of protection against the pneumococcus. These mouse studies have shown that a pneumococcal colonization episode can protect against subsequent colonization (119). In mice, this protection is not dependent on the synthesis of antibodies nor is it serotype specific (*i.e.*, capsular PS specific), but it is dependent on CD4<sup>+</sup> T cells (76, 119). Furthermore, in immunocompetent mice the pneumococcal colonizing episode was cleared in approximately 30 days, while the clearance was much prolonged if the mice were deficient in CD4<sup>+</sup> T cells or Toll-like receptor 2, thus confirming the above-mentioned findings (79, 121). Among these mice, the concomitant antiprotein antibody production resulting from the pneumococcal colonization episodes did not seem to have any role in this naturally acquired immunity, the effector mechanisms of which are still a matter of speculation.

Preliminary epidemiologic evidence suggests that pneumococcal carriage in human infants can also stimulate immunity, preventing recolonization by the same or different serotypes of pneumococci. Whether this cross protection is based on the development of CD4<sup>+</sup> T cells, as in mice, or some other mechanism is not yet known (35). It should be noted that the mice did not produce measurable anti-capsular PS antibodies, and likewise, the human infants were too young to produce an anti-PS response. These data suggest that natural protection against pneumococcal colonization is independent of the production or presence of anti-PS antibodies; however, it is well known that passively administered human bacterial PS immune globulin prevents pneumococcal colonization in infant rats (75). Furthermore, vaccine trials of capsular PS and its derivative PCV, both of which result in the production of serotype-specific anti-capsular antibodies, have proven that serotype-specific immunity is able to affect NP colonization, even if this may not be the predominant mechanism of protection in naturally acquired immunity to colonization.

The classical study that established the protective efficacy of pneumococcal PS vaccine against pneumonia in 1945 also showed an effect of the vaccine on pneumococcal carriage (73). The study was conducted among

adult male army trainees who were randomized to receive the four-valent pneumococcal PS vaccine or a placebo and then observed for up to 6 months. The pneumococcal carriage prevalence of the four serotypes (1, 2, 5, and 7) was 3.26% in the placebo group and 1.79% in the vaccine group, representing a highly significant reduction of 45%. There was no difference in colonization by the nonvaccine serotypes causing colonization (60.8% in the placebo group and 59.1% in the vaccine group), demonstrating the serotype-specific nature of the effect. However, subsequent studies of 14- and 23-valent pneumococcal PS vaccines among other cohorts, all of which involved infants or children, have failed to show any effect of the PS vaccine on protection against vaccine serotype NP carriage (18, 23, 57, 105) or have shown equivocal results (48). The negative or equivocal results in these studies and the positive findings in the army study link the prevention of colonization with a strong immune response to the PS vaccine (i.e., the development of high concentrations of anti-PS antibodies); in the army study, the subjects were young healthy men, known to mount a strong immune response, while the response to the PS vaccines is less robust in most infants and children. This hypothesis of a link was not supported, however, by the experience with 23-valent PS vaccine administration in southern Israel to a whole community which was experiencing a serotype 1 outbreak. The vaccinated community included children and adults of all ages. There was no effect on colonization by serotypes other than the epidemic type 1 when carriage 6 weeks after the immunization was compared to carriage before immunization (17).

The experience with the *Haemophilus influenzae* type b (Hib) vaccine supports the need for a high serum antibody concentration to affect colonization. While the Hib-PS vaccine had been of low immunogenicity in infants and children under 2 years of age and was not found to affect carriage of the Hib bacteria, Hib conjugate vaccines were highly immunogenic even in infants and had a strong effect on carriage (3, 74, 115). Since the only component of the Hib bacteria present in the conjugate vaccine was serotype-specific PS, conjugated to an unrelated protein carrier, the effect on carriage must have been mediated by immunity to the Hib PS. Antibodies to Hib PS, both polyclonal and monoclonal, were furthermore shown to prevent carriage in an infant rat model, and the serum antibody concentration required for the prevention of carriage proved to be 8- to 10-fold higher than that for the prevention of invasive disease (58, 59). Soon after the introduction of the Hib conjugate vaccine in national infant immunization programs, the reduced chances of spread of the infection

due to the reduced carriage were reflected in a reduction of Hib disease among children in age strata that themselves had not received the vaccine.

Mathematical modeling predictions of the indirect effects of the vaccine corresponded closely to the observed effects. This led to the question of whether a similar indirect effect would be observed with pneumococci and PCV administration. This was not a trivial question because of critical differences between the bacteria: there are many serotypes of pneumococci, but only one of *H. influenzae*, that are important causes of disease, and the elimination of only one or a few pneumococcal serotypes by vaccination may have less effect on pneumococcal disease than the Hib conjugate vaccine has on Hib disease. Furthermore, because the prevalence of pneumococcal colonization is much higher than colonization with Hib (115), the elimination of even a portion of the pneumococcal flora may have more profound effects on the local microflora of the nasopharynx than is the case with Hib.

## DIRECT EFFECT OF PCV ON NP CARRIAGE

We define the direct effect of vaccine on carriage as those effects observed among the vaccinated individuals. These effects include the impact on vaccine type (VT) carriage, vaccine-associated serotype carriage, nonvaccine type (NVT) carriage, and nonpneumococcal NP carriage.

When the need to assess the effect of PCV on pneumococcal carriage in clinical trials was recognized, a group of interested vaccine trialists met under the auspices of the WHO to standardize the study methodology to facilitate comparisons of results between trials (92). The "core consensus methods" addressed questions of sampling site and collection method, swabs and media to be used, long-term and short-term storage of samples and isolates, and culture and serotyping methods. The recommendations have well served the purpose of harmonizing the measurement of carriage prevalence; all trials have employed the standard approach, thereby allowing differences in trial observations of NP colonization to be more easily attributable to meaningful biological and epidemiologic differences rather than spurious differences in study design or laboratory methods.

By contrast, there is no agreement on how to measure the efficacy of the vaccine against colonization. Most studies compare the observed cross-sectional carriage prevalence in groups of vaccinated and control children, but there is no consensus on the optimal timing of sample collection relative to vaccine doses, the frequency of sampling, the methods to collect infor-

mation on potential confounding variables, or how to translate such effects into a vaccine efficacy for colonization end point. The major problem is that cross-sectional sampling does not allow observations of point of acquisition and can even miss carriage episodes that start and end between sampling time points. Consequently, carriage acquired after vaccination (and presumed to be prevented by the immunity produced) is usually not distinguished from carriage that had been present already at the time of the vaccination. Only a few studies have assessed the effect of the vaccine on new acquisition by sampling before and after vaccination (15, 32, 129); many more studies have sampled multiple times following vaccination, which is also a strategy for detecting new acquisitions.

The age of the child and the infection pressure from the environment, the two factors most strongly affecting carriage prevalence, are also likely to affect the vaccine impact on both the acquisition and prevalence of colonization. At least two aspects related to the child's age play a role, the maturation of the immune response and the age-dependent prevalence of carriage. A further confounder is the time since last vaccination; the increasing antibody production and maturation within weeks after vaccination and the slow decrease of circulating antibodies thereafter are likely to be reflected in differences in protection from colonization. Therefore, specific, quantitative conclusions tying together results from a variety of trials conducted in different environments with different schedules of vaccination and sampling intervals are very difficult to draw. We are aware of 13 blinded, randomized, controlled trials of NP colonization following PCV (Tables 1 and 2) (16, 18, 20, 21, 24, 60, 77, 88, 90, 93, 100, 120, 122), but the results of only 9 of these are fully published, and of these 9, only 4 studied the effect of a primary PCV series for infants on NP colonization (20, 77, 90, 93). In addition, we are aware of 14 studies which were not blinded, randomized, controlled trials (Tables 3 and 4) (12, 28, 31, 32, 47, 51, 56, 62, 63, 85, 89, 97, 103, 129). In spite of the design differences, taken together, these studies have shown that PCV has a direct effect on the carriage of serotypes covered by the vaccine, and this effect leads to epidemiologically important indirect effects among children and adults not themselves immunized with PCV, as discussed in detail below.

### **Effect on Pneumococcal Carriage of a Primary PCV Series for Infants**

The primary intended use of PCV is within routine infant immunization programs implemented worldwide. The schedules vary between countries and regions, but

all include two or three visits for children between 6 weeks and 6 months of age for the administration of the primary vaccine series. A booster dose of PCV in the second year of life is the recommended practice in economically developed countries but is not included in the schedules of developing countries. From the public health point of view, the primary interest is the effect of PCV on pneumococcal carriage when given on the routine childhood vaccine schedule.

Only four randomized trials with published results have addressed the effect of the primary three-dose PCV series on nasopharyngeal colonization. The PCV products used contained four, seven, or nine serotypes conjugated to tetanus or diphtheria protein or the cross-reactive material CRM<sub>197</sub>, a nontoxic form of diphtheria toxoid (20, 77, 90, 93). A significant reduction in VT colonization was shown in three of these studies (20, 77, 93), and a significantly increased risk of NVT colonization prior to the booster dose was shown only in the South African study (77). It is important to recognize that these studies varied considerably in the vaccine products administered, the number of specimens collected, the timing of specimen collection relative to vaccine doses, and the prevalence of all-serotype pneumococcal carriage in the study population. Thus, observed differences in vaccine efficacy against VT carriage should not necessarily be attributed to PCV product differences.

### **Effect on Carriage of a Pneumococcal Vaccine Booster Dose following PCV Priming**

Either the same PCV used in the primary series or the 23-valent PS vaccine (PS23) has been used as the booster dose product in a variety of both randomized controlled trials and observational studies (Tables 1 to 4). The first experience was in The Gambia, where children received a pentavalent CRM<sub>197</sub>-based vaccine at 2, 3, and 4 months of age and a booster dose of the PS23 at 18 months old; 2 months later, the prevalence of VT carriage in the age-matched control group was 90%, significantly different from the 50% prevalence in the vaccinated group of 26 children and from the 67% prevalence among 30 children who had received only two doses of PCV in infancy without a PS23 booster dose (89). Subsequent reports varied somewhat in their findings regarding VT carriage following the booster dose, with some showing a significant reduction in VT carriage (20, 21, 60) and some finding a reduction not large enough to be statistically significant (93, 100, 129). A number of these studies had small sample sizes, and in some of them, the sampling was done very soon after the administration of the booster dose (20) or

**Table 1** Randomized controlled trials of PCV effect on NP carriage, trial description<sup>a</sup>

Country (reference)	Control group vaccine	Blinded	PCV product(s)	No. of subjects (vaccinees; controls)	Age(s) at PCV	Age (mos) at or time of booster dose (vaccine)	No. of PCV doses	Age(s) at or timing of NP sample collection (no. of specimens per child)
Israel (18)	PS23	N	OMPC-7	263 (97, 98; 68)	12–18 mos	NA	1 or 2	3 mos post-dose 1, 1 mo post-dose 2, 12 mos post-dose 2 (3)
Israel (20)	Placebo	Y	D-4, T-4	75 (25, 25; 25)	2, 4, 6 mos	12 (PS23)	3	2, 4, 6, 7, 12, 13 mos (6)
United States (24)	MnC-C	Y	CRM-9	260 (130; 130)	2, 4, 6, 12 mos	12 (PCV)	4	2, 4, 6, 9, 12 mos (5)
South Africa (77)	Placebo	Y	CRM-9	500 (1:1 randomization)	1.5, 2.5, 3.5 mos	NA	3	6, 10, 14, and 18 wks and 9 mos (5)
Israel (21)	No vaccine	N	D/T-11	269 (185; 84)	2, 4, 6, 12 mos	12 (PCV)	4	18, 24 mos (2)
The Gambia (90)	IPV	Y	CRM-9	207 (103; 104)	2, 3, 4 mos	NA	3	5, 9 mos (2)
Finland (60)	Hepatitis B	Y	CRM-7	1,662 (1:1 randomization)	2, 4, 6, 12 mos	12 (PCV)	4	12, 18 mos (2)
Israel (16)	MnC-C	Y	CRM-9	262 (132; 130)	12–35 mos	NA	1 or 2	Monthly for 1 yr and every 2 mos for 1 year (18)
The Philippines (88)	MnC-AC	Y	D/T-11	180 (60, 60; 59)	Group 1, 1.5, 2.5, 3.5, 9 mos; group 2, 4.5, 9 mos	9 (PCV)	2 or 4	6 wks, 18 wks, and 9 mos (3)
The Netherlands (122)	Hepatitis A or B	Y	CRM-7	383 (190; 193)	1–7 yrs	7 mos post- PCV (PS23)	1 or 2	0, 7, 14, 20, 26 mos following dose 1 (5)
Czech Republic (100)	Hepatitis A	Y	HiD-11	352 (177; 175)	3, 4, 5, 12–15 mos	12–15 (PCV)	4	15–18 mos (1)
Belgium (120)	Hepatitis A	N	CRM-7	74 (38; 36)	1–7 yrs	PS23	1 or 2	0, 7, 14, 20, 26 mos following dose 1 (5)
United States, American Indians (93)	MnC-C	Y	CRM-7	566 (294; 272)	2, 4, 6, 12 mos	12 (PCV)	4	7, 12, and 18 mos and at follow-up with colonized subjects (3–9)

<sup>a</sup>MnC-C, group C meningococcal conjugate vaccine; IPV, inactivated polio vaccine; MnC-AC, group A and C meningococcal conjugate vaccine; OMPC-7, PCV7-OMPC; D-4 and T-4, four-valent PCV, conjugated to diphtheria toxoid and tetanus toxoid, respectively; CRM-9, PCV-9-CRM; D/T-11, 11-valent PCV with PSs of individual serotypes conjugated to diphtheria or tetanus toxoid; HiD-11, 11-valent PCV conjugated to *H. influenzae* protein D; NA, not applicable; N, no; Y, yes.

**Table 2** Randomized controlled trials of PCV effect on NP carriage, trial results<sup>a</sup>

Country (reference)	Control group Pnc prevalence (%) postvaccination	Control group VT prevalence (%) postvaccination	Post-primary series effect on VT carriage	Post-primary series effect on NVT carriage	Post-booster dose effect on VT carriage	Post-booster dose effect on NVT carriage	Effect on VT carriage in toddlers	Effect on NVT carriage in toddlers
Israel (18)	41–49 at 12–18 mos prior to vaccine	18 at 12 mos, 26 at 15 mos, 30% at 18 mos	NA	NA	NA	NA	Reduction	No change
Israel (20)	43 at 7 mos	24 at 7 mos	Reduction	Increase (NS)	Reduction	Greater in controls than vaccinees (NS)	NA	NA
United States (24)	45	21	Reduction (NS)	Increase (NS)	NA	NA	NA	NA
South Africa (77)	61	36	Reduction	Increase	NA	NA	NA	NA
Israel (21)	57	57	NA	NA	No change	No change	NA	NA
The Gambia (90)	92	92	Reduction (NS)	Increase (NS)	NA	NA	NA	NA
Finland (60)	NA	12.2	Reduction (NS)	No change	Reduction	Increase	NA	NA
Israel (16)	NA	40–12	NA	NA	NA	NA	Reduction	Increase
The Philippines (88)	47	NA	Reduction at 9 mos	Increase (NS)	NA	NA	NA	NA
The Netherlands (122)	49	26	NA	NA	NA	NA	Reduction; significant post-booster dose	Increase; significant post-booster dose
Czech Republic (100)	22	11	NA	NA	Reduction (NS)	Reduction (NS)	NA	NA
Belgium (120)	43	26	NA	NA	NA	NA	Reduction	Increase
United States, American Indians (93)	65	26	Reduction	Increase (NS)	Reduction (NS)	NA Increase	NA	NA

<sup>a</sup>Pnc, pneumococcal; NA, not applicable; NS, not statistically significant.

Table 3 Observational studies of PCV effect on NP carriage, study description<sup>a</sup>

Country (reference)	Control group	PCV product(s)	No. of subjects (vaccinees; controls)	Ages at PCV	Age (mos) at booster dose (vaccine)	No. of PCV doses	Age(s) at or timing of NP sample collection (no. of specimens per child)
The Gambia (89)	Y (no vaccine)	CRM-5	240 (56; 184)	2, 3, 4 mos	18 (PS23)	2 or 3	24 mos (1)
Iceland (62)	Y (no vaccine)	D-8, T-8	111 (81; 40)	3, 4, 6 mos	13 (PCV or PS23)	3 or 4	3, 4, 6, 7, 10, 14, 18 mos (7)
United States (28)	N	CRM-7	742	2, 4, 6, 12-15 mos	12-15 (PCV)	4	Cross-sectional (1)
United Kingdom (63)	Y (no vaccine)	CRM-7	581 (267; 314)	2, 3, 4 mos	13 (PS23)	3	1-4 years (1 or 2)
United States (129)	Y (no vaccine)	OMPC-7	81 (49; 32)	2, 4, 6, 12 mos	12 (PCV)	4	2, 6, 7, 12, 13 mos (5)
United States (32)	N	CRM-7	278	2, 4, 6, 12 mos	12 (PCV)	4	2, 4, 6, 9, and 12-15 mos and 2-3 mos post- booster dose (6)
United States (Alaska Natives) (85)	N	CRM-7	1,275	2, 4, 6, 12-15 mos	12-15 (PCV)	4	Cross-sectional (1)
United States (97)	N	CRM-7	275	2, 4, 6, 12-15 mos	12-15 (PCV)	4	All well-child and otitis media sick visits (multiple)
Spain (31)	Y (no vaccine)	CRM-7	695 (238; 457)	6 mos-6 yrs (1-3 doses)	NA	1-3	2-yr period (6)
United States (Alaska Natives) (47)	N	CRM-7	Not stated (>1,800)	2, 4, 6, 12-15 mos	12-15 (PCV)	4	Cross-sectional (1)
United States (51)	N	CRM-7	996	2, 4, 6, 12-15 mos	12-15 (PCV)	4	Cross-sectional (1)
United States (56)	N	CRM-7	106	2, 4, 6, 12 mos	12 (PCV)	1-4 (vaccine shortage)	Medical visits (multiple)
France (13)	N	CRM-7	1,906	2, 3, 4, 12-15 mos	12-15 (PCV)	4	Cross-sectional (1)
United States (103)	Y (no vaccine)	CRM-7	417 (217; 200)	2, 4, 6, 12-15 mos	12-15 (PCV)	1-4 (vaccine shortage)	At study enrollment (1)

<sup>a</sup>Abbreviations are as defined in Table 1.

Table 4 Observational studies of PCV effect on NP carriage, study results<sup>a</sup>

Country (reference)	Control group Pnc prevalence (%) postvaccination	Control group VT prevalence (%) postvaccination	Post-primary series effect on VT carriage	Post-primary series effect on NVT carriage	Post-booster dose effect on VT carriage	Post-booster dose effect on NVT carriage	Overall effect on VT carriage	Overall effect on NVT carriage
The Gambia (89)	94	90	NA	NA	Reduction	Increase	NA	NA
Iceland (62)	44	40	Not reported	Not reported	Not reported	Not reported	Reduction	Increase
United States (28)	26	9	NA	NA	NA	NA	NA	NA
United Kingdom (63)	13 and 31 by season	27 and 41 by season	NA	NA	NA	NA	Reduction (NS)	Increase (NS)
United States (129)	53	28	No change	No change	Reduction (NS)	Not reported	NA	NA
United States (32)	30	14	No change compared with 6 mos	Increase compared with 6 mos	Decrease compared with 12 mos	Increase compared with 12 mos	NA	NA
United States (Alaska Natives) (85)	36–41	18–20	NA	NA	NA	NA	Reduction over time	Not reported
United States (97)	29	22	NA	NA	NA	NA	Reduction from 2000 to 2003	Increase from 2000 to 2003
Spain (31)	67	NA	NA	NA	NA	NA	Not reported	Not reported
United States (Alaska Natives) (47)	Varies by age	Varies by age	NA	NA	NA	NA	Reduction over time	Not reported
United States (51)	26	9	NA	NA	NA	NA	Reduction over time	Increase over time
United States (56)	NA	NA	NA	NA	NA	NA	Increase with increasing dose interval	Not reported
France (13)	70	44	NA	NA	NA	NA	Reduction over time	Increase over time
United States (103)	48	NA	NA	NA	NA	NA	Not reported	Not reported

<sup>a</sup>Abbreviations are as defined in Table 2.

many months or years after the booster dose (93), making generalizations of the booster response challenging. The preponderance of evidence indicates that boosting with either PS23 or PCV results in further reductions in vaccine serotype carriage among vaccinees and may be the critical factor for reducing VT carriage. An increased prevalence of serotypes not covered by the vaccine often but not always accompanied the reduction of VT carriage, and the overall prevalence of pneumococcal carriage generally did not change.

### Effect on Pneumococcal Carriage of PCV Given Later in Childhood

In addition to infants, another target group for PCV is children attending day care centers, where pneumococcal carriage and respiratory infections are very common. Extensive evaluations have been conducted in Israeli day care centers. A pneumococcal heptavalent meningococcal outer membrane protein complex (OMPC) conjugate vaccine was given to children 12 to 18 months old in a study with subjects randomized to receive either one dose of PCV or the PS23 vaccine; another group received two doses, 3 months apart, of the PCV (18). One month after the first vaccine dose, there was no impact on the VT or NVT colonization risk, while a statistically significant reduction in VT carriage from 25% in the PS group to 9% in the PCV groups was seen 3 months after the first vaccine dose and a further reduction to 7% was measured 3 months after the second dose of PCV. The vaccine effect persisted for at least 1 year after the last vaccine dose. In none of the groups was there an effect on serotypes not in the vaccine.

In another large day care center trial, the nine-valent PCV-CRM<sub>197</sub> (PCV9-CRM<sub>197</sub>) vaccine was given in two doses if the child was 12 to 17 months old and only one dose if the child was older (15, 16). Carriage data were obtained at scheduled visits 1 month or, in the second year of follow-up, 2 months apart and analyzed in 1-year age groups. The overall prevalence of the carriage of VT pneumococci was 21% in the control (meningococcal vaccine) group and 13% in the PCV recipients, demonstrating a significant reduction of carriage in all age groups up to 4 years. A significant vaccine effect was shown individually for the vaccine serotypes 6B, 9V, 14, 19F, and 23F and also the vaccine-related serotype 6A but not for serotype 19A. Corroboration of the effect of PCV7-CRM<sub>197</sub> in the day care center context has also been reported from Portugal (31).

The effect of PCV has also been studied among children with recurrent otitis media. In two such studies, from Belgium and the Netherlands, PCV7-CRM<sub>197</sub> was

followed 7 months later by the PS23 vaccine and was given to 1- to 7-year-old children (120, 122). In the Belgian study, a clear reduction in vaccine serotype colonization was seen among the PCV recipients 7 months after the PS booster dose but not before or 13 to 19 months after the booster (120). In the study in The Netherlands, the effect of the PCV-PS23 regimen on vaccine serotypes was statistically significant 7, 13, and 19 months after the booster dose (122).

### Long-Term Effects of PCV on Pneumococcal Colonization

How long do the effects of PCV immunization on nasopharyngeal carriage of pneumococci last? Unfortunately, few data are available, and the existing data are partly contradictory. As cited above, in the day care center context, the reduction of colonization by vaccine serotypes in the PCV recipients compared to that in controls lasted for at least 1 year (16, 19). In the Finnish otitis media study described in chapter 20 (26), a survey 3 to 4 years after the last dose of vaccine (PCV7-CRM<sub>197</sub> given at 2, 4, 6, and 12 months of age) showed 8.5% of the vaccinated children to be VT carriers, compared to 13.6% of those receiving control vaccine (i.e., hepatitis B vaccine), a significant effect (96). The issue of the duration of protection was explored in a third trial in which the study design involved community randomization of American Indian infants and children to receive either PCV7 or a control vaccine (91). The children received PCV7-CRM as three doses in infancy and a booster dose at 12 to 15 months old; the control vaccine was group C meningococcal conjugate vaccine. Carriage was assessed a median of 27 months after the last vaccine dose (82). The prevalence of VT carriage was 17% in the control group and 10% in the PCV recipients, a significant difference. From the results of these randomized studies, it appears that PCV can reduce VT carriage for long periods of time; this observation is supported by findings from observational studies following routine introduction of PCV in the United States (47, 51, 85, 97), but how various factors within the community affect this reduction is not clearly understood.

In contrast with the above-cited studies, a Belgian study found a reduction in VT carriage at 7 months but no longer at 13 to 19 months after the booster PS vaccine (120) and a United Kingdom study found no effect of the vaccine 2 to 3 years after a series of three doses of PCV7-CRM<sub>197</sub> in infancy followed by a booster dose of PS vaccine (63). The latter study, however, was an observational study, not a randomized trial, and the vaccine effect was not assessed at an earlier time.

**Effect of PCV on Nonpneumococcal Colonization**  
Pneumococci are only one part of the mixed flora in the nasopharynx, and the changes in pneumococcal colonization brought about by PCV vaccination may have some effects on the other components of that flora. *H. influenzae* and *Moraxella catarrhalis* are common constituents of the microflora of the human nasopharynx and, after pneumococci, are the most common causes of upper respiratory infection; therefore, they could be expected to be affected by changes in pneumococcal colonization. In the above-mentioned Belgian study of children vaccinated when 1 to 7 years old (one dose of PCV7-CRM<sub>197</sub> followed 7 months later by the PS23 vaccine) and monitored by NP sampling for a total of 26 months, no evidence of a difference between the vaccinated and control (hepatitis A vaccine) children could be found in the prevalence of carriage of *H. influenzae* (76 versus 71%) or *M. catarrhalis* (60 versus 54%) (120). However, when bacterial interference in the nasopharynx was examined by a modeling study with data from 280 Australian children under 2 years of age, positive bacterium-bacterium associations were found, particularly between the pneumococcus and *M. catarrhalis* (54, 55), and a strong negative association between the pneumococcus and *Staphylococcus aureus* was found (124).

The participation of *S. aureus* in interference with the pneumococcus had been suggested by the observation that children with recurrent otitis media who had been vaccinated with PCV when 1 to 7 years old more often had *S. aureus* in their ear discharge than those who had received the control hepatitis vaccine (122). This led to the obvious question of whether this was a reflection of the vaccine's effect on *S. aureus* colonization and to the worry that the use of PCV would further increase the spread of methicillin-resistant *S. aureus*. Two studies surveyed colonization by the pneumococcus and *S. aureus* in a large number of subjects and found a negative association between the two bacteria (6, 101), although the different age distributions of carriage of the two species make comparisons difficult. So far, a negative impact on *S. aureus* disease has not been observed, but sustained surveillance with this possibility in mind is warranted.

### Observational Studies of PCV Effects on Pneumococcal NP Colonization

In the United States, PCV was introduced into the national infant immunization program as soon as it was licensed in 2000, and randomized, controlled carriage studies could no longer be conducted; observational studies, although generally unable to control for ex-

pected secular trends in pneumococcal colonization, are tremendously useful for demonstrating the effects of vaccine when used in the routine use setting. Ghaffar et al. carried out a longitudinal study of 200 children who received Prevnar at 2, 4, and 6 months of age, with a booster dose at 12 months (32). The effect of the vaccine on colonization was assessed by comparing results as the children aged (i.e., comparing samples from children at earlier ages to those from children at later ages). In the samples from children at the age of 12 months, i.e., 6 months after completion of the primary series, the VT colonization prevalence was 18% and thus higher than that observed in the previous sample set from children 6 months of age, consistent with the normal increase of carriage prevalence at this age. Three months after the booster, however, the prevalence of VT carriage had decreased to 9%, which the authors inferred was attributable to the vaccine. Other longitudinal studies have shown that as vaccine coverage has increased, the VT prevalence has decreased (Tables 3 and 4) (47, 51, 85); the inference drawn from these longitudinal observational studies is that the reduction in VT colonization over time can be attributed to PCV use.

The experience accumulated from the large-scale use of PCV has also allowed an analysis of the immunization schedule. During a transient shortage of vaccine, many children received only two or three doses of PCV or had longer intervals than usual between doses. Carriage in a cohort of 106 children was monitored over several health maintenance visits (56). This study showed that if the interval between the second and third doses was greater than 3 months or if that between the third and fourth doses was greater than 8 months, then there was greater VT carriage than if the intervals were shorter.

### Direct Effect of PCV on Antimicrobial Resistance

Since much of the antibiotic resistance and multidrug resistance among *Streptococcus pneumoniae* is found in serotypes included in the vaccines and related serotypes, the reduction of carriage of the vaccine serotypes and related serotypes is expected to be associated with the reduction of carriage of antibiotic-resistant pneumococci. This issue is dealt with in detail by Dagan and Klugman in chapter 25 of this volume.

### Summary of the Direct Effects of PCV on Colonization

The foremost conclusion from the clinical trials and subsequent experience is that PCVs indeed reduce the prevalence of NP colonization by serotypes included in

the vaccines. The effect has been demonstrated for all the serotypes in the PCV7-CRM<sub>197</sub>, and consistently, the effect on serotype 19F has been the lowest (15, 16, 51, 82, 122). The serotypes related to the vaccine serotypes, i.e., belonging to the same serogroups, differ; the vaccine clearly reduces the prevalence of serotype 6A but increases the prevalence of 19A and 23A. The frequency of serotypes not in the vaccine as a rule increase in the vaccinated children relative to those not vaccinated; prominent increases have been reported for types 11A, 15, 16, 17F, 22F, and 29 (51, 82, 122). The final result of these differential effects on VT and NVT pneumococci is that the overall prevalence of carriage (of any pneumococci) is not changed by PCV. The magnitude of the reduction in VT carriage is usually given by comparing the prevalences in vaccinated and control groups, which indicates an approximately 40% protective effect. A more accurate estimate could be obtained by focusing on new acquisitions only.

The effects on colonization appear to be a characteristic of all PCVs, having been seen with different PCVs, including several investigational ones. The effects are equally seen after immunization in infancy and in toddler-age children and after the primary series and a booster dose that can be PCV or PS vaccine. The data concerning the duration of vaccine effects are partially contradictory for children between 1 and 5 years of age. The effector mechanism by which PCV immunization reduces carriage prevalence is not known so far. However, it is clear that the vaccine effect is directed to preventing the new acquisition of colonization, not reducing the duration of colonization (15, 93). The effect is also on the reduction in the density of vaccine serotype colonization (30, 93). Furthermore, it appears that the effect is correlated with the concentration of type-specific immunoglobulin G (IgG) antibodies in serum (15, 83).

## INDIRECT EFFECTS OF PCV VACCINATION

The direct effect of PCV on pneumococcal carriage is to reduce vaccine serotype and increase nonvaccine serotype colonization among vaccinated children. This dramatic effect among the vaccinees results in equally dramatic effects on the transmission of the pneumococcus within households, day cares, and communities as a whole. The effect of PCV on those people who themselves are not immunized is termed the indirect effect. Because the natural transmission of the pneumococcus is from children (mostly toddlers and young children) to other individuals, we expect that the impact of PCV on infant and toddler vaccinees should result in similar pat-

terns of carriage impact among the nonvaccinated community members.

The first evidence that such a phenomenon exists comes from a study with PCV9-CRM<sub>197</sub>, conducted by Givon-Lavi et al. in southern Israel (33). In a double-blind study, 264 day care center attendees aged 12 to 35 months were randomized to receive PCV9-CRM<sub>197</sub> or control vaccine. The children were monitored for 2 years, and NP cultures were obtained every 1 to 3 months. Forty-six young siblings of these children, aged <18 months, were also enrolled and monitored for NP carriage until they reached the age of 18 months or started attending day care centers. Of 306 samples obtained from the younger siblings, 151 (49%) were positive for pneumococci. The prevalence of vaccine serotype *S. pneumoniae* was 21% in younger siblings of vaccinated children, compared with 34% in siblings of the nonvaccinated children ( $P = 0.017$ ). For nonvaccine serotypes, the reverse trend was seen: 44 versus 34%, respectively ( $P = 0.15$ ).

A large NP colonization study of both direct and indirect effects was conducted among American Indians in the course of a phase III community-randomized efficacy trial of PCV7-CRM (93). In that study, the children in the households of study participants were also enrolled in the nested NP study. Those children who lived in households with PCV7-CRM-vaccinated children were less likely to be colonized with vaccine serotype strains, but only if they attended a day care center. Children who were too young to be immunized (i.e., infants less than 2 months old) were also found to be at lower risk for VT carriage if they lived in a household or community of PCV7-CRM-vaccinated infants and children (93). Furthermore, in a cross-sectional study of American Indian adults, immediately following the randomized trial, the adults were found to be at lower risk of VT colonization if they lived in a community where PCV7-CRM<sub>197</sub> was used than if they lived in a community where group C meningococcal conjugate vaccine was administered to the infants (126).

Since the introduction of PCV into the routine immunization schedule, several observational studies of NP colonization among unimmunized community members have been conducted. Detailed information of the effect of universal childhood immunization on pneumococcal carriage in other nonvaccinated age groups derives from Alaska (41, 47, 85). From 1998 through 2004, the Centers for Disease Control and Prevention conducted community-wide surveys of pneumococcal NP colonization in eight rural villages in Alaska during the months of April and May of each year. Hennessy et al. (47) showed

that the carriage of vaccine serotypes decreased in all age groups ( $\leq 1$  year, 2 to 4 years, 5 to 17 years, and  $\geq 18+$  years) in a very similar manner, demonstrating clear indirect immunity to the carriage of vaccine serotypes of *S. pneumoniae*. However, the decrease was still greater in children  $< 5$  years of age than in any other age group. Moore et al. (85) reported that children enrolled in studies after the introduction of the universal immunization program in the United States were at reduced risk of carrying vaccine serotype pneumococci, independent of vaccination status: children enrolled in 2002 were 49% less likely to carry vaccine serotypes than children enrolled in 2000 (adjusted odds ratio, 0.51; 95% confidence interval [CI], 0.30 to 0.87) and were 40% less likely to carry vaccine serotypes than children enrolled in 2001 (adjusted odds ratio, 0.60; 95% CI, 0.37 to 0.97). The decreases in colonization with vaccine serotypes of *S. pneumoniae* in unvaccinated children in 2000, 2001, and 2002 were 54, 48, and 40%, respectively ( $P = 0.057$ ).

Hammitt et al. (41) studied specifically the effect of universal childhood vaccination on the carriage of *S. pneumoniae* in adults (aged  $\geq 18$  years) in Alaska. During the 7 years from 1998 to 2004, a total of 15,598 NP swabs were collected from a mean of 2,228 participants per year. During the study years, the proportion of children of  $< 5$  years that carried *S. pneumoniae* did not change significantly: 59% at baseline (1998 to 2000) versus 61% in 2004 ( $P = 0.91$ ). By contrast, a significant upward trend was observed among adults  $\geq 18$  years of age (13% at baseline versus 26% in 2004;  $P < 0.001$  for trend). Among adults, the proportion colonized with vaccine serotypes decreased from 28 to 5% in the same period ( $P < 0.001$  for trend), and this significant trend was observed for all adult age groups (18 to 24, 25 to 34, 35 to 44, and  $\geq 45$  years of age). At the same time, a marked increase in the proportion of adults with colonization due to nonvaccine serotypes was observed. Single nonvaccine serotypes for which a significant increase was demonstrated were serotypes 12F, 17F, 19A, 23A, and 34. Carriage of these serotypes (with the exception of 12F, for which the sample size was insufficient) also significantly increased among children  $< 5$  years of age. Adults living with a child had a higher likelihood of carrying vaccine serotype pneumococci than did adults who were not living with a child (adjusted odds ratio [adjusted for age of adults and year], 1.81 [95% CI, 1.31 to 2.50]). Adults living in households in which  $\geq 1$  child had been age appropriately vaccinated with PCV had a lower likelihood of carrying vaccine serotypes of *S. pneumoniae* than did

adults living in households with no child who was age appropriately vaccinated with PCV7 (adjusted odds ratio [adjusted for year], 0.49 [95% CI, 0.28 to 0.83]).

In summary, then, both blinded randomized controlled trials and observational studies have shown that PCV immunization of infants and children reduces vaccine serotype colonization in children too young to be immunized and older children and adults who are not eligible for immunization. These studies have also shown an increased risk of NVT carriage among these unimmunized age groups.

## ANTIMICROBIAL RESISTANCE OF NP COLONIZING ISOLATES

PVC has a significant effect on the prevalence of antimicrobial resistance among NP pneumococcal isolates. This important epidemiologic and therapeutic topic is addressed in detail in chapter 25 and is not further described here.

## MECHANISMS OF REPLACEMENT CARRIAGE

The observation of an increase in the prevalence of NVT pneumococcal carriage among children who have been vaccinated and their unimmunized contacts has been demonstrated in numerous studies of NP colonization using a variety of conjugate pneumococcal vaccine products in diverse epidemiologic settings in numerous countries around the world (Tables 1 to 4). This phenomenon has been termed replacement carriage, as it is presumed to reflect a true increase in the risk of NVT pneumococcal NP colonization following the receipt of PCV. This same clinical observation (i.e., that vaccinated subjects are more likely to carry NVT pneumococci than unvaccinated subjects) may also result from an enhanced ability to detect NVT pneumococci in NP specimens of vaccinated children rather than an actual increase in the risk of colonization. The former mechanism is termed unmasking while a true increase in NVT NP colonization risk is termed replacement colonization.

To distinguish whether an observed increase in the risk of NVT carriage among vaccinated subjects results from replacement carriage or unmasking necessitates a highly sensitive assay to detect the presence of multiple-serotype carriage. Most studies have employed the strategy of serotyping a single colony from each NP sample, which offers no opportunity to detect multiple-serotype carriage; serotyping more than one colony has limited ability to detect the true prevalence of multiple-serotype

carriage if the distributions of the various serotypes is very imbalanced (52). Four PCV studies routinely selected more than one colony for serotyping from each NP sample (13, 77, 90, 129). Another study developed and implemented a novel immunoblot method for detecting multiple-serotype carriage (8, 93) which had the ability to detect a second or third serotype even if it constituted only 1 to 3% of the pneumococci in an NP specimen. The latter study, using this highly sensitive method to detect multiple-serotype carriage, also detected an increase in the proportion of vaccinated subjects who carried nonvaccine serotype strains compared with unvaccinated subjects, confirming that this observation results from true replacement colonization and not just the unmasking of nonvaccine serotypes that otherwise were going undetected.

Accepting that replacement carriage is a real phenomenon, there are several possible mechanisms for how such replacement carriage occurs. Which one or combination of these mechanisms is responsible for the replacement phenomenon and with what frequency this occurs has not been clearly established. The three possible mechanisms are (i) capsular serotype switching such that previous VT clones are now NVT clones, (ii) the expansion and propagation of existing NVT clones in the community, and (iii) the introduction of new NVT clones into the community.

Capsular serotype switching among pneumococcal isolates causing invasive disease has been clearly described (95, 99). To demonstrate that such switching has occurred requires an ability to characterize a pneumococcal strain by means other than the capsule. Such strain characterizations have been done using multilocus sequence typing, antibiogram characterization, and ribotyping.

Another mechanism by which replacement disease may occur is through the expansion of NVT clones that are already circulating in communities prior to the introduction of PCV. The biological basis for this hypothesized mechanism is that the immune response to PCV confers protection against the acquisition or proliferation of VT pneumococcal strains in the nasopharynx. The reduction in VT pneumococcal carriage thereby opens a biological niche in the nasopharynx that is filled by NVT pneumococcal strains which were less fit for colonization in the presence of VT strains.

A third mechanism for NVT replacement colonization is the introduction of new NVT clones into the community. The hypothesis is that the elimination of VT strains by using PCV provides a biological niche which can be filled by exposure to circulating NVT strains which were previously less fit to proliferate in the

presence of VT strains. This mechanism would necessitate a community that had ongoing, episodic, or regular interaction with other communities which themselves had a distinct set of serotypes causing colonization or the introduction from another community of an otherwise infrequent pneumococcal strain.

A small number of studies have been conducted which attempt to address the contribution of each of these three mechanisms. An analysis of the pneumococcal NP, invasive, and ear isolates from the American Indian PCV7-CRM<sub>197</sub> efficacy trial concluded that the expansion of existing clones rather than the introduction of new clones or capsule switching was the predominant mechanism of serotype replacement (69). Other studies have reached similar conclusions (42, 95). However, all of these studies are limited by the time period of isolate collection; new clone introduction may take place over a longer time period than the observation period of the studies and may yet prove to be a mechanism by which NVT strain proliferation occurs.

## IMMUNE CORRELATES AND MECHANISMS OF EFFECTS OF PCV ON NP CARRIAGE

As described in "Role of Acquired Immunity in Colonization" above, natural exposure to NP pneumococcal colonization likely results in an antibody response to PSs and proteins and a cellular immune response. In the natural setting, the interaction of these three responses and the relative importance of each by age and setting are not fully understood. Nevertheless, it is clear that PCV administration, which induces antipolysaccharide antibodies, protects immunized individuals from colonization with vaccine serotype strains of pneumococci. Efforts have been directed to characterize the immune mechanisms behind that protective effect.

Three recent studies have assessed the relationship between preexisting circulating IgG and protection against serotype-specific carriage; two of these assessed this relationship among children immunized with PCV (15, 83), and one assessed this relationship among unimmunized adults (34). All three studies demonstrated that, at least for some serotypes, increasing serum antibody concentrations are associated with a statistically significant reduction in the risk of colonization. Combining the findings from all three studies, the serotypes for which this was demonstrated were types 6A, 14, 19F, and 23F. The adult colonization study also assessed whether an antibody concentration of 5 µg/ml (an *a priori* hypothesis) was associated with protection against serotype-specific carriage and found it to be the case for serotype 14. The study of American Indian chil-

dren explored the antibody concentrations observed among PCV-immunized and unimmunized subjects and found that no subjects colonized with type 14 had an antibody concentration above 4 µg/ml. The limited number of colonized subjects did not allow a more detailed analysis of a specific antibody concentration that would predict protection against colonization; however, the similarity of the findings between the adult colonization study and this analysis is striking.

How systemically circulating serotype-specific PS antibodies prevent the subsequent NP acquisition of that serotype, reduce the duration of carriage, or reduce the density of colonization is an important determinant of whether vaccine-induced antibodies will have these effects. Following vaccination and the induction of serotype-specific IgG, it is believed that antibody passively crosses the nasal mucous membrane and blocks the attachment of the organism to receptors on the epithelial cells. This may occur by steric inhibition of pneumococcal protein components necessary for epithelial attachment or possibly by direct inhibition of the colonization enhancing function of PS itself. Mouse studies have shown that small amounts of PS are required for epithelial attachment, suggesting that steric inhibition of pneumococcal cell surface proteins may not be the only mechanism of action of serotype-specific IgG.

It is conventionally held that PCV reduces the risk of acquisition of new pneumococcal strains but does not affect the duration of colonization once established. However, only two studies with published reports have assessed the effect of PCV on the duration of colonization, one among day care center-attending toddlers (15) and one among American Indian infants (93). In neither study did pneumococcal conjugate vaccination affect the duration of colonization. Further studies of this question with other age groups and epidemiologic settings are warranted, as the effect may vary by age at vaccination, age at colonization, and serotype. To fully assess whether PCV diminishes the duration of colonization requires studies with frequent sampling of the NP microflora because carriage episodes can be quite short lived (i.e., days to weeks in duration). Circulating systemic IgG, either naturally acquired or induced by PCV administration, may also affect the density of colonization in addition to the absolute risk of colonization (30, 93). This possibility has been evaluated in only a single published study, in which vaccination with PNC7-CRM<sub>197</sub> led to a reduced risk of VT colonization and, once colonization occurred, those vaccinated with PNC7-CRM<sub>197</sub> had lower densities of colonization with the VTs but not the NVTs than those immunized with control vaccine (93).

## MODELING NP COLONIZATION AND PCV EFFECTS

There are three phases of a nasopharyngeal colonization episode: acquisition, the period of colonization, and clearance of the organism. Because acquisition events are relatively frequent in childhood and generally asymptomatic or, if symptomatic, cause symptoms that are mild and nonspecific in nature, detecting these events requires a sampling frequency that few study participants would agree to. For logistical and study acceptability reasons, most NP studies are able to collect sequential samples from subjects every 2 to 8 weeks over a longitudinal time period. Furthermore, samples are usually collected using a single swab per sampling time period. The sensitivity of a single swab for the detection of pneumococcal colonization is less than 100%, and thus, colonization episodes are certainly left undetected by this method. Once samples are collected, the detection of pneumococci in the lab typically involves standard culture techniques and serotyping of one or, in a limited number of studies, up to five colonies per plate. These study design limitations (i.e., the limited frequency of sampling, the limited sensitivity of the specimen collection technique, and the limited ability of the lab methods to detect more than one cocolonizing serotype) mean that serotypes with long durations of colonization, serotypes dominant in cocolonization episodes, and colonization episodes with high densities are more likely to be detected than other serotypes and colonizing events. The selective detection of episodes with these characteristics necessarily imparts a bias to studies of PCV effects on NP colonization.

If these study design limitations are not accounted for in the analyses of epidemiologic NP studies and, by extension, studies of PCV effects on NP colonization, inferences about the effects of vaccine on the colonization state may be inaccurate or incorrect. One strategy to overcome these vexing limitations of our currently available study procedures is the use of conceptual and statistical models. Outputs from models have been useful for various purposes, including informing the key variables in clinical study design, e.g., defining the maximum sampling interval for longitudinal studies (22, 81) and establishing the importance of lab method sensitivity for the detection of multiple-serotype colonization (67). Models have also been indispensable for estimating acquisition rates from data from cross-sectional longitudinal studies (81, 110), evaluating the intrafamilial transmission of the organism (80, 81), estimating the duration of carriage and its association with the age of the host at colonization and the invasiveness of the organism (2, 25, 109), and understanding the natural

evolution of the genetic population structure of the organism (29).

Statistical models have also been used to infer the effect of PCV on NP colonization by evaluating through the longitudinal observation of multiple samples whether PCV can affect the duration of colonization (15, 93), evaluating the protective effect of preexisting serum antibody on NP acquisition (15, 34, 83), and evaluating individual- and community-level effects of the vaccine (41, 47, 85, 93).

There are efforts, through the PneumoCarr Consortium (see the acknowledgment), to determine whether NP colonization could be used as a primary or adjunct end point for PCV efficacy evaluations and licensure. Currently, the approach for licensing new PCV products or the addition of serotypes to existing PCV products is based on demonstrating noninferiority in achieving a serologic correlate of efficacy against invasive pneumococcal disease (128). This approach has some limitations; the existing correlate of protection is not serotype specific, it does not necessarily correlate with NP protection, and does not necessarily predict that NP protection will be achieved. Protection against VT NP carriage is the mechanism through which the indirect effects of PCV are conferred; these indirect effects are a major benefit of the currently licensed PCV. The approach for licensure of new products or additional serotypes is the best that can be done currently, but there remains considerable scope for exploring improvements to this regulatory pathway with the use of efficacy against NP colonization as an end point.

Assessing the effect of a PCV product on NP colonization requires that we first understand the determinants of NP colonization so that those determinants can be controlled for in analyses of PCV effects. Given the array of host, community, environmental, and pathogen characteristics which determine the experience of an individual with pneumococcal colonization, there is no one study which can independently assess the relationship of these factors in the causal pathway of colonization or their roles as confounders. As a result, we must rely on models to explore and predict the impact of variables on colonization and incorporate that understanding into analyses of the effect of PCV on the ecology of the pneumococcus and, by extension, on pneumococcal disease.

## CONCLUSIONS

In summary, a variety of studies evaluating the effects of PCV on both individual- and community-level colonization have been undertaken in both the prelicensure and

postlicensure settings. The controlled, randomized trials are best able to result in inferences about the causality of effects observed since they control for secular changes in serotype distribution which are known to commonly occur.

From these studies, we have learned that at the level of the vaccinated individual, PCV protects against the acquisition of vaccine serotype carriage while simultaneously increasing the risk of carriage of nonvaccine serotypes that are otherwise observed to be colonizing serotypes. There is no change in the overall risk of pneumococcal carriage among vaccinated individuals as a result of these two opposing effects. To date, there is no evidence that PCV reduces the duration of carriage; however, there are very few studies of this effect. A single study has evaluated the effect of PCV on the density of VT colonization and found that those vaccinated and subsequently colonized are nevertheless colonized with a lower density of VT organisms than those not vaccinated.

At the community level, PCV also provides protection against VT colonization of unimmunized individuals and results in replacement colonization with NVT serotypes. These effects have been observed among children too young to be vaccinated, unvaccinated age-eligible children, and adults in the community. These profound effects on the community-level circulation of pneumococcal strains are self-perpetuating, as the broad use of the vaccine may drop the circulation of vaccine serotypes to almost 0, even though in controlled trials the efficacy against VT colonization is only about 50%.

NP studies have also demonstrated that preexisting circulating serotype-specific antibody is associated with a reduced risk of colonization, at least for some serotypes. The concentration of antibody required to exert this effect is likely to be 4 to 5 µg/ml, as evaluated in two studies. Although other mechanisms of protection from NP colonization have been shown in animal studies and in human natural history studies or challenge studies, circulating IgG has now been well established to be a key protective mediator through PCV studies.

The complexity of the NP-pneumococcal ecosystem interaction and its determinants necessitate the use of models to fully explore and thereby account for covariates and confounders relevant to the design and analysis of PCV effects. The key to understanding and ultimately predicting the effect of PCV on disease is having an in-depth understanding of how these many factors interact to affect colonization from which invasive disease emerges. The challenges in the future rest on establishing the maximum public health impact of vaccine dose

schedules at the time of country-level introduction, introduction strategies (i.e., catch-up or no catch-up regimens), and vaccine dose regimens following the introduction phase so that the maximum impact of PCVs are felt in the target population and throughout the community beyond those children who are age eligible for vaccination.

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Terhi Kilpi  
Lode Schuerman

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# Acute Otitis Media and Its Sequelae

## BURDEN OF PNEUMOCOCCAL AOM

Acute otitis media (AOM) is one of the most commonly diagnosed infections of childhood in the industrialized world. In the United States alone, it accounts for more than 20 million pediatric medical visits per year (30). AOM is also a major source of health problems in children in developing countries (3). AOM is most prevalent in children under 2 years of age, with the peak of disease incidence in children between 6 and 18 months of age. It has been reported that approximately 60% of children have had at least one episode of AOM by 1 year of age (16, 28, 58).

The pathophysiology of mucosal pneumococcal infections such as AOM is different from that of invasive pneumococcal diseases (IPD) such as bacteremia and meningitis. The entry of pneumococci and other bacterial pathogens from the nasopharynx through the Eustachian tube into the middle ear is facilitated by the negative pressure in the middle ear cavity. This entry likely occurs more easily than the entry of pneumococci

into the lungs or into the bloodstream. It is probably because of this difference in the pathophysiology of AOM, compared to that of IPD such as bacteremia and meningitis, that vaccine efficacy estimates obtained for pneumococcal conjugate vaccines (PCV) in randomized clinical trials were systematically lower for protection against AOM (14, 16, 27, 50) than for protection against IPD (4, 31, 43). Moreover, due to the polymicrobial nature of AOM disease, protection against pneumococcal AOM only, even if high efficacy could be obtained, would result in only a limited impact on the overall burden of AOM disease.

Several studies have shown *Streptococcus pneumoniae* and *Haemophilus influenzae* (mainly the noncapsulated or “nontypeable” (*H. influenzae* [NTHI] form) to be the two leading bacterial pathogens causing AOM, followed by *Moraxella catarrhalis*, *Staphylococcus aureus*, and *Streptococcus pyogenes* (33).

Although *S. pneumoniae* was already recognized during the 19th century as an important cause of AOM

(2), the early development of the first killed whole-cell vaccines and the later capsular polysaccharide vaccines was almost abandoned when antibiotic drugs became available in the first half of the 20th century. Once it became clear that antibiotics would not prevent the sequelae of bacterial infections, and with the emergence of antibiotic resistance, interest in the pneumococcal vaccine development was renewed, leading to a number of clinical trials to evaluate the protective efficacy of capsular polysaccharide vaccines against otitis in infants and young children (24, 25, 34, 35, 54, 57). Results of these trials were disappointing, but they contributed to our global understanding of polysaccharide vaccines as T-cell-independent antigens that are unable to stimulate

a satisfactory antibody response in recipients in early infancy and childhood.

### PROTECTIVE EFFICACY OF PCV AGAINST AOM AND ITS SEQUELAE

A number of studies have evaluated the potential of PCV to prevent AOM and its complications. When evaluating the results of these studies, it is important to make a distinction between studies in which the subjects were vaccinated in early infancy and studies that enrolled older children with a history of previous attacks of otitis media. The effects of PCV appear to be different in these two populations.

**Table 1** Randomized, double-blind, controlled trials evaluating the efficacy of PCV immunization initiated in the first year of life against various types of otitis media<sup>a</sup>

Study, country(ies), yr begun	PCV(s), no. of recipients	Control vaccine, no. of recipients	Ages (mos) at vaccination	Duration of efficacy follow-up
Kaiser (NCKP), United States, 1995	PCV7-CRM, 18,927	MCV-C-CRM, 18,941	2, 4, 6, and 12–15	From enrollment (starting October 1995) until April 1999
FinOM, Finland, 1995	PCV7-CRM, 831, and PCV7-OMP, 835	HBV vaccine, 831	2, 4, 6, and 11–14	Until 24 mos of age for each child
POET, Czech Republic and Slovakia, 2000	PCV11-PD, 2,489	HAV vaccine, 2,479	3, 4, 5, and 12–15	Until 24 mos of age for each child

<sup>a</sup>MCV-C-CRM, *N. meningitidis* serogroup C conjugated to CRM<sub>197</sub>; HAV, hepatitis A virus; HBV, hepatitis B virus.

### Efficacy of PCV Immunization when Initiated during the First Year of Life

The efficacy of PCV primary immunization in the first year of life followed by a booster dose in the second year of life was evaluated in four different cohorts. As shown in Table 1, these trials varied in design, objectives, and case definitions. Per-protocol (PP) and intention-to-treat (ITT) analyses were performed in all studies. The PP follow-up always started 14 days after the third primary dose of PCV, whereas the ITT follow-up began the day of randomization and receipt of the first PCV dose. In all studies, the conclusions of the ITT analysis were in line

with those from the PP analysis, and therefore only PP results are discussed in this chapter.

Protective efficacy against otitis media was first evaluated for the PCV7-CRM vaccine. The first and largest trial, performed by the Northern California Kaiser Permanente (NCKP) health maintenance organization in the United States, was designed to demonstrate efficacy of PCV7-CRM against IPD due to vaccine serotypes, but allowed evaluation of the vaccine impact on overall otitis episodes and visits using clinical data registered in the NCKP databases (4, 16). A study in Finland was designed to evaluate the efficacy of two seven-valent

Primary end point	Secondary end points	Case definition for AOM	Case ascertainment
IPD due to pneumococcal vaccine serotypes	Otitis visits, clinical otitis episodes, recurrent otitis episodes, tympanostomy tube placements, first clinical otitis episode	No standardized clinical case definition. The otitis diagnosis was left to the clinical judgment of the treating physician	Otitis visits were identified using the computerized diagnoses of various types of otitis media as registered within the Kaiser health care system. No attempt to enhance visits due to otitis symptoms or to standardize diagnostic practices
Culture-confirmed pneumococcal AOM episodes due to vaccine serotypes	First episode of culture-confirmed pneumococcal AOM due to vaccine serotype, pneumococcal AOM due to any serotype, clinical otitis episodes, OME	Abnormal tympanic membrane in terms of color, position, or mobility suggesting middle ear effusion and at least one of the following signs or symptoms: fever, ear pain, irritability, diarrhea, vomiting, acute otorrhea not caused by otitis externa, or other symptoms of respiratory infection	Parents were encouraged to bring their child to the study clinic for evaluation of symptoms suggesting respiratory infection or AOM. Myringotomy and aspiration of MEF were performed at the study clinic if AOM was diagnosed
First episode of culture-confirmed pneumococcal AOM due to vaccine serotype	First episode of culture-confirmed AOM due to NTHI, first clinical otitis episode, culture-confirmed pneumococcal AOM due to vaccine serotypes, clinical otitis episodes, recurrent otitis episodes	Either the visual appearance of the tympanic membrane (i.e., redness, bulging, loss of light reflex) or the presence of effusion in the middle ear (as demonstrated by simple or pneumatic otoscopy or by microscopy). The presence of at least two of the following signs or symptoms was also required: ear pain, ear discharge, hearing loss, fever, lethargy, irritability, anorexia, vomiting, or diarrhea	Parents were advised to consult their pediatrician if their child was sick or had ear pain or spontaneous ear discharge. As per routine practice in the study areas, children with suspected AOM were referred to an ENT specialist for confirmation of the clinical AOM diagnosis and subsequent MEF sampling via tympanocentesis

PCV vaccines (PCV7-CRM and PCV7-OMP [*Neisseria meningitidis* outer membrane protein complex]) against culture-confirmed pneumococcal AOM episodes due to vaccine serotypes (14, 27). The reduction in AOM of any cause associated with the use of PCV7-CRM was remarkably similar in the U.S. and Finnish trials: 7 and 6%, respectively (14). Results of both trials are summarized in Table 2.

The routine collection of middle ear fluid (MEF) samples obtained through myringotomy in the Finnish otitis media (FinOM) study has provided insight into the overall impact of vaccination on otitis media episodes. Table 3 shows the results for both seven-valent PCV tested in the FinOM study (14, 27). The serotype-specific efficacy estimates for the seven pneumococcal serotypes contained in both vaccines were very similar, as were the overall effects against AOM episodes caused by all pneumococcal vaccine serotypes combined. The efficacy estimates for end point of the first AOM episode due to vaccine serotypes were 52% (95% confidence interval [CI], 39 to 63%) and 50% (95% CI, 36 to 61%) for PCV7-CRM and PCV7-OMP, respectively, and those for the end point of any vaccine serotype AOM episode were 57% (95% CI, 44 to 67%) and 56% (95% CI, 44 to 66%), respectively. For PCV7-CRM, the pneumococcal serotype-specific efficacy estimates reached statistical significance for serotypes 6A, 6B, 14, and 23F, and for PCV7-OMP, additionally those for serotypes 9V and 19F but not for 6A reached significance (Table 3). The similarity of estimates of serotype-specific vaccine efficacy against pneumococcal AOM for both vaccines is remarkable given the lower

post-primary antibody responses to serotypes 6B, 9V, 14, 18C, and 23F elicited by PCV7-OMP (14, 27). A single enzyme-linked immunosorbent assay (ELISA) antibody threshold that correlates to AOM protection could, however, not be identified (23), and opsonophagocytic activity was recently reported to be more closely related to efficacy against AOM than ELISA-determined antibody concentrations (53). The lower concentrations and functional capacities (13) of antibodies elicited by PCV7-OMP could also explain why PCV7-OMP, unlike PCV7-CRM, did not confer protection against serotypes that cross-react with the vaccine serotypes (Table 3).

The lowest efficacy was observed for serotype 19F for both PCV tested in the FinOM study. Consistent with the low protective efficacy against 19F observed in the FinOM study, all six PCV7-CRM vaccine failures in children with spontaneously draining ears in the NCKP trial in the United States were caused by serotype 19F.

The most recent study evaluated the efficacy of an 11-valent PCV using the *H. influenzae*-derived protein D as the carrier protein (PCV11-PD) against culture-confirmed AOM episodes caused by pneumococcal vaccine serotypes and NTHI (Table 4) (50). Protein D is highly conserved among capsulated and noncapsulated *H. influenzae* strains and has proven to be immunogenic, and data from animal studies have demonstrated its protective potential against NTHI AOM (42, 48).

A substantial protective efficacy of the PCV11-PD vaccine was demonstrated against the first and all pneumococcal AOM episodes caused by vaccine serotypes: 52.6% (95% CI, 35.0 to 65.5%) and 57.6% (95% CI,

**Table 2** Impact of PCV7-CRM on overall AOM in infants<sup>a</sup>

Type of AOM episode <sup>b</sup>	No. of "episodes" in NCKP trial among:		NCKP trial VE		No. of "episodes" in FinOM trial among:		FinOM trial VE	
	Vaccinees (n = 11,849)	Controls (n = 11,897)	%	95% CI	Vaccinees (n = 811)	Controls (n = 821)	%	95% CI
Clinic visit for otitis media	22,478	24,914	8.9	5.8–11.8	1,597	1,735	7	–4–16
AOM episode	16,124	17,405	7.0	4.1–9.7	1,251	1,345	6	–4–16
First AOM episode	7,126	7,411	5.4	2.3–8.4	512	562	12	1–22
Recurrent otitis <sup>c</sup>	1,647	1,809	9.3	3.0–15.1	123	149	16	–6–35
Tympanostomy tube placement <sup>d</sup>	157	198	20.1	1.5–35.2	178	189	4	–19–23
Otitis with pneumococcal vaccine serotypes isolated <sup>e</sup>	4	12	66.7	NC	107	250	57	44–67

<sup>a</sup>VE, vaccine efficacy; NC, not calculated.

<sup>b</sup>See Table 1 for case definitions and methods of case ascertainment.

<sup>c</sup>Defined as at least three episodes in 6 months or four episodes in 12 months.

<sup>d</sup>Data are expressed as the number or percentage of children with at least one tympanostomy tube placed during the NCKP study and as the number or percentage of any tympanostomy tube placements during the FinOM study. Intention-to-treat analysis for the FinOM study.

<sup>e</sup>Based on cultures obtained from spontaneously ruptured tympanic membranes in the NCKP study and from MEF obtained via myringotomy in the FinOM study (4, 14, 49).

**Table 3** Impact of PCV7-CRM and PCV7-OMP in infants on culture-confirmed bacterial AOM end points in the FinOM study<sup>a</sup>

Type of AOM episode <sup>b</sup>	No. of “episodes” among:		VE of PCV7-CRM			No. of “episodes” among:		VE of PCV7-OMP		
	PCV7-CRM recipients (n = 786)	Controls (n = 794)	%	95% CI		PCV7-OMP recipients (n = 805)	Controls (n = 794)	%	95% CI	
Any clinical AOM episode	1,251	1,345	6	-4	16	1,365	1,345	-1	-12	10
All <i>S. pneumoniae</i> AOM	271	414	34	21	45	314	414	25	11	37
First AOM episode due to a vaccine serotype	89	177	52	39	63	NC	NC	50	36	61
All vaccine serotype AOM	107	250	57	44	67	110	250	56	44	66
Serotype 4 AOM	2	4	49	-176	91	1	4	75	-122	97
Serotype 6B AOM	9	56	84	62	93	12	56	79	58	89
Serotype 9V AOM	5	11	54	-48	86	2	11	82	12	96
Serotype 14 AOM	8	26	69	20	88	11	26	58	10	80
Serotype 18C AOM	7	17	58	-4	83	8	17	53	-28	83
Serotype 19F AOM	43	58	25	-14	51	37	58	37	1	59
Serotype 23F AOM	33	82	59	35	75	40	82	52	28	68
All AOM due to cross-reactive serotypes	41	84	51	27	67	89	84	-5	-47	25
Serotype 6A AOM	19	45	57	24	76	53	45	-17	-85	26
Serotype 9N AOM	2	8	75	-24	95	11	8	-37	-272	50
Serotype 19A AOM	17	26	34	-26	65	22	26	16	-53	54
Serotype 23A AOM	1	4	75	-151	97	2	4	50	-210	92
AOM due to other pneumococcal serotypes	125	95	-33	-80	1	121	95	-27	-70	6
<i>H. influenzae</i> AOM	315	287	-11	-34	8	315	287	-9	-32	10
<i>M. catarrhalis</i> AOM	379	381	-1	-19	15	444	381	-16	-36	2

<sup>a</sup>VE, vaccine efficacy; NC, not calculated.<sup>b</sup>See Table 1 for case definitions and methods of case ascertainment (4, 14, 27, 49).

41.4 to 69.3%), respectively. The use of *H. influenzae*-derived protein D as a carrier protein for pneumococcal polysaccharides resulted in 35.3% (95% CI, 1.8 to 57.4%) protection against AOM episodes caused by NTHI. It has been speculated that the observed reduction of *H. influenzae* AOM episodes may have been the result of an indirect effect following the reduction of pneumococcal AOM episodes, which would make children less vulnerable to subsequent infection by *H. influenzae*, rather than a direct effect of the carrier protein D. This hypothesis is not supported by the findings of the FinOM study, where an increase in AOM episodes due to *H. influenzae* was observed following both seven-valent PCV, despite the reduction of pneumococcal AOM episodes (Table 3). Concomitant reduction in the prevalence of nasopharyngeal carriage of NTHI in the Pneumococcal Otitis Efficacy Trial (POET) study lends further support to the argument that the carrier protein induced a real protective effect (50).

Overall, the PCV11-PD reduced the total number of ear, nose, and throat (ENT) specialist-confirmed clinical AOM episodes by 33.6% (95% CI, 20.8 to 44.3%), ir-

respective of the etiology (Figure 1), a remarkably higher vaccine efficacy estimate than those from previous studies of PCV.

The pneumococcal serotype-specific efficacy estimates reached statistical significance for serotypes 6B, 14, 19F, and 23F, but surprisingly no protection could be demonstrated against serotype 3. Despite the relatively high ELISA-measured levels of post-primary dose antipolysaccharide antibody against serotype 3 (50), the measured concentrations of antibody against serotype 3 polysaccharide following a fourth consecutive PCV11-PD dose at 12 to 15 months of age remained below post-primary dose antibody levels (50). The poor efficacy results for serotype 3 have led the vaccine manufacturer to remove serotype 3 from its final pediatric PCV formulation. It is currently not known why serotype 3 polysaccharide-specific antibodies were not able to provide protection against AOM. Serotype 3 pneumococci are known to be different from other serotypes: they have a slimy morphology on culture media and produce an abundant amount of capsular polysaccharide that is able to induce an immune response

**Table 4** Impact of PCV11-PD in infants on overall AOM and culture-confirmed bacterial AOM end points in the POET study<sup>a</sup>

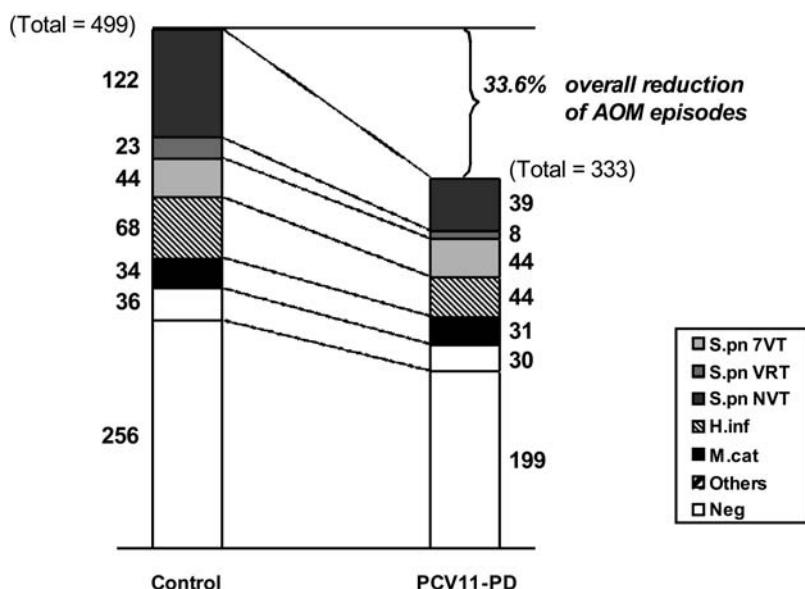
Type of AOM episode <sup>b</sup>	No. of episodes among:		VE	
	PCV11-PD recipients (n = 2,455)	Controls (n = 2,452)	%	95% CI
Any ENT specialist-confirmed clinical AOM episode	333	499	33.6	20.8–44.3
First ENT specialist-confirmed clinical AOM episode	242	359	34.5	22.9–44.3
Recurrent AOM episodes <sup>c</sup>	8	18	55.6	–1.9–80.7
Tympanostomy tube placement <sup>d</sup>	4	10	60.3	–26.7–87.5
All <i>S. pneumoniae</i> AOM	92	189	51.5	36.8–62.9
First vaccine serotype AOM	57	118	52.6	35.0–65.5
All 11-valent vaccine serotype AOM	60	141	57.6	41.4–69.3
All seven-valent vaccine serotype AOM	39	122	68.2	53.5–78.2
Serotype 4 AOM	0	3	100	–27.8–100
Serotype 6B AOM	3	24	87.6	58.4–96.3
Serotype 9V AOM	3	8	62.6	–40.6–90.1
Serotype 14 AOM	1	22	95.5	66.0–99.4
Serotype 18C AOM	3	5	40.1	–176.6–87.0
Serotype 19F AOM	24	43	44.4	8.3–66.3
Serotype 23F AOM	5	18	72.3	24.8–89.8
Additional vaccine serotype AOM				
Serotype 1 AOM	1	1	0.2	–1,495–93.8
Serotype 3 AOM	20	17	–17.1	–126.5–39.5
Serotype 5 AOM	0	0		
Serotype 7F AOM	0	1	100	–283.6–100
All cross-reactive serotype AOM	8	23	65.5	22.4–84.7
Serotype 6A AOM	4	11	63.7	–13.9–88.4
Serotype 9N AOM	3	4	25.5	–231.2–83.2
Serotype 19A AOM	1	3	67.4	–208.7–96.6
Serotype 23A AOM	0	2	100	–91.8–100
Other pneumococcal serotype AOM	23	25	8.5	–64.2–49.0
All <i>H. influenzae</i> AOM episodes	44	68	35.6	3.8–57.0
NTHI AOM episodes	41	63	35.3	1.8–57.4
<i>M. catarrhalis</i>	31	34	9.4	–52.5–46.1
Group A streptococcus AOM	6	8	24.7	–117.3–73.9
<i>S. aureus</i> AOM	24	28	14.6	–50.9–51.7

<sup>a</sup>VE, vaccine efficacy.<sup>b</sup>See Table 1 for details on case definitions and case ascertainment.<sup>c</sup>Defined as at least three episodes in 6 months or four episodes in 12 months.<sup>d</sup>Data are expressed as the number or percentage of children with at least one tympanostomy tube placed during the POET study (50).

earlier in life than those of other serotypes (32). It is also possible that serotype 3 pneumococci are able to down-regulate *in vivo* their capsular polysaccharide expression and/or take advantage of biofilms in order to escape the protective host mechanisms triggered by capsular polysaccharide antibodies (22, 39, 59).

Comparisons among the results of the clinical trials of PCV efficacy are hampered by differences in baseline

epidemiology and disease incidence, study design, case definitions, case ascertainment, and local clinical practice. It should be noted that the observed incidence of AOM episodes in the control group of the POET study (0.13 episodes/person year) was only about 1/10 the incidence rates seen in children less than 2 years of age in the NCKP (1.72 episodes/person year) (49), and FinOM (1.24 episodes/person year) (14) control groups and in



**Figure 1** Number of AOM episodes in the POET study groups by bacterial AOM pathogen and impact of PCV11-PD. S. pn, *S. pneumoniae*; 7VT, *S. pneumoniae* serotype 4, 6B, 9V, 14, 18, 19F, and 23F; VRT, 7VT-related serotypes; NVT, other serotypes; H. inf., *H. influenzae*; M. catarrhalis; others, other bacterial AOM pathogens; neg, culture-negative AOM episode.

other U.S. and Finnish studies (1, 28, 58). By contrast, incidence rates much closer to the POET rate have been observed in The Netherlands (0.2 to 0.3 general practitioner consultations/person year for children less than 2 years of age) (46) and the United Kingdom (0.24 to 0.25 general practitioner consultations or hospitalizations because of AOM/person year for children less than 5 years of age) (40).

The impact of a protein D conjugate vaccine on the overall incidence of AOM outside of the POET study setting is difficult to estimate, since it is largely influenced by the local epidemiology of otopathogens causing AOM. Therefore, the relative risk reduction will vary across countries. However, the observed efficacy of the protein D conjugate vaccine against AOM due to NTHI holds promise for a higher impact on otitis media than what has been seen so far.

### Efficacy of PCV Immunization Initiated after the First Year of Life

The efficacy of PCV immunization in children in older age groups was evaluated in three smaller clinical studies. The design and objectives of these studies can be found in Table 5.

The protective efficacy of PCV9-CRM against otitis media was evaluated in healthy Israeli toddlers of 12 to 35 months of age attending day care centers. Two vaccine doses were administered to children below 18

months of age and one single vaccine dose was administered to older children (11). Although the clinical diagnosis of otitis relied on parental interviews and was not confirmed or validated by medically skilled people, a vaccine effect of 17% risk reduction (not statistically significant) was observed. This was accompanied by a 20% reduction in the number of days with antibiotic treatment for otitis. These results following the immunization of healthy toddlers seem to confirm the protective potential of PCV when administered to infants in their first year of life. Therefore, the question was raised whether PCV would also allow the prevention of recurrent disease in older children already suffering from complicated or refractory otitis episodes.

Two studies were conducted that addressed this question. They evaluated the impact of PCV7-CRM when administered to toddlers and young children with established middle ear disease, either recurrent otitis media (61, 62) or chronic otitis media with middle ear effusion (60). No differences between vaccinated and nonvaccinated groups were observed in terms of overall otitis rates, the severity or duration of subsequent AOM episodes, or rates of tympanostomy tube placement. Nasopharyngeal carriage of vaccine pneumococcal serotypes was greatly reduced in the PCV7-CRM recipients, but immediate and complete replacement by nonvaccine pneumococcal serotypes occurred. A possible protective trend observed before the 23-valent pneumococcal

**Table 5** Randomized, controlled trials evaluating the impact of PCV immunization initiated after the first year of life<sup>a</sup>

Study, country(ies), yr begun, blinding	PCV, no. of recipients	Control vaccine, no. of recipients	Ages at and timing of vaccination	Target group, duration of efficacy follow-up
Israel, double blind	PCV9-CRM, 131	MCV-C-CRM, 130	12–17 mos, 2 doses 2–3 mos, apart; 18–35 mos, single dose	12–35-mo-old toddlers attending day care center, 2-yr follow-up
OMAVAX study, The Netherlands, 1998, double blind, and Belgium, 1998, single blind	PCV7-CRM, 190 (The Netherlands) and 38 (Belgium)	HBV or HAV, 193 (The Netherlands) and 36 (Belgium)	12–24 mos, 2 doses of PCV7-CRM 1 mo apart and 1 dose of PPSV23 6 mos later or 3 doses HBV; 25–84 mos, 1 dose of PCV7-CRM and 1 dose of PPSV23 7 mos later or 2 doses of HAV	1–7-yr-olds with history of at least two AOM episodes in the last year, 18-mo follow-up
OME study, The Netherlands, 2000, open	PCV7-CRM, 80	No vaccine, 81	Single dose of PCV7-CRM 3–4 wks before tympanostomy tube placement and 1 dose of PPSV23 4 mos later	2–8-yr-olds referred to ENT surgeons for persistent (at least 3-mo-duration) bilateral OME; follow-up until August 2003

<sup>a</sup>MCV-C-CRM, *N. meningitidis* serogroup C conjugated to CRM<sub>197</sub>; HAV, hepatitis A vaccine; HBV, hepatitis B vaccine; PPSV23, 23-valent polysaccharide capsular vaccine.

polysaccharide vaccine (PPSV23) booster dose disappeared after the booster dose (62). Whether a PPSV23 booster dose might have attenuated the protective effect of the PCV priming remains unclear but is not confirmed by the observations of the FinOM study, in which some of the children primed with PCV7-OMP received a booster dose of PPSV23 without jeopardizing protective efficacy (27).

The observation of a trend for an increased risk of otitis due to *S. aureus* and *Pseudomonas aeruginosa* in the PCV7-CRM recipients (62) needs to be interpreted with caution because all positive *S. aureus* and *P. aeruginosa* samples were obtained from spontaneously draining ears (75% of these children having tympanostomy tubes).

Following these studies, it has been hypothesized that early pneumococcal AOM results in inflammation and

damage to the middle ear and Eustachian tube mucosa, which then predisposes infants to subsequent recurrent AOM episodes. PCV vaccination before the start of this cascade of pathophysiological events may play a significant role by preventing otitis-prone children from suffering from recurrent and complicated infections, but once the cascade of events has started, PCV vaccination will have little or no effect.

#### Effect of PCV on Prolonged or Severe Clinical Course, Complications, and Sequelae of AOM

In an uncomplicated case of AOM, the signs and symptoms of the acute infection are usually relieved within a few days after the onset of the attack and the potential initiation of antimicrobial therapy, and the middle ear effusion (21) resolves within a few weeks (6, 15). If the illness takes a complicated course, symptoms and signs

Primary end point	Secondary end points	Case definition for AOM or OME	Case ascertainment
Nasopharyngeal carriage of vaccine serotypes	Upper respiratory tract infections, lower respiratory tract problems, otitis media episodes, other illnesses not associated with respiratory problems or otitis, antibiotic drug usage	No specific definitions	Information on disease episodes was obtained through parental interviews that occurred monthly during the first year of follow-up and bimonthly thereafter. The diagnoses reported by the parents were not medically validated by a physician.
Clinical AOM episodes	Culture-confirmed pneumococcal AOM episodes due to vaccine serotypes, nasopharyngeal carriage of pneumococcal vaccine serotypes	AOM, abnormal tympanic membrane on otoscopy (red, dull, or bulging) or otorrhea and at least one of the following signs or symptoms of acute infection: acute earache, new-onset otorrhea, irritability, fever of $>38.5^{\circ}\text{C}$ measured rectally or axillary reading of $>38.0^{\circ}\text{C}$	Parents were instructed to visit the study clinics or their family physician, ENT specialist, or pediatrician within 24 h after the onset of symptoms suggesting AOM. Bacterial cultures from MEF samples were obtained only once from every child.
Recurrence of bilateral OME within 6 mos after spontaneous extrusion of tympanostomy tube		OME, either a type B (flat) tympanogram or a type C2 (middle ear pressure, $<-200$ dPa) tympanogram with otoscopic evidence of middle ear effusion	Follow-up visits were scheduled every 3 mos until recurrent bilateral OME was determined, until 6 mos after spontaneous extrusion of the ventilation tubes, or until the predetermined closing date of the study.

may persist or recur, middle ear effusion may persist for months, a suppurative complication may develop, or spontaneous perforation of the tympanic membrane can occur (6). The persistence of middle ear effusion without symptoms and signs of acute infection (except for those of upper respiratory tract infection), referred to as otitis media with effusion (OME), may also develop without a preceding episode of AOM (15). A vaccine preventing pneumococcal AOM should not only reduce the uncomplicated episodes of AOM but also decrease the burden of more severe and persistent disease.

PCV7-CRM reduced recurrent AOM (defined as at least three episodes in 6 months or four episodes in 12 months) by 9.3% (95% CI, 3.0 to 15.1%) in the NCKP trial (4) and by 16% (95% CI, -6 to 35%) in the FinOM study (Table 2) (14). The corresponding efficacy of PCV11-PD in the POET study was 55.6% (95% CI,

-1.9 to 80.7%; Table 4) (50). The efficacy of both PCV7-CRM and PCV11-PD tended to be higher against tympanostomy tube placements than against clinical episodes of AOM (Table 2 and 4). In the FinOM study, the efficacy of PCV7-CRM against tympanostomy tube placements was seen only after the trial follow-up had ended, when the children were 24 months old (44). The rate of surgical intervention during the FinOM trial was significantly higher (19.3% of children by 2 years of age) than that in other studies, including the NCKP study (3.9% of children by 3.5 years of age) (16). The lower clinical threshold for tube placements may have diluted the impact of PCV7-CRM on tympanostomy tube placements in the FinOM study. During the 2 to 3 years after the trial follow-up, PCV7-CRM reduced tympanostomy tube placements by 39% (95% CI, 4 to 61%) (44).

PCV7-CRM did not reduce the prevalence of OME in children at the age of 7 or 24 months in the FinOM study (55), but according to data from parental interviews performed when the children were 4 to 5 years old, 5% of the children in the PCV7-CRM group had been monitored for OME since the age of 24 months compared to 9.9% of the children in the control group (vaccine efficacy, 50% [95% CI, 15 to 71%]) (44).

The reduction of recurrent AOM, tympanostomy tube placements, and OME demonstrated in the PCV otitis media efficacy studies indicates that PCV may have the strongest impact on complicated AOM.

## ECOLOGY OF VACCINE IMPACT

AOM is considered to result from the interplay between the microbial load (viral and bacterial) and the immune response (52). The complex relationships between viral and bacterial infections and the nasopharyngeal carriage of bacterial pathogens and the roles of these pathogens in middle ear inflammation are poorly understood. The multifactorial pathogenesis of AOM makes it difficult to predict, understand, or even assess the impact of a vaccine targeting only some types of a single bacterial pathogen involved. PCV obviously changes the microbial density and alters the ecological balance both in the microenvironment of the nasopharynx and in the macroenvironment of the surrounding community, and it is the newly formed balance after the introduction of wide-scale immunization that outlines the ultimate effects of the vaccine on AOM and its sequelae.

### Relation of Effects of PCV on Nasopharyngeal Carriage and on AOM

Since AOM is a mucosal rather than invasive infection, the effect of a pneumococcal vaccine on nasopharyngeal carriage is expected to correlate better with the impact of the vaccine on AOM than with its efficacy against more invasive forms of pneumococcal disease. The prevention of the acquisition of new pneumococcal serotypes in the nasopharynx is considered to be an important mechanism for the prevention of AOM disease with PCV (26). It is, however, important to realize that even the prevention of a mucosal disease such as AOM is not solely dependent on the prevention of carriage. PCV have generally reduced the prevalence of nasopharyngeal carriage of vaccine serotypes by 40 to 50% (10, 29, 36, 50, 62), which is a little less than the highly consistent reduction of 56 to 58% in AOM due to vaccine serotypes associated with the use of three different PCV in randomized controlled trials. In the FinOM trial, full efficacy against AOM due to vaccine serotypes was seen

from 6.5 months onwards, but the reduction in carriage of vaccine serotypes did not become apparent until 18 months of age (29). Since the estimated mean duration of carriage in this age group is 3 months (12, 18, 19, 41), one would have expected the difference between the PCV7-CRM and control groups to be seen earlier than 18 months of age if the vaccine had prevented new acquisitions of vaccine serotypes in the nasopharynx as early and as effectively as it prevented AOM caused by the vaccine serotypes. It is also noteworthy that PCV given after the first year of life did reduce the nasopharyngeal carriage of vaccine type pneumococci, although this had no effect on AOM disease (62).

### Replacement of Vaccine Serotypes by Nonvaccine Serotypes

Several studies have demonstrated a compensatory increase in nasopharyngeal carriage of nonvaccine pneumococcal serotypes in PCV-vaccinated children, resulting in little or no effect from PCV immunization on pneumococcal carriage overall (29, 36, 62). Not surprisingly, when the replacement phenomenon was associated with a disease for the first time, the disease was AOM. In the FinOM trial, the rate of episodes caused by pneumococcal serotypes other than the vaccine or cross-reactive serotypes was 33% (95% CI, -1 to 80%) higher in the PCV7-CRM group and 27% higher (95% CI, -6 to 70%) in the PCV7-OMP group than in the control group (14, 27). This finding has raised the concern that pneumococcal serotypes not included in the vaccine, or even other bacterial pathogens, may eventually replace the vaccine serotypes as causes of AOM and thereby diminish the overall impact of the vaccine. After the introduction of PCV7-CRM in the United States, pneumococcal serotypes not included in PCV7-CRM and NTHI have shown a relative increase among MEF isolates (5, 7). The magnitude of the absolute increase, if any, cannot be elucidated from these studies.

The POET study that evaluated the efficacy of PCV11-PD against AOM did not show the replacement of vaccine serotypes by nonvaccine serotypes in either carriage or AOM (50). A lower bacterial load in the environment offers perhaps the most plausible explanation for this finding. The 22.3% prevalence of nasopharyngeal carriage of *S. pneumoniae* seen in the control group at 15 to 18 months of age was lower than the carriage rate in children at comparable ages in practically any other recent study. Moreover, while the incidence of any AOM episodes in the control group was only 10% of the corresponding incidence in the FinOM study, the incidence rates for AOM episodes due to *H. influenzae* and *M. catarrhalis* were only 7 and 2% of the FinOM

rates, respectively. It is possible that in the POET study areas, there were no other bacterial pathogens readily available to replace the pneumococcal serotypes erased from the scene by PCV11-PD. However, one would not expect vaccine serotypes to be replaced by NTHI since the vaccine provided protection against nasopharyngeal carriage of this particular pathogen (50).

### Herd Effect

An unexpected and important effect of the conjugate vaccines, including PCV, has been their ability to provide indirect protection from disease for those not vaccinated. After 5 years of widespread use of PCV for infant immunization in the United States, more cases of IPD are prevented through indirect effects than through vaccine-induced immunity in those vaccinated. So far, no reduction of AOM has been seen among the unvaccinated children in the United States. In fact, according to the National Ambulatory Medical Care Survey, the rates of ambulatory-patient visits for otitis media among unvaccinated 3- to 6-year-old children have increased rather than decreased since the introduction of PCV during the period from 2000 to 2003 (20). Considering the fluctuations of AOM incidences observed in populations over the years, a potential herd effect may easily be embedded in the normal variation.

### Antimicrobial Resistance

In the last decade, resistance to antimicrobials has increased dramatically, due largely to the overuse of antibiotics for common respiratory infections, such as otitis media. Since antimicrobial resistance is most prevalent among vaccine serotypes, the wide use of PCV provides an opportunity to break the vicious cycle in which antibiotic use and antimicrobial resistance boost each other. Since the introduction of PCV, nonsusceptibility to penicillin among pneumococcal strains isolated from MEF samples has decreased in the United States (5, 38).

## IMPACT OF ROUTINE CHILDHOOD IMMUNIZATION WITH PCV ON AOM INCIDENCE

The clinical trials reported so far have not managed to elucidate conclusively what the benefit of infant PCV vaccination on the overall otitis media disease burden would be. Consequently, otitis prevention on its own has been considered by some as insufficient to justify universal vaccination (55, 56). These conclusions were drawn based on the analysis of pooled data from infant and toddler immunization studies; although PCV im-

munization given before the first middle ear infection may have beneficial effects, these effects can no longer be obtained once the first otitis episode has occurred. In addition, since the introduction of universal childhood immunization with PCV7-CRM in the United States, several groups have reported reductions in overall AOM episodes and visits that in some cases exceeded the vaccine effect observed in the previously discussed randomized clinical trials. For example, a 6% reduction of otitis media reported in Tennessee was reported, but the same study observed a reduction of 20% in upstate New York (47), and more recently, a 20% reduction in otitis media visits was reported in ambulatory medical care statistics in the United States (20). It should be noted, however, that the conclusion of reduced incidence of otitis media in these studies relies on risk ratios of risk ratios (otitis media in children less than 2 years old versus that in children 3 to 6 years old and otitis media during the pre-PCV7 versus the post-PCV7 period). Such ratios could be affected by several confounding factors. According to the national medical care statistics in the United States, the incidence of otitis media visits for children less than 2 years of age decreased more during the period from 1994 to 1999 than from 2000 to 2003 and increased slightly in children 3 to 6 years of age from 2000 to 2003. It is therefore clear that the introduction of PCV7-CRM is not the only factor that might have influenced these trends (20).

Microbiological surveillance of AOM disease due to *S. pneumoniae* and other pathogens since the introduction of PCV7-CRM has been done by extrapolating from selected cases in observational studies, because tympanocentesis is not performed routinely when children present to their primary care physicians for evaluation of AOM. Therefore, surrogate markers for typical and recurrent AOM are obtained from MEF from children with spontaneously draining ears and tympanostomy tube placement, respectively, or from MEF samples obtained through tympanocentesis in children with severe or refractory AOM. These data have shown that the proportion of otitis cases due to vaccine pneumococcal serotypes was reduced since the introduction of PCV7-CRM by nearly 50% (from 70 to 36%) in 7- to 24-month-old Kentucky children with severe or refractory AOM (5). This finding was confirmed by another study (38) in which the overall percentage of AOM cases due to vaccine pneumococcal serotypes in U.S. children with spontaneous drainage of the middle ear decreased from 76% in 1999 (before universal immunization with PCV) to 52% in 2002 (after PCV7-CRM introduction). This reduction was significant for serotypes 6B, 14, and 23F. However, the proportion of AOM

in children due to serotype 19F remained unchanged (21% in 1999 versus 28% in 2002), revealing a lack of protective efficacy for this serotype. In addition, the proportion of AOM cases due to cross-reactive pneumococcal serotypes 6A and 19A remained unchanged between 1999 and 2002, but a trend towards a higher incidence of AOM caused by vaccine-related serotypes in correlation with the number of PCV7-CRM doses received was observed (i.e., in children having received at least two doses compared to those having received less than two doses) (37, 38). This trend was also observed in Kentucky, where a statistically significant increase in AOM due to vaccine-related serotypes 6A and 19A (from 8 to 32%) was observed in children having received at least three vaccine doses compared to children in the prevaccine era (5). Finally, an increase of the proportion and possibly even the absolute numbers of AOM cases due to nonvaccine pneumococcal serogroups has been observed by several groups (5, 38).

From the FinOM study, it was already known that following PCV vaccination, vaccine pneumococcal serotypes might be replaced as bacterial causes of AOM, not only by nonvaccine pneumococcal serotypes (serotype replacement), but also by other bacterial pathogens such as *H. influenzae*. A study performed with children from Rochester, NY, also observed that following the introduction of PCV7-CRM into the United States, *H. influenzae* became the predominant causative pathogen of persistent AOM and AOM linked to treatment failures (increased from 38% of the isolates in 1995 to 1997 to 57% in 2001 to 2003), although *S. pneumoniae* was still important (48% in 1995 to 1997 and 31% in 2001 to 2003) (7). Similar observations were made in Kentucky, where the proportion of pneumococcal pathogens decreased by more than one-third (from 48 to 31%) while that of *H. influenzae* increased from 41 to 56% and that of *M. catarrhalis* remained unchanged (5). Whether this increase in AOM episodes caused by cross-reactive and nonvaccine pneumococcal serotypes and *H. influenzae* is entirely attributable to the use of PCV7-CRM or whether other confounding factors such as changes in the routine practice of standard antibiotic treatment and/or the spread of specific pneumococcal clones have also contributed remains to be determined.

Universal PCV immunization results in a nasopharyngeal microbiological shift (17, 51) and subsequent changes in the bacterial pathogens causing ear infections, with increased nonvaccine pneumococcal serotype and nonpneumococcal bacterial pathogens (9, 45). New PCV providing improved efficacy against pneumococcal serotypes included in the current vaccines and

broader protection against the most relevant pneumococcal serotypes and nonpneumococcal bacterial pathogens such as *H. influenzae* would have a greater impact on AOM and its sequelae than current PCV and may even prevent this microbiological shift. The alternative scenario is that new pathogens will always emerge into the nasopharynx to replace the ones removed by the vaccine. Nevertheless, the currently available data indicate that even if nonvaccine type pneumococci so far have occupied the ecological niche in the nasopharynx left vacant by the vaccine type pneumococci, they have not managed to do the same in the middle ear. PCV have induced a genuine reduction of pneumococcal AOM. This finding should encourage the development of new vaccines that would confer better protection against AOM through a wider range of antigens targeting bacteria and viruses causing AOM (8).

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Keith P. Klugman, Felicity Cutts, Richard A. Adegbola,  
Steven Black, Shabir A. Madhi, Katherine L. O'Brien,  
Mathuram Santosham, Henry Shinefield, Jonathan A. C. Sterne

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# Meta-Analysis of the Efficacy of Conjugate Vaccines against Invasive Pneumococcal Disease

The efficacy of pneumococcal conjugate vaccine against invasive pneumococcal disease (IPD) has been tested in four large-scale clinical trials to date (2, 3, 9, 14). Common features of these trials include the definition of the invasive disease end point and the use of similar vaccines conjugated by a single technology to CRM<sub>197</sub> protein (a mutant nontoxic diphtheria protein) and produced by a single vaccine manufacturer (Wyeth Pharmaceuticals, Pearl River, NY). The trials differ in the populations at risk, the spectrum of severity of IPD, the concomitant and control vaccines, the valency of the conjugate vaccines, the schedules of immunization, and the use of cluster randomization in one trial (14). This chapter will review the results of the four trials and explore the protection achieved against aggregate and individual serotypes using the common end point of IPD in all four trials. It will also discuss these results in terms of the different populations at risk and

the schedules of vaccination. There are no data to date to suggest that the differences in the control vaccine, the valency of the conjugate, or the study design of cluster versus individual randomization are likely to profoundly alter the trial observations of vaccine efficacy. As human immunodeficiency virus (HIV) infection increases the burden of pneumococcal infection and decreases vaccine efficacy (9), it is important that estimates of vaccine effectiveness include populations in which HIV is prevalent. In the analyses, the aggregate intent-to-treat data are therefore analyzed both by excluding HIV-infected children to make the data sets more comparative and by including these children to give an estimate of the impact on vaccine efficacy of just 2,500 HIV-infected children in an aggregate at-risk population of around 100,000 infants. This review also allows a comparison of the safety data from the four trials (2, 3, 9, 14).

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Keith P. Klugman, Hubert Department of Global Health, Rollins School of Public Health, Emory University, Atlanta, GA 30322, and Medical Research Council, University of the Witwatersrand Respiratory and Meningeal Pathogens Research Unit, Johannesburg, South Africa. Felicity Cutts and Richard A. Adegbola, Medical Research Council (UK) Laboratories, Fajara, The Gambia. Steven Black, Dept. of Pediatric Infectious Diseases, Stanford University, Palo Alto, CA 94304. Shabir A. Madhi, Medical Research Council, University of the Witwatersrand Respiratory and Meningeal Pathogens Research Unit, Johannesburg, South Africa. Katherine L. O'Brien and Mathuram Santosham, Center for American Indian Health, Dept. of International Health, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD 21205. Henry Shinefield, Dept. of Pediatrics, University of California, San Francisco, San Francisco, CA 94143. Jonathan A. C. Sterne, Dept. of Social Medicine, University of Bristol, Bristol, United Kingdom.

## METHODS

### Population Sites

The California trial (2) studied individually randomized infants (about 50% white and also including significant numbers of Hispanic, Asian, and black infants) at 23 medical centers served by the Northern California Kaiser Permanente medical care program. Subjects in the American Indian trial (14) were group randomized and comprised infants in 36 community clusters in the Navajo nation and 2 community clusters on the White Mountain Apache reservation. The South African trial (9) utilized individually randomized urban African infants at 21 clinics in Soweto, South Africa, while the Gambian trial (3) studied similarly randomized rural African infants at 15 fixed facilities and 110 outreach sites in the Central and Upper River Divisions of The Gambia.

### Vaccines Administered and Schedules

In the two trials conducted in the United States (2, 14), a seven-valent conjugate was given to infants at 2, 4, and 6 months of age, with a booster at 12 to 15 months. In the community-randomized American Indian trial, a catch-up vaccine schedule was also employed (children less than 7 months of age received doses according to the schedule described above; children aged 7 to 11 months received two doses 2 months apart and the booster, while those children 12 to 23 months of age received just two doses 2 months apart). In both of these studies, the control vaccine was meningococcal C polysaccharide conjugated to the same CRM<sub>197</sub> diphtheria toxoid as the pneumococcal conjugate itself. Concomitant vaccines in these studies varied; all children received a *Haemophilus influenzae* type b (Hib) conjugate and a hepatitis B vaccine. The children initially randomized in California received oral polio and diphtheria-tetanus-whole-cell pertussis (DTwP) vaccines, while children randomized later received inactivated polio and diphtheria-tetanus-acellular pertussis (DTaP) vaccines; children in the American Indian trial received inactivated or oral polio and DTaP vaccines. Older children received measles-mumps-rubella and varicella vaccines in both trials. In the African studies (3, 9), a nine-valent conjugate was given in three doses at least a month apart, starting when infants were 6 weeks of age, with no booster. Both trials used a true placebo, and all children received Hib conjugate, hepatitis B, oral polio, and DTwP vaccines. The vaccine used in The Gambia was reconstituted with DTwP and Hib conjugate vaccines, while in South Africa these vaccines were given sepa-

rately and the pneumococcal conjugate was reconstituted with diluent. The children in the trials in Africa received *Mycobacterium bovis* BCG at birth.

### Analysis

Trials reported either intent-to-treat or per-protocol analyses and, in some instances, both analyses; where serotype-specific efficacy was not reported in the original trial, the data were obtained from the authors. The Gambian trial originally reported results by using incidence rates and rate ratios; for simplicity, we have presented risk ratios for all trials here. The efficacy data from South Africa include data from extended follow-up for 6.2 years (10). The safety analyses include all children randomized in the four trials (2, 3, 9, 14) and follow-up safety data from the northern California and South African studies. The data from California include postmarketing data from 65,927 children who received a primary series of three doses of pneumococcal conjugate between March 2000 and November 2002 (S. Black, E. K. France, E. Lewis, J. Hansen, K. Center, K. Schwalbe, B. Fireman, J. Graepel, B. Hilton, and D. Isaacman, presented at the 12th International Congress of Infectious Diseases, Lisbon, Portugal, 15 to 18 June 2006). We used an adapted version of the metan command for Stata statistical software (Stata Corporation, College Station, TX) to conduct Mantel-Haenszel fixed-effect meta-analyses of the risk ratios for IPD in each trial. We performed meta-analyses of results from intent-to-treat analyses, modified to include children who received at least one dose of vaccine or placebo. For selected analyses in which there was evidence of between-trial heterogeneity, we also conducted DerSimonian and Laird random-effects meta-analyses (5) and displayed the results of both fixed- and random-effects analyses in forest plots. We did not allow for the cluster-randomized design of the American Indian trial (14); in this trial, the average number of cases of IPD per cluster was less than 1, and so adjustments for clustering would have little effect. We quantified heterogeneity by using the I<sup>2</sup> statistic, which can be interpreted as the proportion of the total variation in estimated risk ratios that is due to between-trial heterogeneity (7). Vaccine efficacy was calculated as follows: 100 × (1 – risk ratio).

## RESULTS

### Efficacy

The intent-to-treat efficacy for the seven vaccine types common to all four trials (4, 6B, 9V, 14, 18C, 19F, and 23F) was calculated both including and excluding the

HIV-infected population from South Africa. The calculation included data from extended follow-up with children to age 6 years in the South African study (10) and with all children enrolled in the American Indian study (including the catch-up groups) who received at least one dose of vaccine (14). The estimated efficacy was noticeably lower (28%) for children in South Africa known to be HIV infected than for non-HIV-infected children in the same trial or in the other trials (Fig. 1). For 100,608 children (excluding children in South Africa known to be HIV-infected), the efficacy (Fig. 1) determined using the Mantel-Haenszel analysis was 85% (95% confidence interval [CI], 75 to 91%). This estimate may be compared to the case-control estimate of vaccine efficacy in the United States of 96% (95% CI, 93 to 98%) (15). The inclusion of HIV-infected children in South Africa lowers the aggregate vaccine efficacy slightly to 72% (95% CI, 60 to 80%) (Fig. 1). There was evidence of between-trial heterogeneity when HIV-infected children were included ( $P = 0.001$ ) but not when they were excluded ( $P = 0.25$ ).

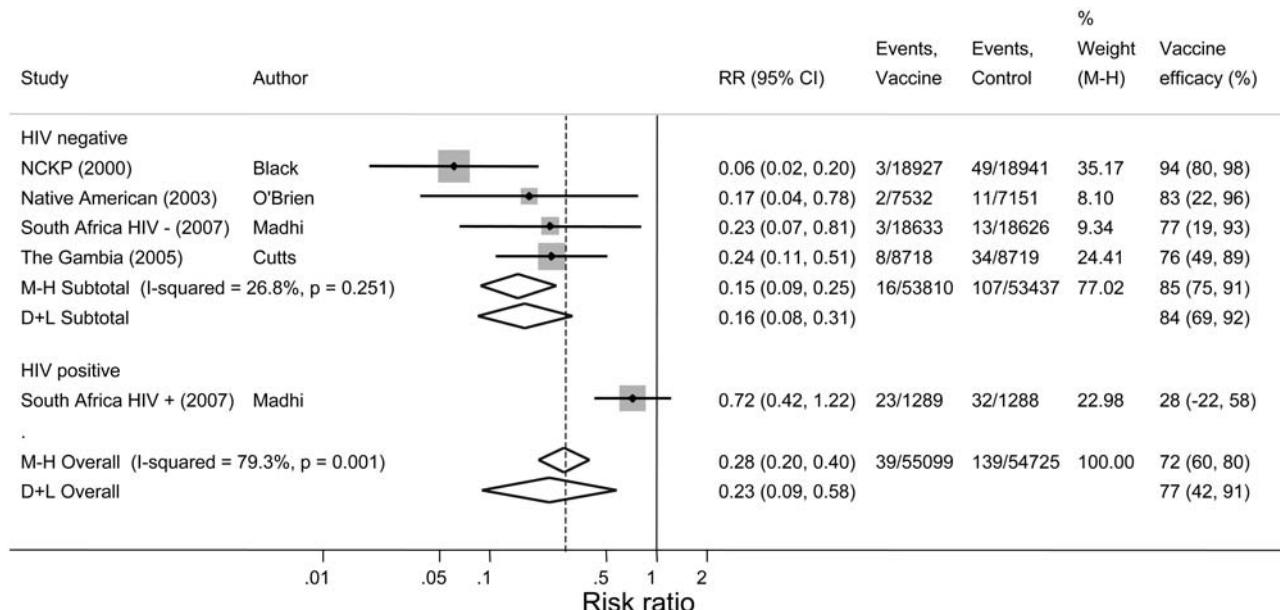
Efficacy against all IPD also varied between trials (heterogeneity;  $P = 0.007$ ), with clearly greater efficacy in northern California (89%; 95% CI, 75 to 95%) than

in the other trials (Fig. 2). The overall efficacy (random-effects analysis) was 56% (95% CI, 30 to 72%). The exclusion of HIV-infected children had little effect on the overall efficacy (60%; 95% CI, 21 to 80%) (Fig. 2).

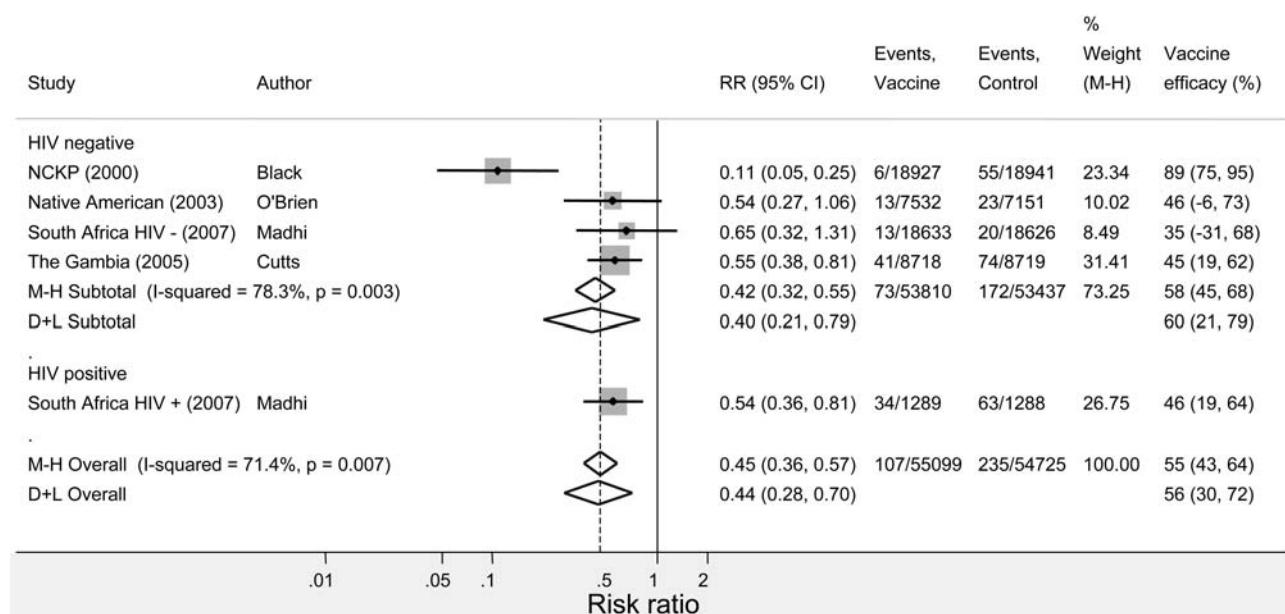
The vaccine efficacy against individual serotypes is shown in Fig. 3. These data show that for the seven vaccine types in the currently licensed vaccine, the vaccine appears to protect against IPD, with aggregate point estimates of vaccine efficacy ranging from 50 to 81%. There was little evidence of between-trial heterogeneity for any individual serotype, even with the inclusion of HIV-infected children in the analysis.

Figure 4 shows that there was clear evidence of protection against serotype 5 from the two African nine-valent vaccine studies (vaccine efficacy, 83%; 95% CI, 26 to 96%). This result was driven mainly by clear efficacy in The Gambia (9 of 10 cases of serotype 5 disease occurred among controls). In contrast, there were six serotype 1 cases in The Gambia and six in South Africa, with little evidence of vaccine efficacy (27%; 95% CI, -120 to 76%) (Fig. 5).

Figure 6 shows that there was evidence of cross-protective efficacy against serotype 6A, driven by an efficacy of 79% in South African HIV-infected children



**Figure 1** Intent-to-treat efficacy against serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F, both including and excluding the HIV-infected children in the South African trial. IPD, invasive pneumococcal disease; 7v, seven-valent; DL, DerSimonian and Laird analysis; M-H, Mantel-Haenszel analysis; RR, relative risk; NCKP, Northern California Kaiser Permanente;  $I^2$ , statistic measuring between-trial heterogeneity (7). References: NCKP (2); Native American (14); South Africa (10); The Gambia (3).



**Figure 2** Intent-to-treat efficacy against all pneumococcal serotypes including and excluding the HIV-infected children in the South African trial. Abbreviations and references as in Fig. 1.

(overall, 23 of 32 cases of disease were in controls) (10). The efficacy is similar to that predicted by the U.S. case-control effectiveness study (15). Note that there were few serotype 6A cases, and little evidence of vaccine efficacy, in non-HIV-infected children.

There is little evidence of cross protection against serotype 19A (Fig. 7), again in concordance with U.S. effectiveness data (15). Figure 8 shows that there is no evidence of vaccine efficacy against nonvaccine types and no evidence of replacement disease.

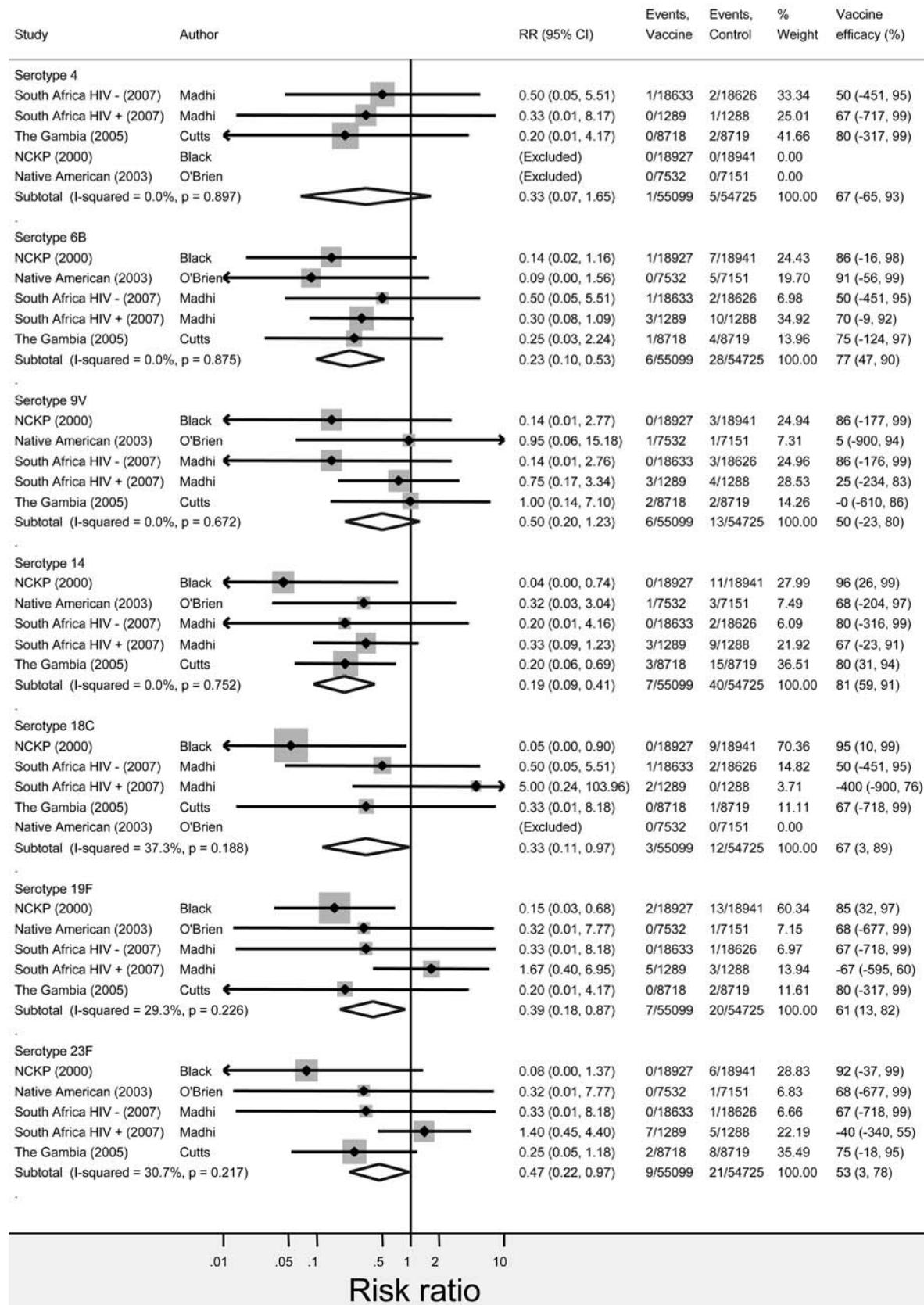
## Safety

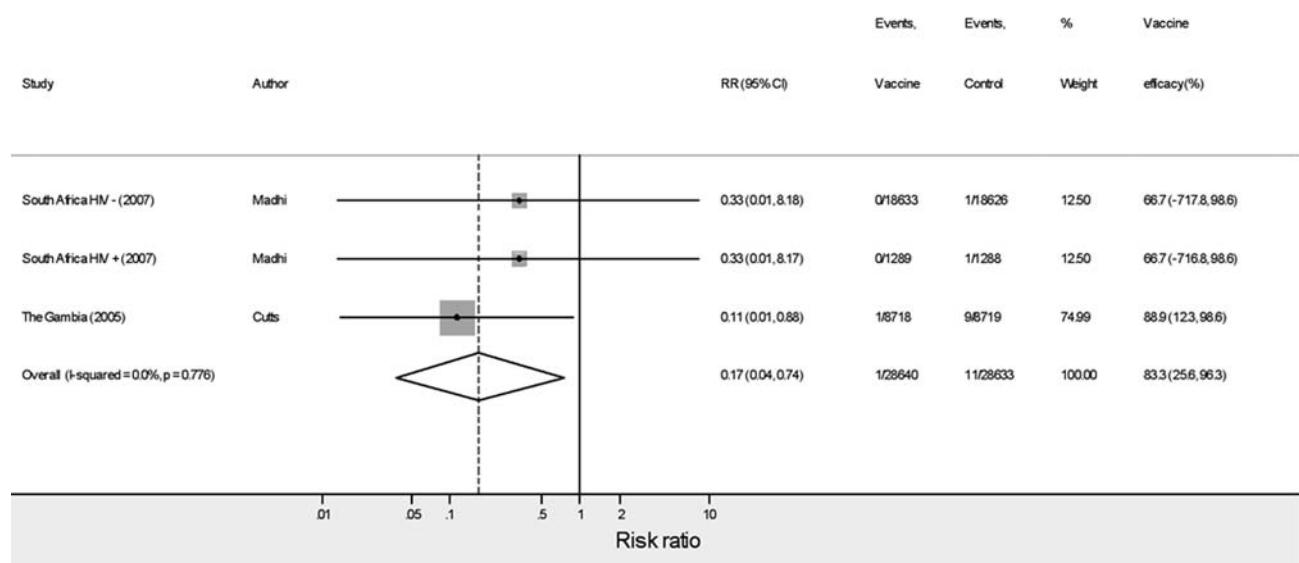
### Californian Trial (2)

Local reactions among children receiving pneumococcal conjugate vaccine were not significantly different from those found among children receiving the control vaccine when coadministered with DTwP, but minor increases in swelling as well as redness and tenderness in response to the pneumococcal vaccine compared to the meningococcal C conjugate were found in recipients of

DTaP. Fever of  $>38^{\circ}\text{C}$  was also more common in the primary series in that study. Febrile seizures not clustered within 3 days of immunization were more common in DTwP recipients (7:1;  $P = 0.039$ ). In multiple comparisons of other adverse events in children presenting to emergency rooms, only breath holding (5:0;  $P = 0.031$ ) was more common in pneumococcal vaccine recipients in that trial. In the postlicensure study in California (Black et al., presented at the 12th International Congress of Infectious Diseases), fever was more common in vaccine recipients mainly within 2 days after the second, third, and fourth doses but also up to a month after the fourth dose. Seizures were more common within 1 to 2 weeks postimmunization after the third dose, while wheezing and diagnoses of bronchiolitis and bronchitis were more common in the 2 to 4 weeks after the first and second doses. Breath holding was more common in vaccine recipients within 4 weeks after the first dose. Asthma was not found to be significantly increased in the follow-up of the trial cohort (4).

**Figure 3** Intent-to-treat efficacy against serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F, including the HIV-infected children in the South African trial. Abbreviations and references as in Fig. 1.





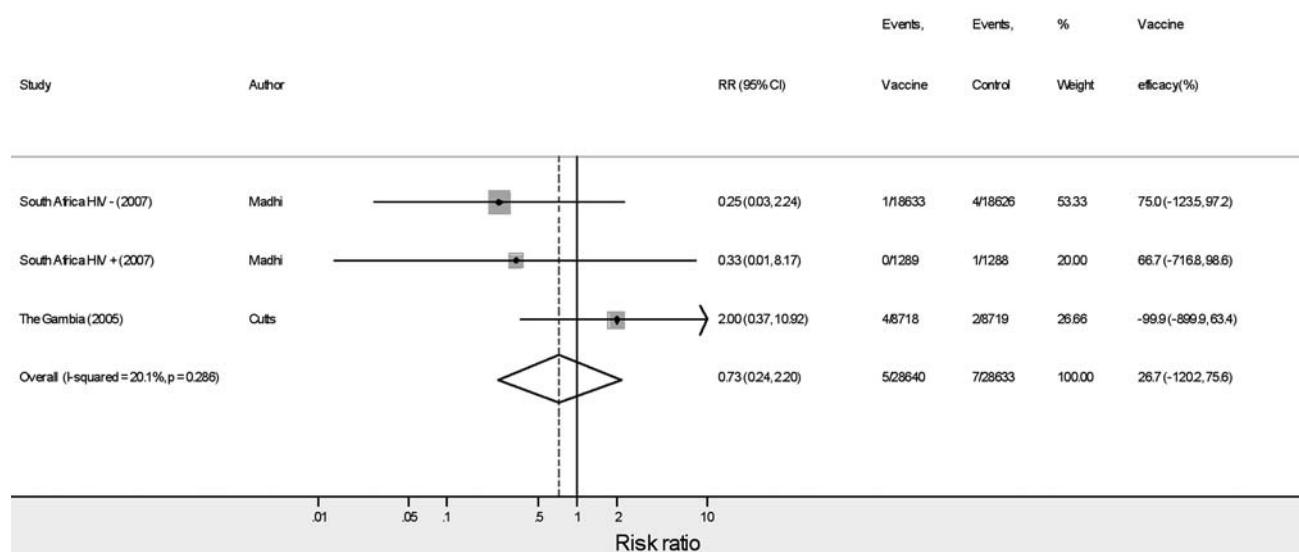
**Figure 4** Intent-to-treat efficacy against serotype 5, including the HIV-infected children in the South African trial. Abbreviations and references as in Fig. 1.

#### American Indian Trial (14)

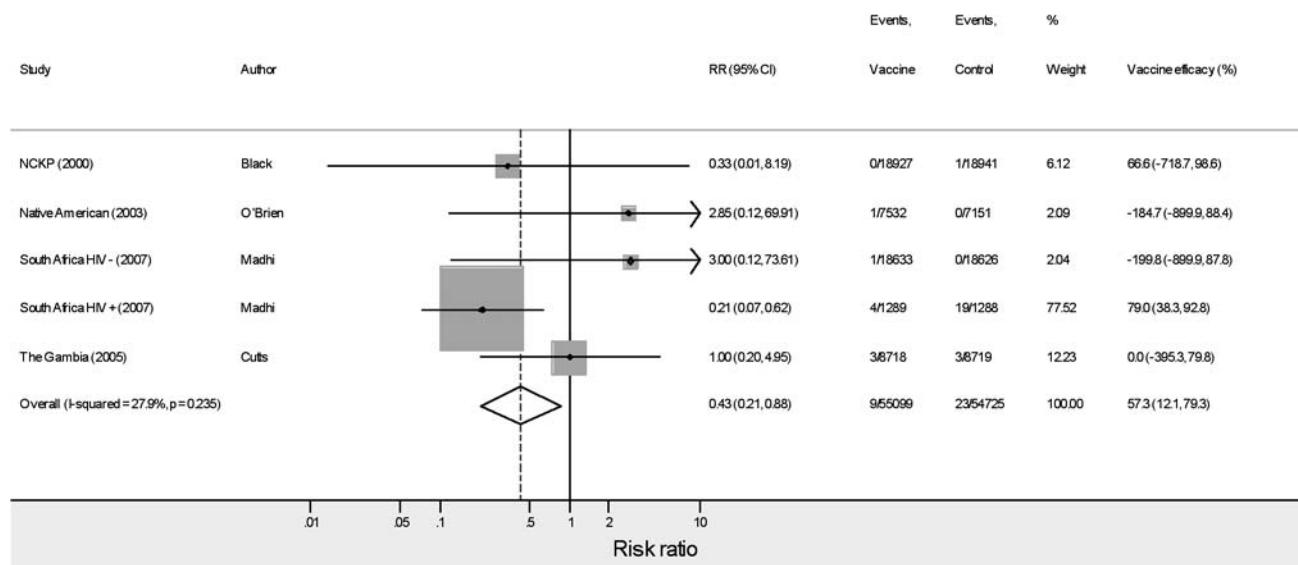
Among specific diagnoses, there was an excess ( $P < 0.01$ ) of otitis media in the 12- to 23-month catch-up group (12:2), but no increase in disease due to any specific etiology was defined.

#### South African Trial (9)

No excess of major local reactions was found. Children who received the conjugate pneumococcal vaccine had an excess of viral pneumonias in the 1 to 4 days postimmunization (18:6;  $P = 0.02$ ) that persisted to day 8



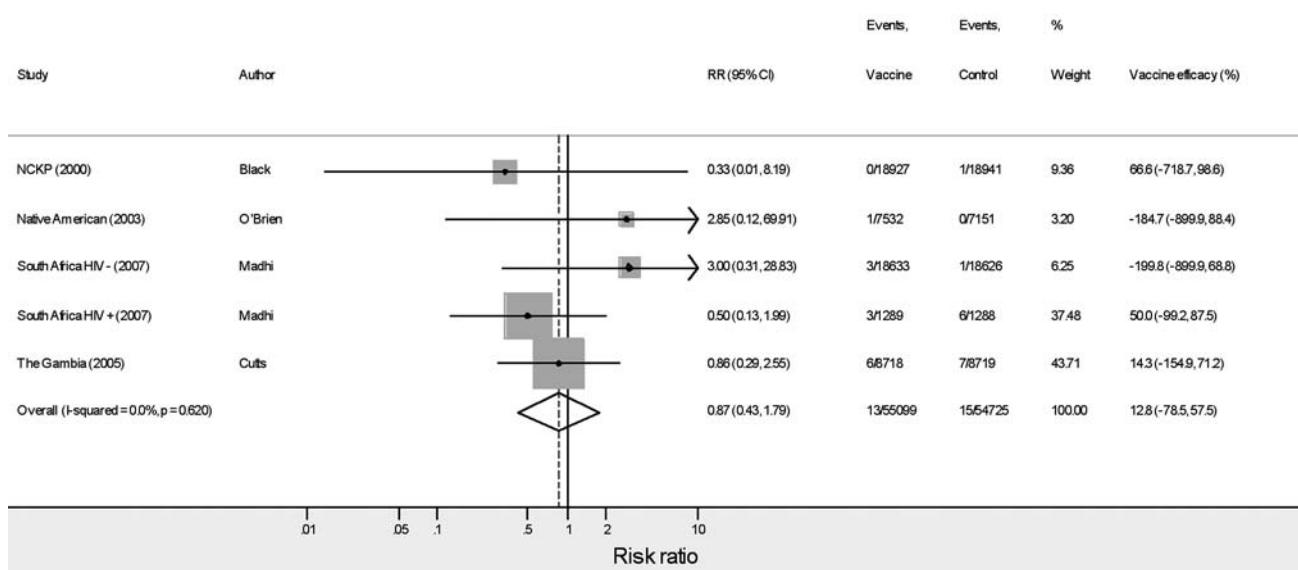
**Figure 5** Intent-to-treat efficacy against serotype 1, including the HIV-infected children in the South African trial. Abbreviations and references as in Fig. 1.



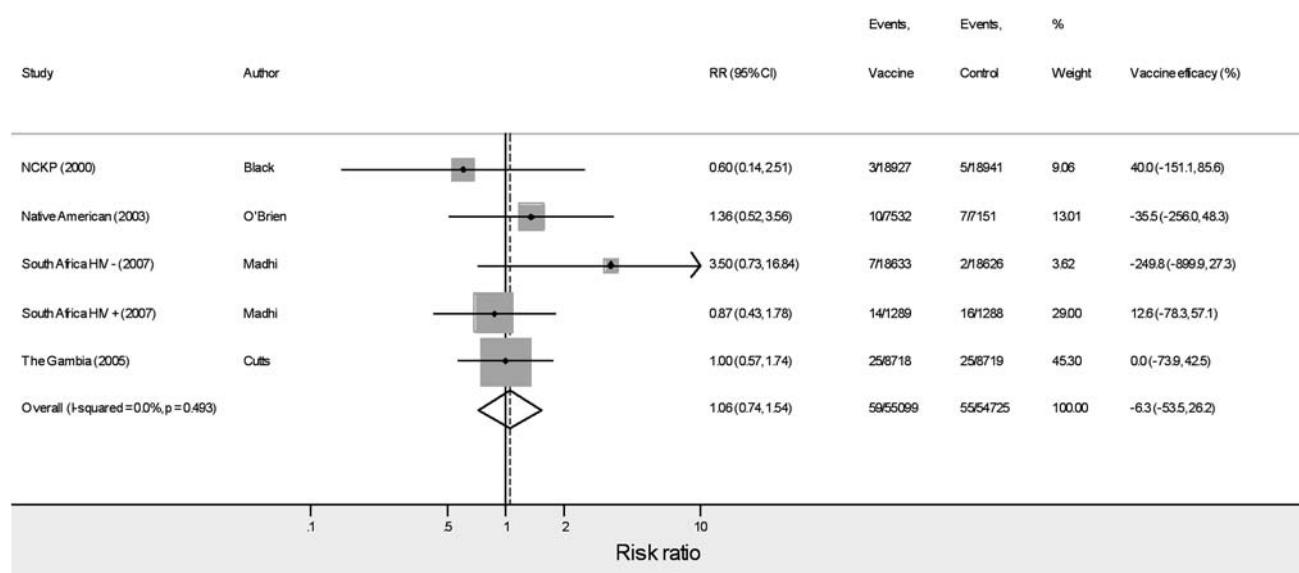
**Figure 6** Intent-to-treat efficacy against serotype 6A, including the HIV-infected children in the South African trial. Abbreviations and references as in Fig. 1.

(31:15;  $P = 0.03$ ), but the excess was reduced by day 31 (83:71;  $P = 0.37$ ). These early viral pneumonias were caused mainly by respiratory syncytial virus (RSV; 83% in vaccinees were caused by RSV, and 80% in controls were caused by RSV). By the end of the 2-year follow-up, the incidence of viral pneumonias was significantly lower in the pneumococcal vaccine group (160:231;  $P$

= 0.0004) (11). The overall rate of seizures was not increased (9). Asthma was increased in the first 2 years of follow-up among vaccinees (59:33;  $P = 0.009$ ), including just those presenting after 1 year of age (42:22;  $P = 0.02$ ). The absolute increase in the risk of asthma was 57/100,000 person years, with a background of a low rate of detected disease in the controls of 71/100,000



**Figure 7** Intent-to-treat efficacy against serotype 19A, including the HIV-infected children in the South African trial. Abbreviations and references as in Fig. 1.



**Figure 8** Intent-to-treat efficacy against all non-vaccine types excluding serotype 6A, including the HIV-infected children in the South African trial. Abbreviations and references as in Fig. 1.

person years in this community (4). The extent of the increase was similar to that of the nonsignificant increase of 53/100,000 person years with a background rate of 707/100,000 person years in Californian infants. Surveillance for asthma-related hospitalizations was extended in the South African study beyond the end of the follow-up on 15 November 2001 through June 2005, and the difference in asthma risk was reduced overall (132:115;  $P = 0.31$ ) and among non-HIV-infected children (92:73;  $P = 0.14$ ) (S. A. Madhi and K. P. Klugman, unpublished data).

### The Gambian Trial (3)

An excess of outpatient visits was noted among vaccinees for the first week only after dose 1 (105:71). The diagnoses were varied and included pneumonia (16:6) and diarrhea (14:6). There were no other safety issues in the Gambian trial, and overall, admissions to the hospital and all-cause mortality were significantly reduced among pneumococcal conjugate vaccine recipients.

## DISCUSSION

The demonstration of substantial conjugate pneumococcal vaccine efficacy in large clinical trials in four disparate regions of the world encourages the view that the vaccine is likely to prevent IPD in most countries in which vaccine serotypes are an important cause of IPD

among infants. The highest estimate of vaccine efficacy against vaccine type and all IPD was found in the trial of Californian children (2). For vaccine type IPD, however, there was little evidence of between-trial heterogeneity when the South African HIV-infected children were excluded. The higher point estimate of vaccine efficacy in the northern California study than in the other three trials may thus be due to chance, but the findings may also suggest the possibility of lower vaccine efficacy against severe disease than against mild disease or of various degrees of vaccine efficacy against different disease syndromes. Most cases of IPD in the American Indian, South African, and Gambian trials were among children admitted to the hospital with pneumonia or meningitis (3, 9, 14), while most cases in northern California were bacteremias in children managed as outpatients. The pathogenesis of both pneumonia and meningitis may involve contiguous spread from the nasopharynx to the lungs and the meninges, respectively, through the cribiform plate, and it is plausible that the prevention of such infections, like that of otitis media (8), may require higher levels of antibody than are needed to prevent primary bacteremia.

Pneumococcal conjugate vaccine has been considered as a probe to determine the burden of disease due to pneumococci in contexts such as pneumonia (3, 12). To estimate the preventable fraction of pneumococcal disease, an overall estimate of vaccine efficacy (for all sero-

types) is used and multiplied by the proportion of invasive disease in the geographic area of interest that is due to the serotypes included in the vaccine. If vaccine efficacy varies by disease syndrome and/or by serotype, however, the estimates of the preventable burden of disease so derived may be artificially low or high. The point estimates for the pooled analyses of vaccine efficacy against the seven serotypes common to all four trials varied from 50% for serotype 9V to 81% for serotype 14. The numbers of cases due to each individual serotype were small, however, even in the combined analyses, and it is not possible to determine with confidence whether there is significant variation in efficacy among serotypes. It should be noted, however, that for otitis media (8), for which there is more statistical power, such differences have been found. The U.S. case-control study showed high vaccine effectiveness against all seven serotypes included in Prevenar (15). For serotype 1, the two trials that examined vaccine efficacy showed no strong evidence of protection. This serotype is a major cause of invasive disease in many developing countries but is more common in infants and older children (6) than adults, and unfortunately the two studies of infants (3, 9) included too few cases to detect an efficacy of less than 80%. Postmarketing surveillance of the impact of vaccination on IPD in different regions of the world will be important to estimate serotype-specific effectiveness and to investigate further whether this effectiveness varies according to disease syndrome and severity.

Consideration of the impact of the vaccine on all IPD, as depicted in Fig. 2, suggests that the vaccine-preventable fraction of IPD due to serotypes in the seven- or even nine-valent conjugate is lower in developing countries than in California. As discussed above, more-detailed data on the serotype distribution of different disease syndromes in different countries, coupled with postmarketing surveillance of vaccine impact on serotype-specific disease and disease syndrome, will help to elucidate the reasons for the difference between the northern California trials and other trials. The similarity in the overall efficacy (about 45%) against all IPD in the American Indian and African trials is striking, despite the use of nine-valent vaccine in Africa. These data suggest that a significant burden of IPD remains to be prevented in developing countries, despite a nine-valent conjugate. On the other hand, it is important to emphasize that the higher overall burden of disease in Africa means that the vaccine-attributable reduction in disease burden is greater than that in the United States, despite a lower proportionate serotype coverage by the vaccine in Africa (1).

Although the numbers of serotype-specific IPD cases remain modest even when aggregated across the trials, the serotype-specific vaccine efficacy estimates by intent-to-treat analyses are similar to those of the U.S. effectiveness study (15). Slightly lower protection of about 60 to 70% against serotype 6A is suggested by both the efficacy and effectiveness data, although the former was driven almost entirely by the results for the South African HIV-infected children. The data show little evidence of protection against serotype 19A and little evidence of serotype replacement disease, but apart from the cluster-randomized trial (14), the studies were not designed to detect replacement in a setting of herd immunity.

Some common themes are apparent from the analysis of vaccine safety. In general, the conjugate pneumococcal vaccine seems to be well tolerated, with few major local or systemic issues detected. The apparent signal of an increase in asthma detected in the South African study (9) does not seem to have evolved into a significant excess of cases over longer-term follow-up in that community. There is, however, a suggestion from three of the four trials (2, 3, 9) that there may be a transient excess of viral pneumonias in the first week or two after mainly the first dose of vaccine. This is a biologically plausible event that may reflect an effect of vaccine of the commitment of circulating B cells to the vaccine, thus reducing the pool of available cells able to commit to pneumococcal infection prior to the development of specific antibody over the 2-week postimmunization period. It is also possible that vaccination reduces the maternal antibody directed against vaccine serotypes. The excess of viral pneumonias may therefore be explained by an excess in the first few days after the first immunization of vaccine type pneumococcal superinfection leading to hospitalization with both pneumococcal and viral pneumonia. Once pneumococcal antibodies have been established after immunization, the incidence of these pneumococcal and viral pneumonias in immunized infants is reduced compared to that in controls (11, 13).

Pneumococcal conjugate vaccine has greatly reduced the burden of IPD due to vaccine serotypes in the United States. It reduces disease due to cross protection against serotype 6A, the nine-valent conjugate protects against serotype 5, and protection against vaccine types remains evident among both HIV-infected and uninfected children through the first 5 years of life; yet, a significant fraction of IPD due to both vaccine and nonvaccine types remains to be prevented in developing countries.

The results of these trials form a basis for consensus about the value of conjugate pneumococcal vaccine for both the developed and developing world. At a time

when innovative funding mechanisms are being developed to accelerate the introduction of the vaccine into developing countries, these data support those efforts. The introduction of vaccine requires well-designed surveillance studies to understand the impact of the vaccine on the range of disease in populations in which it is introduced.

*We wish to acknowledge Ross Harris, who amended the metan command in Stata to display the vaccine efficacies in the graphs.*

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Shabir A. Madhi  
Keith P. Klugman

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# Efficacy and Safety of Conjugate Pneumococcal Vaccine in the Prevention of Pneumonia

## BURDEN OF PNEUMONIA

Ninety percent of the estimated 1.9 million (95% confidence interval [CI], 1.6 to 2.2 million) annual deaths due to acute respiratory infections in children less than 5 years of age occur in developing countries, and these deaths are due mainly to bacterial pneumonia (7). Seventy percent of pneumonia deaths occur in Africa and Asia. A detailed description of the epidemiology of pneumonia and the challenges of making a pathogen-specific diagnosis of bacterial pneumonia in children is discussed in chapter 8 (1).

## CHALLENGES IN DETERMINING THE BURDEN OF PNEUMOCOCCAL PNEUMONIA

Etiologic studies, including those in which lung aspirates were obtained, conducted mainly in developing countries suggest that *Streptococcus pneumoniae* may be the most common bacterial cause of pneumonia (12, 40, 42, 47). This possibility is corroborated by findings of studies of ambulant and hospitalized children that

used newer investigational methods, including serological tests and whole-blood PCR-based assays, for diagnosing pneumococcal infections (19, 32, 48). *S. pneumoniae* was identified in 27 to 37% of children with pneumonia in the United States and Finland, and 40% of children with evidence of *S. pneumoniae* were identified as having viral coinfections (19, 48). The molecular mechanisms by which respiratory viral infections predispose to superimposed pneumococcal infection, as well as related animal model studies supporting the concept of pneumococcal coinfection's resulting in more severe virus-associated pneumonia, have been recently reviewed (39).

The World Health Organization (WHO) clinical criteria for managing acute respiratory tract infections are sensitive for detecting episodes of severe pneumonia and have been useful in improving the management of childhood pneumonia in developing countries and contributing to the reduction of childhood mortality (3). The clinical criteria are, however, unable to discriminate between nonbacterial and bacterial causes of lower-respiratory-tract infections (LRTI). This inability has in

Shabir A. Madhi, Respiratory and Meningeal Pathogens Research Unit, Medical Research Council/University of the Witwatersrand, and Dept. of Science and Technology/National Research Foundation: Vaccine Preventable Diseases, Bertsham, Gauteng, South Africa. Keith P. Klugman, Rollins School of Public Health, Emory University, Atlanta, GA 30322, and Respiratory and Meningeal Pathogens Research Unit, Medical Research Council/University of the Witwatersrand, Johannesburg, South Africa.

part led to the need to investigate proxy markers that are better able to discriminate between bacterial and nonbacterial pneumonia. The validation of the sensitivity and specificity of these proxy markers of bacterial infection, including results of serological assays such as those measuring C-reactive protein (CRP), procalcitonin, and other cytokines (33, 44), is impeded by the absence of a suitable "gold standard" for the diagnosis of bacterial pneumonia. Although some studies have found procalcitonin to be more useful than CRP in discriminating between bacterial and nonbacterial infections, many of these studies have included a broad range of syndromes associated with bacterial infections, hence limiting any inference specific to pneumonia episodes (43). Additionally, studies limited to children with pneumonia have surmised that CRP may not be useful in discriminating between bacterial and viral pneumonia (36). The absence of a sensitive assay to identify possible occult bacterial infection in children in whom a respiratory virus has been identified, however, limits the interpretation of these studies.

The absence of uniform and objective criteria for making a bacterial etiological diagnosis of pneumonia has implications not only for determining the true burden of pneumonia in children but also for defining the effects of interventions such as vaccination against specific bacterial pathogens. This challenge was addressed by a working group under the auspices of the WHO that aimed to standardize suitable proxy markers of pneumococcal pneumonia that could be used to evaluate the efficacy of pneumococcal conjugate vaccines (PCV) against pneumococcal pneumonia (8).

In The Gambia, a *Haemophilus influenzae* type b conjugate vaccine (HibCV) was shown to reduce the overall burden of LRTI by only 4.4% (95% CI, -5.0 to 12.9%) while simultaneously reducing radiologically confirmed pneumonia by 21.1% (95% CI, 4.6 to 34.9%) (34). This result provided the impetus to use a radiological outcome as a proxy marker to measure the efficacy of PCV against pneumonia. A reduction in radiologically confirmed pneumonia, albeit with a modification of the definition used in the Gambian *H. influenzae* type b (Hib) study and with diagnosis by the adjunctive use of clinical signs of bronchial breathing and elevated erythrocyte sedimentation rates, was also associated with a 22% (95% CI, -7 to 43%) reduction in overall pneumonia in Chile (22). Similarly, reductions of 31 to 55% in radiologically confirmed pneumonia have been observed in case-control studies, which may be biased in favor of vaccinees, in other Latin American countries (10, 11). A randomized study in Indonesia failed to find a reduction (efficacy, -5%;  $P > 0.05$ ) in cases of radio-

logically confirmed pneumonia requiring hospitalization, despite a significant overall reduction in LRTI (efficacy, 4%;  $P < 0.05$ ) (14). The absolute burden of LRTI prevented in Indonesia was, however, 3.2-fold greater than that in The Gambia; numbers of vaccine-prevented LRTI episodes were 2.64 per 1,000 child years versus 0.83 per 1,000 child years, respectively. Possible reasons for the contradictory results of these studies may be differences in study design. Specific factors that differed between these studies included more active case surveillance in Indonesia, as well as probably greater access to antibiotics among Indonesian study participants. Thus, cases of hospitalization in Indonesia may have been restricted largely to children with pneumonia due to viruses or to bacteria unresponsive to antibiotics available in the community. Although surveillance was mainly hospital based in The Gambia, access to antibiotics was not enhanced in the community, and therefore, the hospitalized patients may have included a large fraction of patients with bacterial pneumonia. Another possible reason for the lack of efficacy of HibCV against radiologically confirmed pneumonia in Indonesia compared to other countries may relate to geographic differences in the epidemiology of pneumonia among countries or regions, with Hib possibly causing a lower proportion of pneumonia in Indonesia than elsewhere.

## PATHOGENESIS AND SPECTRUM OF CHEST RADIOLOGICAL FEATURES ASSOCIATED WITH BACTERIAL PNEUMONIA

Although the visibility of a homogeneous infiltrate on chest radiographs is commonly associated with bacterial infection, the sensitivity and specificity of chest radiographs in diagnosing bacterial pneumonia are uncertain (13, 15, 46). That bacterial pneumonia may be associated with a spectrum of radiographic changes is not surprising considering the histopathological processes during bacterial infection of the lung, the consequences of which are seen on the radiograph (21). The chest radiograph may be normal in the initial 2 days following infection, during which time the histopathological changes are associated primarily with local capillary congestion with leukocytes and minimal fibrin deposition in the alveoli (21). In the absence of effective antimicrobial treatment or an adequate immune response, there is subsequent fluid and cellular infiltration into the alveoli and further fibrin deposition. This results in the type of homogeneous infiltrate that is frequently associated with bacterial pneumonia. Occasionally, pneumococci may infect the lung through hematogenous dissemination in the body, rather than contiguous spread

down the airway, and such infections classically present as "round pneumonia" on chest radiographs (45).

The timing of the chest radiograph in relation to the onset of the lung infection, the initiation of antibiotics, and the immune response being mediated by the host may therefore influence the radiological presentation associated with bacterial pneumonia. Although chest radiographs are subject to limitations as a proxy marker to diagnose pneumococcal pneumonia, a working group of the WHO agreed to a standardized method for reporting evidence of alveolar consolidation on chest radiographs, which was to be used in determining the efficacy of PCV against pneumonia (8). The definition used in categorizing chest radiographs as having alveolar consolidation was as follows: "presence of a dense opacity that may be a fluffy consolidation of a portion or whole of a lobe or of the entire lung, often containing air bronchograms and sometimes associated with pleural effusion, or a pleural effusion in the lateral pleural space associated with a pulmonary infiltrate/or an effusion large enough to obscure such an opacity." Although not absolute as a measure of pneumococcal pneumonia, such an outcome at least improves the specificity of a clinical diagnosis of LRTI as an outcome for measuring PCV efficacy against pneumococcal pneumonia. Additionally, the use of a standardized outcome measure among trials provides a benchmark against which results of different studies can be compared, although studies are subject to innate differences in design, including potential differences in access to health care among the study populations. Furthermore, the use of a proxy marker such as chest radiographs which is available in most developing countries also provides a useful tool which may be used in the course of epidemiological studies to estimate the potential burden of pneumonia of any definition that may be preventable by vaccination.

## EFFICACY OF PCV AGAINST PNEUMONIA

In addition to studies in the United States involving the currently licensed seven-valent PCV (PCV-7; Prevnar) (6, 38), the efficacy of PCV against pneumonia has involved clinical trials evaluating a nine-valent vaccine (PCV-9, including serotypes 1 and 5 in addition to those included in the seven-valent vaccine) in Africa (9, 20). The polysaccharides of the various serotypes in PCV-7 and PCV-9 are individually conjugated to CRM<sub>197</sub>, a nontoxic mutant form of diphtheria toxoid, as a carrier protein. Another vaccine that is unlikely to become commercially available was evaluated in the Philippines for efficacy against pneumonia and included polysaccharides of 11 serotypes that were conjugated to

either tetanus toxoid or diphtheria toxoid as a carrier protein (2). Additionally, a conjugate vaccine containing 10 serotypes, including serotypes 1, 5, and 7 in addition to the serotypes included in PCV-7, in which individual serotypes are conjugated to *H. influenzae*-derived protein D, has not yet been evaluated for efficacy against pneumonia caused by either pneumococci or *H. influenzae*.

Significant reductions in WHO-defined radiologically confirmed pneumonia associated with PCV-CRM<sub>197</sub> in children not infected with human immunodeficiency virus (HIV) or in populations with a minimal risk of HIV infection were observed in studies from the United States and Africa (Tables 1 and 2), with the vaccine efficacy point estimate ranging from 20 to 37%. A sustained reduction in the burden of radiologically confirmed pneumonia of 20% (95% CI, 10 to 30%) by 2003 to 2004, i.e., the period following the introduction of PCV in the United States, compared to the burden in the immediate period (2000 to 2001) of vaccine introduction has been observed (35). An outlier among these results is the data from the only study that used a cluster-randomized design, among American Indians in the United States, in which PCV-7-CRM<sub>197</sub> was compared to a meningococcal conjugate vaccine administered to controls (38). The efficacy of PCV-7 against vaccine serotype-specific invasive pneumococcal disease (efficacy, 82.6%; 95% CI, 21.4 to 96.1%) among the American Indian infants was similar to that observed in African and Californian children. Surprising was the absence of PCV-7 efficacy against radiologically confirmed pneumonia; however, the analysis of the radiological outcome was conducted only among children who required hospitalization (K. O'Brien, personal communication). The studies from northern California and The Gambia included both inpatient and outpatient episodes of radiological pneumonia, while that from South Africa, although including only cases in children requiring hospitalization, did include some cases in children with mild pneumonia that were admitted primarily for overnight observation.

Some of the key demographic and study-specific characteristics of the published studies are summarized in Table 1. The initial published report on efficacy against pneumonia from California differed in its criteria for evaluating chest radiographs from the standardized reporting undertaken in the other studies (6). Subsequent reanalysis of the Californian data by following the standardization for reporting of chest radiographs recommended by the WHO provided a higher, although not significantly higher, point vaccine efficacy estimate (18 versus 26%) (17).

**Table 1** Key demographic and study design differences among studies in which PCV has been evaluated for efficacy against pneumonia<sup>a</sup>

Study site (reference[s])	Mean age (wks) of subjects (SD or range) at:				Study population(s)
	First dose	Second dose	Third dose	Booster dose	
California (6)	8.6 <sup>b</sup>	17.3	25.9	65.4–78.4	Urban American
South Africa (20, 29)	6.6 (SD, 1.2)	11.2 (SD, 2.5)	15.9 (SD, 3.8)	No booster given	Urban African; approx 6.47% HIV infected
The Gambia (9)	10.7 (8.4–15.5)	17.4 (13.9–23.8)	24.2 (19.4–32.2)	No booster given	Rural African
United States (37)	Varied <sup>b</sup>	Varied <sup>b</sup>	Varied <sup>b</sup>	Varied <sup>b</sup>	Residents on Indian reservations
Philippines (ARIVAC) (2) <sup>j</sup>	6	10	14	No booster given	Urban, semiurban, and rural

<sup>a</sup>Adapted in part from reference 37.<sup>b</sup>Exact ages at vaccination of study cohort were not reported. Data are based on extrapolation of vaccination schedule of 2, 4, 6 months and booster at 15 to 18 months.<sup>c</sup>Seven-valent PCV including serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F.<sup>d</sup>DBRCT, double-blind randomized controlled trial.<sup>e</sup>MnCV, meningococcal type C polysaccharide-protein conjugate vaccine.<sup>f</sup>Includes serotypes 1 and 5 in addition to the seven serotypes included in the seven-valent PCV.<sup>g</sup>Subanalysis based on WHO clinical criteria for mild and severe or very severe pneumonia.<sup>h</sup>Vaccine doses varied depending on the ages of children at enrollment. Children aged 6 weeks to <7 months were scheduled to receive three doses during the primary series and a booster dose at 12 to <24 months of age. Children aged 7 to <12 months were scheduled to receive two doses by 12 months of age and a further booster between 12 and <24 months of age. Children aged 12 to <24 months at enrollment were scheduled to receive two doses at least 60 days apart before the age of 24 months.<sup>i</sup>Personal communication from K. O'Brien.<sup>j</sup>Information as per protocol registration. The report of actual study data is pending.<sup>k</sup>Includes serotypes included in the nine-valent PCV as well as serotypes 3 and 7F. Individual serotypes are conjugated to either diphtheria toxoid (DT) or tetanus toxoid (TT).

Comparing the estimates of the efficacy of PCV from different studies provides insight into the global potential of conjugate vaccines to prevent pneumococcal pneumonia, although the findings of such an undertaking need to be interpreted with caution. There are a

number of speculative reasons that may explain the higher efficacy of PCV-CRM<sub>197</sub> against radiologically confirmed pneumonia in children from The Gambia (37%; 95% CI, 27 to 45%) than in children from the United States (26%; 95% CI, 7 to 40%) and non-HIV-

Study vaccine	Study design	Definition and/or criteria for identification of LRTI or pneumonia and/or diagnostic method	Indication for chest radiograph	Type of surveillance
7-Valent PCV-CRM <sub>197</sub> <sup>c</sup>	Individual DBRCT <sup>d</sup> ; MnCV <sup>e</sup> as control	Clinical and/or radiological diagnosis of pneumonia by attending physician	Attending physician discretion	Passive physician-centered surveillance of inpatients, emergency room patients, and outpatients
9-Valent PCV-CRM <sub>197</sub> <sup>f</sup>	Individual DBRCT; true placebo	Diagnosis of LRTI by study physician based on clinical and/or radiological findings <sup>g</sup>	Study physician diagnosis of LRTI	Passive surveillance of hospitalized subjects
9-Valent PCV-CRM <sub>197</sub>	Individual DBRCT; true placebo	Overall, cough for <14 days and tachypnea or lower chest wall indrawing; severe, study physician assessment of presence of lower chest wall indrawing	Tachypnea; lower chest wall indrawing; fever (subjects enrolled early in the study)	Passive outpatient and inpatient surveillance at study hospital or health center; subsequent addition of active community clinic surveillance, with referral of cases to study center
7-Valent PCV-CRM <sub>197</sub>	Group randomized; MnCV as control	Diagnosis of LRTI by attending physician using clinical and radiologic findings <sup>i</sup>	Attending physician discretion	Active surveillance of all hospitalized study children <sup>j</sup>
11-Valent PCV-DT or PCV-TT <sup>k</sup>	Individual DBRCT; true placebo	Modified WHO clinical criteria for pneumonia, i.e., cough or difficulty breathing and tachypnea, chest indrawing, cyanosis, or inability to drink, and/or radiological findings	Presence of symptoms listed above or physician suspicion of pneumonia	Passive outpatient and inpatient surveillance in public and private health care facilities

infected South African children (20%; 95% CI, 3 to 35%). Some of the most compelling differences among the study in The Gambia and those in the United States (California) and South Africa are geographic access of the study populations to health care services, the availability of antibiotics, and the surveillance systems used in the studies to detect cases of pneumonia (Table 1). The South African study focused exclusively on identifying cases of LRTI based on the hospitalization of study subjects at the study hospital, including some children that could have been managed on an outpatient basis but were rather admitted overnight for observation. In the United States (California) and The Gambia, surveillance of pneumonia outcomes was extended to involve hospitalized subjects and children treated on an

outpatient basis. The criteria used for performing a chest radiograph differed among the studies (Table 1). Furthermore, in the United States, the study was integrated into parents' normal health-seeking behavior, children may have presented soon after developing symptoms of a respiratory tract infection, and many children who subsequently developed pneumonia may already have been started on a course of antibiotics. Conversely, because of difficulty with geographic access to health care, coupled with possible cultural differences in the health care-seeking behavior of parents and a probably higher threshold of disease associated with starting antibiotics, treatment of a respiratory tract infection may have been delayed in The Gambia. In South Africa, an emerging-economy country, the study population

**Table 2** Summary of conjugate pneumococcal vaccine efficacy (VE) and burdens of pneumonia prevented in efficacy trials in the United States, South Africa, and The Gambia<sup>a</sup>

Country of study (population or relevant characteristic) (reference)	All clinically diagnosed LRTI			Radiologically confirmed pneumonia <sup>b</sup>		
	VE (95% CI)	Incidence in controls <sup>c</sup>	VAR <sup>d</sup>	VE (95% CI)	Incidence in controls <sup>e</sup> (proportion) <sup>f</sup>	VAR <sup>d</sup> (proportion) <sup>f</sup>
United States (6)	6.0 (-1.5-11)	4,580	230	18 (5-29)	1,010 (22%)	180 (78%)
United States (WHO definition of VE used) <sup>g</sup> (17)				26 (7-40)	Not calculated	
South Africa (non-HIV- infected children) (29)	7.0 (-1-14)	2,566	172	20 (3-35)	491 (19%)	100 (59%)
South Africa (HIV-infected children) (29)	15 (6-24)	16,724	2,573	13 (-7-28)	6,996 (42%)	909 (35%)
The Gambia protocol (9)	7.0 (1-12)	24,850	1,700	37 (27-45)	4,090 (17%)	1,490 (88%)
United States (American Indians) (K. O'Brien, personal communication)	Not available	Not available	Not available	-21.2 (-61.5-9.0) <sup>b</sup>	Not available	Not available

<sup>a</sup>Intent-to-treat data from the U.S., South African, and American Indian studies are included. Data are adapted in part from reference 38.

<sup>b</sup>Pneumonia diagnosed by using chest radiographs showing alveolar consolidation.

<sup>c</sup>Number of cases per 100,000 child years in the control group of the trial.

<sup>d</sup>Expressed as number of cases per 100,000 child years.

<sup>e</sup>Percentages in parentheses are the proportions of placebo recipients clinically diagnosed with LRTI that had evidence of radiologically confirmed pneumonia.

<sup>f</sup>Percentage of VAR for radiologically confirmed pneumonia compared to VAR for clinically diagnosed LRTI.

<sup>g</sup>Vaccine efficacy based on using the methodology for interpretation and reading of results suggested by a WHO working group and used in South Africa and The Gambia (8).

<sup>b</sup>Efficacy estimates for children included in the primary efficacy cohort, i.e., children enrolled in the study at between 6 weeks and 6 months of age.

involved children living in an urban area with free access to health care in which there is a very low threshold for treating with antibiotics. In Soweto, 19 to 34% of parents of children hospitalized for pneumonia volunteer that their children have already been started on antibiotics prior to their admission (31), which in all likelihood underestimates the actual number of children that have been started on antibiotics by as much as 40% (41).

The probable delayed presentation and decreased likelihood of being started on antibiotics prior to the performance of chest radiographs in The Gambia compared to California and South Africa may have resulted in a greater proportion of episodes of radiologically confirmed pneumonia in The Gambia being due to *S. pneumoniae*. Conversely, many episodes of pneumococcal pneumonia may have been successfully treated with antibiotics even prior to the performance of chest radiographs or perhaps at a stage when there were minimal changes visible on the chest radiographs in California and South Africa. Consequently, episodes of pneumonia progressing to manifest as radiologically confirmed pneumonia in South African and Californian children may have been less likely to be due to *S. pneumoniae* than to viruses or bacteria that did not respond to empirical antibiotic treatment.

The specificity of radiologically confirmed pneumonia as a marker of pneumococcal pneumonia would therefore be higher in settings such as The Gambia than in settings with health care similar to that provided within the context of the South African and Californian studies. The net result of a change in the specificity of the outcome used to measure vaccine efficacy would be a proportional change in the vaccine efficacy estimate. Similarly, a number of other bacteria, respiratory viruses, and fungi may cause an alveolar consolidation interpreted as evidence of radiologically confirmed pneumonia more often in children infected with HIV than in those not infected with HIV (24). Therefore, even if the vaccine protection against pneumococcal pneumonia was the same between children infected and those not infected with HIV, the poorer specificity of radiologically confirmed pneumonia as a proxy of pneumococcal pneumonia among HIV-infected children would result in a lower vaccine efficacy estimate.

In addition to affecting the vaccine efficacy estimate, differences among studies also limit firm comparisons from being made regarding the absolute burden of pneumonia prevented by PCV. Table 2 indicates that although the proportions of LRTI episodes that were radiologically confirmed pneumonia among controls or

placebo recipients were similar (17 to 22%) among the three studies in which vaccine efficacy was demonstrated, the absolute incidence of radiologically confirmed pneumonia differed vastly between the study populations (491 to 4,090 cases per 100,000 child years). Although this may reflect true differences in the incidence of radiologically confirmed pneumonia, it is also indicative of the influence that the study design has on such measurements.

Despite the variation in estimates of vaccine efficacy against radiologically confirmed pneumonia, the estimates of the efficacy of PCV in reducing the incidence of clinically diagnosed LRTI were almost identical (efficacy, 6.0 to 7.0%) among the studies for non-HIV-infected low-risk children. The lower vaccine efficacy against LRTI than against radiologically confirmed pneumonia was expected considering the poorer specificity of LRTI as a proxy of pneumococcal pneumonia. The value of measuring the effect of the vaccine against LRTI is, however, underpinned by differences that may exist between this outcome and that of radiologically confirmed pneumonia in estimating the overall burden of LRTI that is prevented by PCV, at least in some settings. Although radiologically confirmed pneumonia detected a level of prevention 88% of that detected by using LRTI in The Gambia, the percentage was lower in the study in California (78%) and even lower in the study in South Africa (35 to 59%) (Table 2). These differences may be explained by differences among the study populations in the factors that may influence the spectrum of radiographic features associated with pneumococcal infections, as previously described. The difference in study power (87 versus 30%, respectively) is the most likely explanation for the observation of a significant reduction in LRTI in HIV-infected children despite a nonsignificant reduction in radiologically confirmed pneumonia (29).

### IMPROVING OUR UNDERSTANDING OF THE TRUE EFFICACY OF PCV AGAINST PNEUMOCOCCAL PNEUMONIA

Although an outcome of radiologically confirmed pneumonia provides a useful tool to measure the efficacy of PCV, improving the specificity of the outcome measure may enhance our understanding of the true efficacy of PCV against pneumococcal pneumonia. Blood cultures provide a more specific marker of pneumococcal pneumonia than does chest radiology, but the limitations of blood cultures include a sensitivity of only 5 to 30% (1, 29), as well as a lack of availability in resource-constrained countries. Additionally, the clinical spec-

trum of bacteremic pneumococcal pneumonia may not reflect the same severity as nonbacteremic pneumococcal pneumonia (18). Microbiological confirmation of the etiology of pneumonia may be improved through the use of lung aspirates, despite the associated limitations as discussed above. The vaccine efficacy determined by using blood and/or lung aspirate culture-confirmed vaccine serotype-specific pneumococcal pneumonia was higher in California (efficacy, 87.5%;  $P = 0.04$ ), in The Gambia (efficacy, 70%; 95% CI, 31 to 88%), and in South Africa (efficacy: overall, 61% [95% CI, 16 to 82%]; non-HIV-infected children, 67% [95% CI, -65 to 93%]; and HIV-infected children, 59% [95% CI, 1 to 83%]) than the efficacy determined by using the radiographic outcome measure (6, 9, 29). In South Africa, only 2.6% (95% CI, 1.0 to 5.3%) of pneumococcal pneumonia episodes in control children not infected with HIV were found to be bacteremic. The sensitivity of blood cultures in detecting pneumococcal pneumonia was greater (18.8%; 95% CI, 17.3 to 20.3%) among children infected with HIV than in non-HIV-infected children. The latter observation may indicate impaired local immune responses in the lung parenchyma and consequent inadequate localization of the bacterium to the lung among these immunocompromised individuals (29).

The availability of an effective vaccine against pneumococcal disease importantly also provides an opportunity of probing the clinical, radiological, and epidemiological aspects of pneumococcal pneumonia and pneumonia in general. Alternate approaches to improve the specificity of radiologically confirmed pneumonia as a proxy for pneumococcal pneumonia would be to further restrict the inclusion of episodes of radiologically confirmed pneumonia to those most likely to be due to a bacterial etiology as an outcome measure. The absence of a sensitive test for diagnosing bacterial pneumonia contributes to the conflicting data regarding the usefulness of procalcitonin and CRP in discriminating between bacterial and viral pneumonia. A substudy performed in South Africa showed that the adjunctive use of CRP and procalcitonin improved the estimate of the efficacy of PCV against pneumococcal pneumonia. Vaccine efficacy in children not infected with HIV increased from 21% (95% CI, 1 to 37%) for radiologically confirmed pneumonia to 64% (95% CI, 23 to 83%) for only those episodes of radiologically confirmed pneumonia that were associated with a CRP level of  $\geq 120$  mg/liter and a procalcitonin level of  $\geq 5.0$  ng/ml (25). While the higher vaccine efficacy probably resulted from improving the specificity of the proxy outcome measure of pneumococcal pneumonia, the outcome was

nevertheless associated with reduced sensitivity in detecting the burden of pneumococcal pneumonia prevented compared to radiologically confirmed pneumonia. The latter outcome was, however, 5.3-fold more sensitive in detecting the burden of pneumococcal pneumonia prevented than an outcome of pneumococcal bacteremic pneumonia. Similarly, higher vaccine efficacy estimates were observed in children infected with HIV when the outcome of radiologically confirmed pneumonia was coupled with that of a CRP level of  $\geq 120$  mg/liter and a procalcitonin level of  $\geq 5.0$  ng/ml (efficacy, 9% [ $P = 0.38$ ] versus 52% [ $P = 0.004$ ]). The value of CRP and procalcitonin in improving the specificity of radiologically confirmed pneumonia needs to be tested a priori in future studies to confirm the usefulness thereof, especially in areas where malaria is endemic.

CRP at a threshold of  $\geq 40$  mg/liter was also associated with an improvement in the specificity and hence the vaccine efficacy estimates for other outcome measures used to measure vaccine efficacy against pneumococcal pneumonia. While the vaccine efficacy against any clinically diagnosed LRTI was only 1.6% ( $P = 0.78$ ), considering only those episodes of LRTI associated with CRP levels of  $\geq 40$  mg/liter yielded a vaccine efficacy estimate of 31.5% ( $P = 0.007$ ). Similarly, using episodes of LRTI which were associated with CRP levels of  $\geq 40$  mg/liter in the presence of radiographically confirmed pneumonia or of nonconfluent infiltrate on chest radiographs was more informative than using the outcome measure of radiologically confirmed pneumonia as a measure of vaccine efficacy (15% [95% CI, -6 to 32%] versus 22% [95% CI, 7 to 35%], respectively) (28). Additionally, the outcome of radiographically confirmed pneumonia plus a CRP level of  $\geq 40$  mg/liter associated with the presence of nonconfluent infiltrate was found to be more sensitive in detecting the burden of pneumococcal pneumonia prevented (vaccine-attributable reduction [VAR], 350 cases [95% CI, 111 to 557] per 100,000 child years) than the outcome of radiologically confirmed pneumonia alone (VAR, 134 cases [95% CI, -54 to 286] per 100,000) (27).

Notwithstanding the advances made in potentially reducing childhood morbidity due to pneumonia through the use of HibCV and PCV, studies in developed and developing countries identify respiratory viruses as the dominant pathogens identified from children with pneumonia. These data may be biased because of the higher sensitivity of detection of respiratory viral pathogens than of the causative agents of bacterial pneumonia. Additionally, largely because of the poor sensitivity in detecting bacterial infections, the role of concurrent bacterial infections in children with pneumonia in

whom a respiratory viral pathogen is identified remains controversial. Nevertheless, epidemiological evidence, including the influenza epidemics in 1918 and 1957, indicates that superimposed bacterial infections are important in the subsequent complications of respiratory viral infections (16). Additionally, animal model studies support the notion that viral infections are more severe when followed by a subsequent bacterial infection (39). More recently, investigational assays for diagnosing bacterial infections, particularly pneumococcal infections, have also shown that approximately one-third of children with community-acquired pneumonia in whom a respiratory virus is identified have concurrent pneumococcal infection, as discussed in chapter 8 (19, 32, 48).

The role of pneumococci in hospitalization for pneumonia in which a virus is identified was also investigated in the South African vaccine efficacy trial by using PCV-9 as a probe (26). Although vaccination with PCV-9 would be unlikely to have biologically changed the risk of children of being infected with the respiratory virus in the community, the incidence of hospitalization for pneumonia associated with the identification of one of the studied respiratory viruses was decreased by approximately one-third. Specifically, fully vaccinated PCV-9 recipients were found to have a lower incidence of hospitalization for pneumonia associated with the identification of the following viruses: influenza virus (45%; 95% CI, 14 to 64%), respiratory syncytial virus (22%; 95% CI, -3 to 41%), parainfluenza virus type 1 to 3 (44%; 95% CI, 8 to 66%), and human metapneumovirus (58%; 95% CI, 34 to 73%) (26, 30). The inference from this observation was that there was a reduced risk of superimposed pneumococcal infection, through PCV immunization, following infection with one of the studied viruses. This result may have been due to protection against the nasopharyngeal acquisition of vaccine serotype pneumococci among PCV recipients, as well as possible protection against mucosal invasion by pneumococci following viral exposure in vaccinees. Consequently, there were probably alterations in the clinical courses of the respiratory viral infections among vaccinees. Placebo recipients remained susceptible to superimposed bacterial infections, especially due to *S. pneumoniae*, following the viral infection that may have precipitated severe illness among these children.

## SAFETY OF PCV AND RESPIRATORY ILLNESSES

An intriguing finding from the study in South Africa was the transiently increased susceptibility to confirmed viral pneumonia, due predominantly to respiratory syn-

cytial virus, in the immediate period of 8 days following vaccination among PCV recipients ( $P = 0.03$ ). Hypothetically, the rationale for the heightened risk of viral pneumonia in these children may have been associated with the induced activation and subsequent depletion of circulating B cells by PCV-9 stimulation, which caused a period of greater susceptibility to pneumococcal infection. Additionally, vaccination may theoretically have been associated with the formation of complexes of pre-existing maternally derived circulating serotype-specific pneumococcal antibodies with antigen included in the vaccine. This infection by respiratory viruses during the immediate postvaccination period may have rendered these children more susceptible to developing superimposed, albeit unconfirmed, pneumococcal pneumonia. Additionally, PCV recipients were more likely to be hospitalized for hyperreactive airway disease and/or asthma ( $P = 0.009$ ) following the initial phase of follow-up (20). This difference in the incidence of wheezing episodes had, however, subsided after 6 years of follow-up with the South African cohort (S. A. Madhi and K. P. Klugman, unpublished data). Another intriguing finding has come from the postmarketing safety assessment of PCV in the United States. Black et al. reported that PCV vaccinees ( $n = 65,927$ ) had a 1.6-fold-increased risk of reactive airway disease compared to historical controls ( $n = 35,549$ ) to age 30 months. There was a 20% ( $P < 0.02$ ) increase in risk after adjustment for confounders in that study (4).

Similarly, increased susceptibility to wheezing episodes (results ratio, 1.5 to 1.6;  $P < 0.02$ ) was observed in the immediate period (0 to 14 or 0 to 30 days) following either the first or second dose of PCV compared to the period from 31 to 60 days postvaccination, with each individual being used as his or her own control, in the United States (5). Although this observational study did not perform testing for viruses, it is likely that the majority of the bronchiolitis and bronchitis episodes were virus induced. The study from the United States did not, however, find any significant increase in asthma or reactive airway disease during the immediate post-vaccination period compared to the later period (31 to 60 days) postvaccination.

Investigators involved in researching PCV recently concluded, "While more data are always helpful, there is a compelling case for pneumococcal vaccination in developing countries now. In light of the high burden of pneumococcal disease, consistent proof of conjugate vaccine protection against vaccine-type pneumococcal invasive disease, pneumonia, and meningitis, and the impressive reduction in all-cause mortality in The Gambia, use of pneumococcal conjugate vaccines, beginning

with the available 7-valent product, should be initiated as soon as possible in developing countries" (23). The effect that the introduction of PCV would have on the epidemiology of pneumococcal pneumonia in developing countries may be truly appreciated only following the widespread use of the vaccine in such countries.

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Robert C. Kohberger  
Jukka Jokinen  
George R. Siber

23

## Establishing Immune Correlates of Protection

The establishment of immune correlates of protection is critical for the development of new and improved pneumococcal vaccines. These vaccines may come from new manufacturers or manufacturing improvements to an existing vaccine. Once a vaccine is recommended for general use, a placebo-controlled trial with clinical disease end points is problematic because the use of a placebo may no longer be ethical. The reference group for new vaccines must then be the existing vaccine, and such trials require noninferiority approaches to their design and analysis. With clinical disease end points and relatively rare events such as invasive pneumococcal disease (IPD), noninferiority trials are often impractically large. An accepted immune correlate of protection is required as an end point for such trials to allow for a feasible trial design. In addition to licensing considerations, the establishment of protective correlates has intrinsic value for understanding immune protective mechanisms and aiding the development of improved vaccines.

Pneumococcal conjugate vaccines (PCV) present unique difficulties in establishing a correlate. The current seven-valent vaccine (Prevnar; Wyeth) has demonstrated efficacy in reducing IPD, acute otitis media (AOM), pneumonia, and nasopharyngeal colonization. Each of these syndromes has a different pathogenesis and most likely involves different protective mechanisms. Additionally, the current vaccine consists of seven distinct serotypes which also may have different protective requirements. Thus, with the current first-generation vaccine, a total of 28 different models could theoretically be proposed. Because of the limited number of IPD cases, the available data do not support the evaluation of all different possibilities. Some serotype-specific models are available for colonization and AOM. Only a serotype-pooled model is available for IPD, and as yet, no protective model exists for pneumonia, mainly because reliable serotype-specific diagnostics are not available for this disease.

This chapter will discuss the possible immune measurements that could be used to develop a correlative model, the functional forms of statistical models that have been used, and the results of correlative models for IPD, AOM, pneumonia, and colonization.

## IMMUNE MEASUREMENTS

The most common immune measurement that is used in the development of protective correlates is the enzyme-linked immunosorbent assay (ELISA) for immunoglobulin G (IgG) class anticapsular polysaccharide antibodies (19). This is because this assay (i) measures a relevant response by IgG, the class of antibody mediating primarily protective activity, (ii) has been validated for use for infants, (iii) is standardized and has completed an interlaboratory validation process, and (iv) is efficient, allowing large numbers of samples to be analyzed at a reasonable cost.

There are other measurements that may be as good as or better than the IgG ELISA in their predictive capability. These include functional antibody assays (i.e., opsonophagocytosis) and avidity and mucosal or cell-mediated measurements. While these measurements may be better than the IgG ELISA, they lack ease of use and standardization, and in any event, all of the models so far developed have used IgG ELISA. Jódar et al. (11) note that as the standardization and specificity of the assays have increased, the correlation between the results of IgG ELISA and opsonophagocytic assays for vaccinated subjects has increased. An example is given for serotype 4, for which the correlation is 0.92, and it is noted that similar correlations are found for serotypes 6B, 9V, 14, 18C, and 23F.

It is important to note that any changes to the IgG ELISA or the use of other immune measurements requires a bridge from the original correlate model to either establish equivalence or justify adjustment of the model.

All of the correlate models developed to date use a single IgG ELISA measurement taken approximately 1 month after the completion of a primary immunization series. The definition of a primary series depends on the trial and the age of the subjects in the trial and differs among the examples to be presented here. This is in distinction from the definition of a case, which can occur at any time after the immune measurement up to a defined end of follow-up. Therefore, the immune measurement does not represent the antibody concentration just prior to the case event, and thus, these models cannot be used to predict the precise risk of an event for an individual subject at any time during follow-up. They

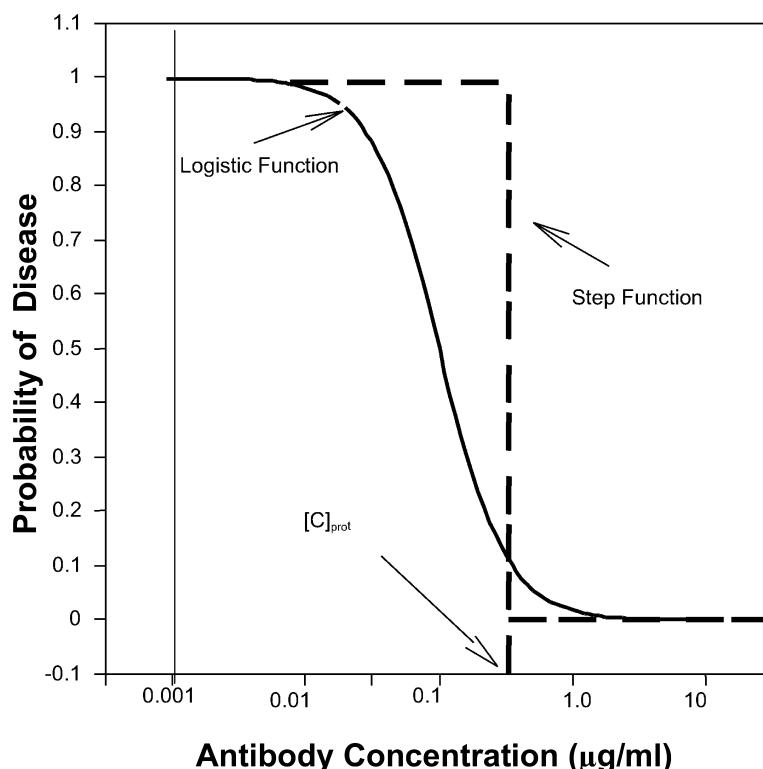
can, however, be used to predict and compare immune responses of populations after vaccination and relate these to clinical efficacy.

## STATISTICAL MODELS

The models to be discussed here are all correlative models rather than surrogate models. The statistical literature defines a validated surrogate end point as one where all of the treatment effect is explained by the surrogate (8). This is a very high standard to achieve, and there are few end points validated with this measure. Fleming (8) gives a hierarchy for end points with the following levels: level 1, the clinical disease outcome itself; level 2, a validated surrogate measure; and level 3, a measure correlated with the clinical end point and “reasonably likely to predict clinical benefit.” The IgG ELISA immune measurement is, therefore, a level 3 surrogate in Fleming’s hierarchy. It does not explain all of the PCV’s effect on disease prevention. The memory response to the conjugate vaccines is but one example of a property not completely captured by an IgG ELISA measurement taken 1 month after the primary series. Thus, the generalization of these models to vaccines of a different type from Prevnar, which has been the only type studied clinically, must be done with caution. Generalization to other polysaccharide-based conjugate vaccines regardless of the carrier protein(s) is most likely acceptable, but the models have uncertain validity for nonconjugate pneumococcal vaccines that are protein or polysaccharide based or for populations that differ in important ways from those studied in the clinical efficacy trials, such as infants infected with human immunodeficiency virus.

The statistical models relating immune response to protection are of two types, depending on whether the outcome measure is binomial (disease versus no disease) or time dependent, in which the time after vaccination when the event occurs is taken into account. The latter end point is modeled using survival analysis techniques and is described by Chan et al. (2) for a varicella vaccine example. All of the pneumococcal protective models use a binomial outcome. While a binomial model has been used in the past, survival models that estimate the point prevalence of events over time will be important, especially in the detailed modeling of colonization rates.

In order to develop these binomial models, there must be paired observations of the immune response and the clinical end point. Because the immune response of the control group (placebo recipients) is often essentially zero, in several of the models, only observations from the vaccinated group are used. There are several models



Theoretical relationship between risk of disease and concentration of protective antibodies. The step function represents the simplifying assumption required to calculate a protective concentration,  $C_{\text{prot}}$ .

that relate a continuous variable to a binomial response. To fit a model, a minimum number of vaccine failures are required. Colonization and AOM have a sufficient number of serotype-specific vaccine failures to make it possible to calculate the disease incidences for individuals with different immune response and then fit such models for at least some serotypes. IPD does not have a sufficient number of outcomes, and a different approach must be used.

The statistical models used for the analysis of binomial end points all follow the generalized linear model (GLM). GLM is very powerful and allows for a wide variety of outcome variables, including continuous, binomial, and rates of events. Logistic regression is one form of the GLM which is in widespread use. Logistic regression models the probability of an event as follows:

$$\text{probability of disease} = 1/(1 + e^{-u})$$

where

$$u = a + b \cdot x,$$

$a$  and  $b$  are constants to be estimated, and  $x$  is the immune measurement, usually log transformed. If the constant  $b$  is significantly different from 0, then the immune measurement is significantly related to the probability of disease.

When there are not a sufficient number of vaccine failures, a different approach must be used. It is assumed that for each subject, the relationship of the immune response and the probability of the event is a step function (Fig. 1). Below some level, the protective level ( $C_{\text{prot}}$ ), the probability of disease is relatively high and constant regardless of the immune response, and above this level, the probability of disease is much lower or absent and also constant. This step function can be linked to vaccine efficacy (VE) by using the step function probability and the immune responses in a population that is representative of the one for which the VE was calculated.

Let

- (i)  $p_v$  be the percentage of subjects  $< C_{\text{prot}}$  in the vaccinated group,
- (ii)  $p_c$  be the percentage of subjects  $< C_{\text{prot}}$  in the control group,

- (iii)  $a$  be the probability of an event at  $< C_{\text{prot}}$ , and
- (iv)  $b$  be the probability of an event at  $\geq C_{\text{prot}}$

Then

$$\text{probability of event given vaccination} = ap_v + b \cdot (1 - p_v)$$

$$\text{probability of event given control status} = ap_c + b \cdot (1 - p_c)$$

(Note that the latter equation represents the probability of disease in an unvaccinated cohort.)

Since

$$VE = 1 - (\text{probability of event given vaccination} \div \text{probability of event given control status})$$

$$VE = [(a - b) \cdot (p_c - p_v)] \div [b + p_c(a - b)]$$

we will assume that

$$VE \approx 1 - (p_v \div p_c)$$

In other words, the relative risk of disease is the same as the relative risk of being below  $C_{\text{prot}}$ . This will hold exactly if  $b$  is 0; i.e., if the probability of disease at  $\geq C_{\text{prot}}$  is 0.0. By knowing the VE and the distribution of immune responses in the vaccinated and control groups,  $C_{\text{prot}}$  may be estimated.

Each of these binomial approaches models protection on an individual basis, as a continuous function of antibody level for the GLM approaches and as a step function for the IPD model.

## IMMUNE CORRELATE MODELS FOR IPD

A WHO working group has proposed a level of 0.35  $\mu\text{g}$  of IgG anticapsular antibody/ml as measured by ELISA as a protective level for IPD (reference 11 by Jódar et al. and WHO Technical Report [20]). The detailed derivation of this level was recently described by Siber (17).

The proposed protective level is based on results from three trials. The Northern California Kaiser Permanente (NCKP) trial and the trial conducted in the United States with American Indian infants both used a seven-valent PCV conjugated with a nontoxic mutant form of diphtheria toxin (PCV7-CRM [Prevnar] from Wyeth Vaccines). Subjects were given the vaccine on a schedule of 2, 4, 6, and 12 months. The third trial was conducted in South Africa using PCV9-CRM on a

schedule of 6, 10, and 14 months. These trials were large, with approximate sizes of 38,000 subjects (NCKP), 8,000 subjects (U.S. American Indians), and 40,000 subjects (South Africa). Subjects were randomized 1:1 into vaccine and control groups. For all of these trials, antibody concentrations were measured approximately 1 month after the third dose and used to estimate the correlate of protection. The clinical efficacy and safety results for these trials are described in individual reports (see Black et al. [1], O'Brien et al. [16], and Klugman et al. [14]). Both the NCKP and South African trial found a significant vaccine protective effect for IPD. The trial with U.S. American Indians had a small number of cases and did not reach significant efficacy for IPD in the per-protocol analysis but did in the intent-to-treat analysis.

Siber et al. (17) derived a protective level by pooling cases across serotypes and using the step function approximation. Both VE and immunogenicity results were pooled across the serotypes. A protective level was estimated for each trial as well as the three trials combined. Three pooling methods were used: (i) simple pooling, in which all observations were combined; (ii) weighted pooling, in which immunogenicity data were weighted by the number of subjects in the trial; and (iii) weighted-protective-level pooling, in which each trial's protective level was weighted by its estimated variability. The results are given in Table 1 and as a visual presentation in Fig. 1. The results are similar regardless of the pooling method and suggest a protective level of 0.35  $\mu\text{g}/\text{ml}$ .

The limited number of vaccine failures precludes estimating a protective level without making several major assumptions, which include that (i) protection is related to the IgG ELISA antibody measurement through a step function model, (ii) protective levels are similar across serotypes, and (iii) protective levels are similar across trials. Because of the small sample sizes, either these assumptions cannot be statistically tested (i) or such tests are of limited power to detect differences (ii and iii). The assay itself adds a further complication. The results are based on a single-absorption assay with cell wall polysaccharide which has been standardized through international collaborative studies. Since that time, a second absorption with type 22F polysaccharide has been introduced in order to remove additional non-specific antibodies and enhance the specificity of the anticapsular polysaccharide antibodies that mediate protection. Henckaerts et al. (10) reported the effect of this second absorption of the samples (3) with type 22F and reported a decline of the protective estimate from 0.35 to 0.20  $\mu\text{g}/\text{ml}$  using sera from infants immunized with

**Table 1** Three controlled double-blind efficacy trials of PCV used in meta-analysis of protective pneumococcal antibody concentration<sup>a</sup>

Study (yr) and authors (reference)	No. of evaluable controls (vaccine)	No. of evaluable vaccinees (vaccine)	No. of IPD cases due to PCV serotypes among:			$C_{\text{prot}}$ ( $\mu\text{g/ml}$ ) (95% CI)
			Controls	Vaccinees	VE % (95% CI)	
NCKP (2000), Black et al. (1)	10,995 (MnCC)	10,940 (7vPnC)	39	1	97.4 (82.7–99.9)	0.20 (0.03–0.67)
Native American (2003), O'Brien et al. (16)	2,818 (MnCC)	2,974 (7vPnC)	8	2	76.8 (−9.4–95.1)	1.00 (0.25–>50.00)
South African (2003), Klugman et al. (14)	18,550 (placebo)	18,557 (9vPnC)	10	1	90 (29.7–99.8)	0.68 (0.03–6.00)
Total						
Pooling 1 (simple)	33,363	32,471	57	4	93.0 (81–98.2)	0.35 (0.09–0.89)
Pooling 2 (data weighted by trial size)						0.35 (0.11–0.85)
Pooling 3 (data weighted by variability of $C_{\text{prot}}$ )						0.38

<sup>a</sup>Adapted from Siber et al. (17). Numbers of evaluable patients are for per-protocol analysis. MnCC, meningococcal group C conjugate; 7vPnC, seven-valent PCV; 9vPnC, nine-valent PCV.

an experimental PCV. Siber et al. reassessed sera from the three efficacy trials with Prevnar and found much less impact of 22F absorption, which resulted in a minimal decline in the protective estimate, from 0.35 to 0.32  $\mu\text{g/ml}$  (17). The different results of these two studies underscore the need for rigorous design and control of bridging protocols.

Although IgG ELISA antibody cannot be viewed as a formal surrogate of protection for IPD, this does not preclude its use in immunogenicity trials. Although Prevnar has been shown to be a highly effective vaccine, its efficacy is less than 100%. Examining the reverse cumulative distribution (RCD) curves in Fig. 2 shows that 0.35 is at a point where the percentage of subjects responding just starts to decline below 100%, i.e., where with lower antibody levels it is expected that some breakthrough cases would occur, but at a low rate. Higher protective levels would predict too high a breakthrough rate, while lower levels would correspond to the flat portion of the RCD curve and predict too low a breakthrough rate. While not a perfect surrogate, the level of 0.35  $\mu\text{g/ml}$  is a useful benchmark for comparisons in immunogenicity trials.

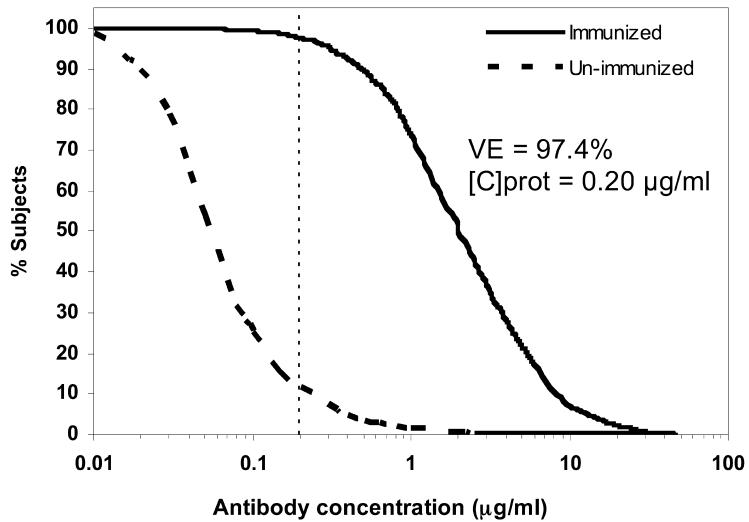
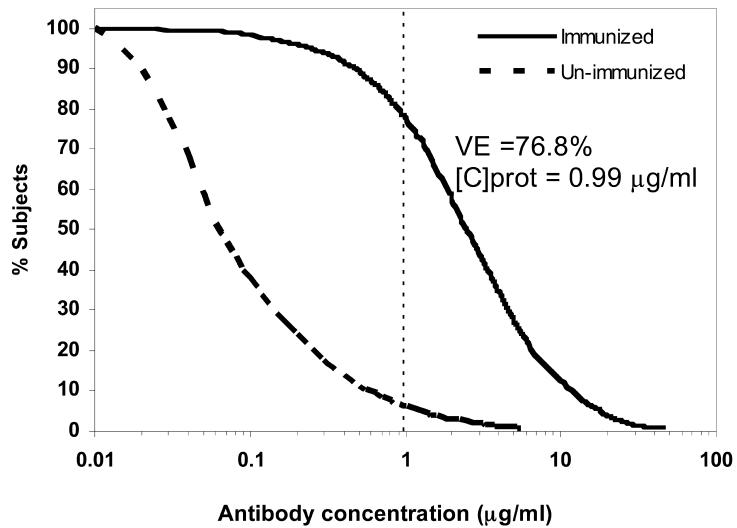
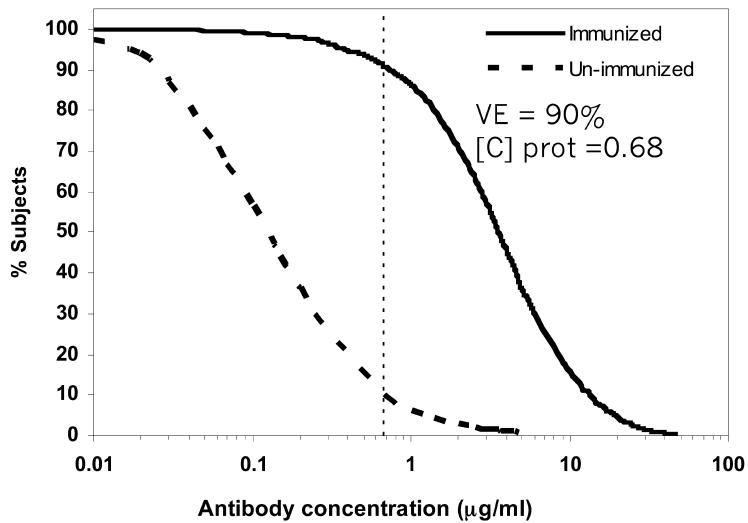
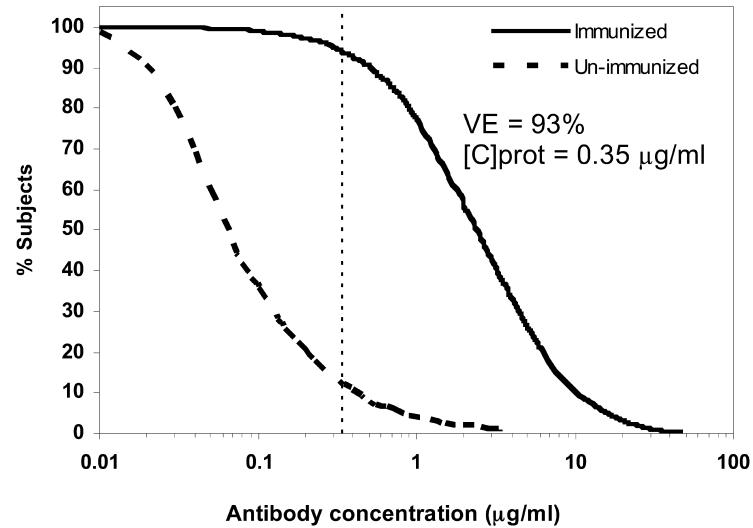
## IMMUNE CORRELATE MODELS FOR AOM

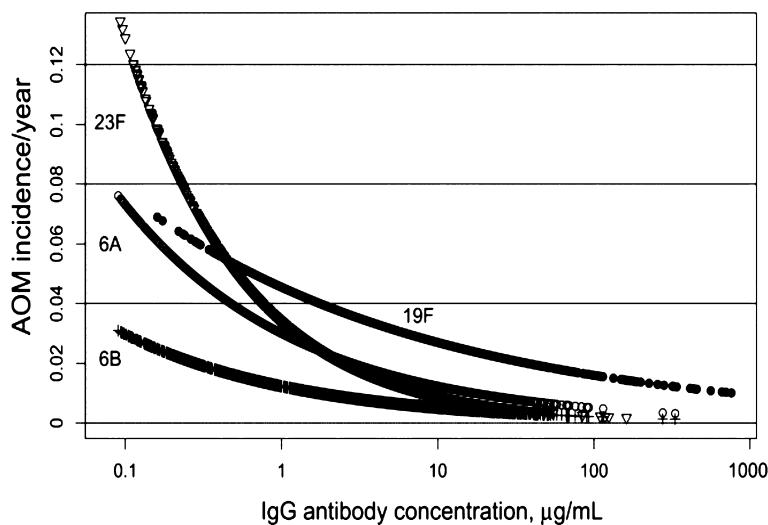
Jokinen et al. (12) have reported on a serological correlate of protection for AOM. The results are based on a trial conducted in Finland and reported by Eskola et al. (6) and Kilpi et al. (13). This randomized double-blind trial enrolled 2,497 subjects into three groups: (i) PCV7-

CRM (Prevnar, Wyeth) recipients, (ii) recipients of a seven-valent pneumococcal vaccine conjugated to meningococcal outer membrane protein complex (PncOMPc; experimental Merck Sharp & Dohme vaccine), and (iii) controls (recipients of a hepatitis B vaccine; Merck, Sharpe, Dohme). Subjects were vaccinated at 2, 4, 6, and 12 months of age. Serology samples were obtained at either 7 months or 13 months (samples were obtained from one half of the subjects at each time point). Antibody measurements were obtained by IgG ELISA methods. The trial found a serotype-specific efficacy against the first occurrence of vaccine type AOM of 57% (95% confidence interval [CI], 44 to 67%) for PCV7-CRM and 56% (95% CI, 44 to 66%) for PCV-OMPc, pooled for all vaccine serotypes. Efficacy was statistically significant for individual serotypes 6B, 14, 23F, and 6A.

The diagnosis of AOM was based on symptoms and pneumatic otoscopy findings. When AOM was diagnosed, myringotomy with aspiration of middle ear fluid was performed. This middle ear fluid was cultured, and if the culture was positive for pneumococci, the serotype was determined. For the evaluation of protective correlates, follow-up intervals were defined as 5 months from the date the serology sample was taken. For the 7-month sample, follow-up stopped at the date of the 12-month vaccination if earlier than 5 months from the date of serology sampling.

For each serotype, subjects were classified into two groups depending on whether at least one serotype-specific episode of AOM occurred. Because of the limited

**A. NCKP****B. American Indian****C. South African****D. Pool of 3 studies (weighted)**



**Figure 3** Effect of antibody concentration on the yearly incidence of serotype-specific AOM. Fitted values are from the GLM for each serotype. The risk of AOM caused by serotype 6A is associated with the concentration of cross-reactive 6B antibodies. Data are from Jokinen et al. (12).

number of AOM events available, only types 6A, 6B, 19F, and 23F were examined. The paired form of the data (each subject having an immune response measurement and outcome) allowed for a continuous model to be fit for each of the four serotypes. For 6A AOM results, the events were fit to the 6B immune responses. Both pneumococcal vaccines were included without any differentiation. The model fit to the data was a GLM with a complementary log-log link. The GLM with this link function obtains a fit that is similar in shape and form to a logistic regression model but may model the extreme ends of the curve better than logistic regression and can be used to estimate incidence rates.

The fit of the model to the four serotypes is shown in Fig. 3. Only immunized children were included in this model fit. Nearly all of the antibody levels in the control group were below those in the immunized group, and they had no effect on the risk of AOM. From the slope of the curves, the association of IgG antibody response was strongest for 23F and weakest for 19F. Statistical significance for the individual curves was not presented, but significance for 6A and 23F can be inferred from the significantly reduced relative risk associated with a 10-fold increase in antibody concentration. All serotypes show a difference in geometric mean concentrations (GMCS) of antibodies between subjects with and with-

out AOM (significant for 6A and 23F) and a reduction in risk (Table 2).

In a study using data from the same trial, Ekstrom et al. (N. Ekstrom, H. Lehtonen, H. Kayhty, T. Kilpi, J. Jokinen, and the FinOM Study Group, Fifth Int. Symp. Pneumococci Pneumococcal Dis., 2006) report the correlate results using opsonophagocytic activity (OPA) as the immune response. Their conclusion is that the effect of a 10-fold increase in antibody measured by IgG ELISA and that measured by OPA have similar impacts on reducing the risk of AOM but that differences could be seen for the two different pneumococcal conjugate vaccines in the trial. Only two serotypes (19F and 23F) were evaluated. For 23F, the risk reductions were similar between vaccines and response measurements, but for 19F, while reductions were similar for response measurements, they were different between vaccines, with PCV-OMP having a greater impact on AOM reduction (Table 3).

The results of these two studies demonstrate that IgG ELISA antibody is a correlate of protection for AOM. Incidence rates at an antibody level of between 5 and 10 µg/ml are quite low and, except for 19F, do not greatly decline at levels greater than 10 µg/ml. Because of the small sample sizes, differences among serotypes were not statistically demonstrated. The results suggest that the

**Figure 2** RCD curves for IgG antipneumococcal capsular polysaccharide antibody concentrations aggregated for the seven vaccine types in three controlled PCV efficacy studies and the studies with pooled data weighted for the number of study subjects (17).

**Table 2** Number of children with and without 6A, 6B, 19F, or 23F AOM events and GMCs of corresponding IgG antibodies among children immunized with PCV<sup>a</sup>

Serotype	No. of children:		GMC (95% CI) in children		RR (95% CI) associated with 10-fold increase in antibody concn
	Without AOM	With AOM	Without AOM	With AOM	
6A	1,559	17	2.43 (2.2–2.7)	0.81 (0.4–1.6)	0.41 (0.21–0.80)
6B	1,569	7	2.42 (2.2–2.6)	0.58 (0.1–3.4)	0.41 (0.15–1.17)
19F	1,555	21	5.35 (5.0–5.7)	3.86 (2.3–5.5)	0.59 (0.25–1.42)
23F	1,558	18	2.42 (2.3–2.6)	0.81 (0.4–1.7)	0.26 (0.12–0.58)

<sup>a</sup>Adapted from Jokinen et al. (12). GMCs are expressed as micrograms per milliliter. RR, relative risk.

relationship of immune response and protection differs across serotypes, with 23F corresponding to the strongest relationship and 19F to the weakest.

## IMMUNE CORRELATE MODELS FOR PNEUMONIA

Two large-scale, randomized, double-blind trials have evaluated PCV9-CRM efficacy against radiologically confirmed pneumonia (studies by Klugman et al. [14] and Cutts et al. [4]). Both found significant efficacy. In South Africa (14), per-protocol efficacy was estimated to be 25%, and that in The Gambia (4) was estimated to be 37%. Neither study correlated immune response with efficacy against pneumonia.

Esposito et al. (7) evaluated pneumococcal capsular antibodies and pneumonia in children aged 2 to 5 years admitted to the hospital with signs or symptoms of community-acquired lower-respiratory-tract infection and radiological findings consistent with pneumonia. Serum samples were obtained at admission and 4 to 6 weeks after admission. Pneumonia associated with pneumococcal infection (cases) was defined by radiological findings consistent with pneumonia and a ≥ 3-fold increase in the concentration of IgG antibodies from admission to 4 to 6 weeks after admission. Controls were those subjects not meeting these criteria.

For most serotypes, levels of ≥1.0 µg of IgG/ml measured by ELISA were associated with substantial reductions in pneumonia (88.6%), and for type 14, even levels of >0.15 µg/ml were associated with protection.

Several caveats in interpreting these levels are that (i) the children had not received conjugate vaccine and thus had acquired their immunity through natural exposures, (ii) the serologic diagnosis of nonbacteremic pneumonia used in this study has not been validated, (iii) the control group consisted of hospitalized subjects and may not be representative of the general population, and (iv) the case definition and outcome measure, IgG antibodies, are confounded. The possible biases in these results make definitive conclusions concerning protective levels problematic. Nevertheless, it is plausible that the level of antibody needed for protection from pneumonia is higher than that for bacteremic IPD.

## IMMUNE CORRELATE MODELS FOR COLONIZATION

Dagan et al. (5) reported the results of relating serum IgG antibody concentrations after vaccination with nasopharyngeal acquisition of pneumococci. Day care center attendees in Israel aged 12 to 35 months in a randomized study received either PCV9-CRM or, as a con-

**Table 3** Reduction in risk of AOM associated with a 10-fold increase in ELISA-measured antibody concentration or OPA<sup>a</sup>

Immune response measure	RR (95% CI) for serotype:			
	19F		23F	
	PCV-CRM	PncOMPC	PCV-CRM	PncOMPC
IgG antibody	0.8 (0.2–2.8)	0.26 (0.1–0.8)	0.31 (0.1–1.0)	0.42 (0.2–1.0)
OPA	1.03 (0.4–2.8)	0.19 (0.1–0.7)	0.56 (0.3–1.2)	0.40 (0.1–1.1)

<sup>a</sup>Adapted from Ekstrom et al., Fifth Int. Symp. Pneumococci Pneumococcal Dis. RR, relative risk.

**Table 4** Results of the logistic regression model of the probability of a new acquisition of pneumococcal serotypes 6A, 9V, 14, 19F, and 23F during follow-up of 129 subjects who received PCV9-CRM<sup>a</sup>

Serotype	No. of events observed among:		Logistic coefficient (95% CI)	P
	PCV9-CRM recipients	Controls		
6A	50	49	-0.63 (-1.14 to -0.15)	0.009
9V	9	21	-0.16 (-1.59 to 1.36)	0.83
14	16	31	-1.09 (-1.98 to -0.30)	0.006
19F	38	50	-0.68 (-1.33 to -0.05)	0.03
23F	25	45	-0.28 (-0.90 to 0.30)	0.34

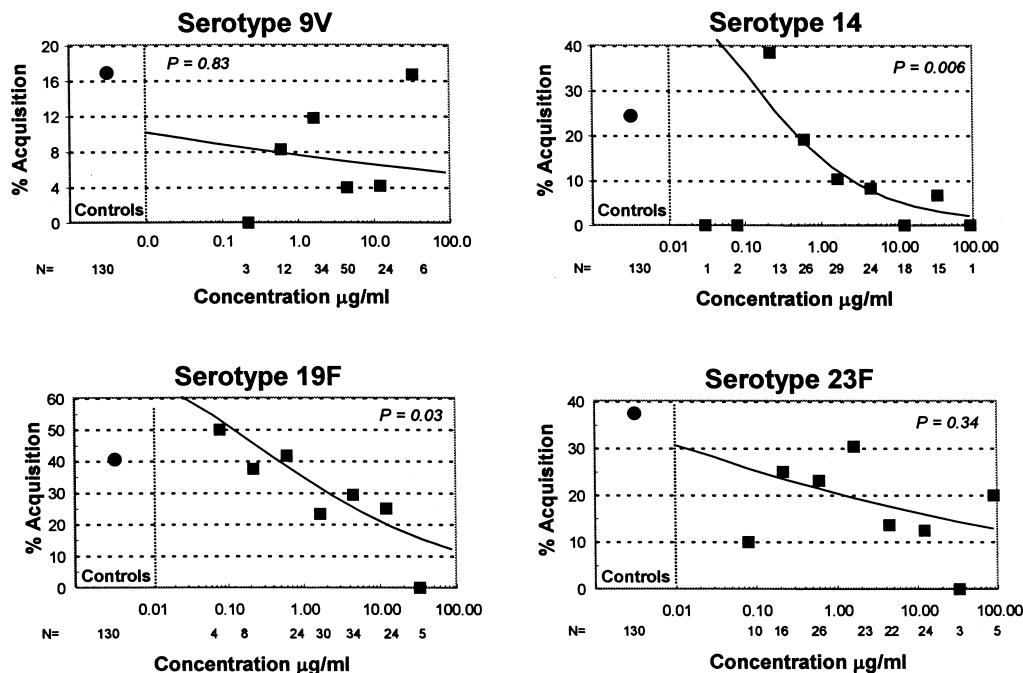
<sup>a</sup>Adapted from Dagan et al. (5).

control, a meningococcal type C conjugate vaccine. PCV9-CRM is a nine-valent extension of the seven-valent Prevnar vaccine (Wyeth) which includes serotypes 1 and 5. Subjects 12 to 17 months of age received two vaccinations 2 to 3 months apart, and subjects 18 to 35 months of age received a single vaccination.

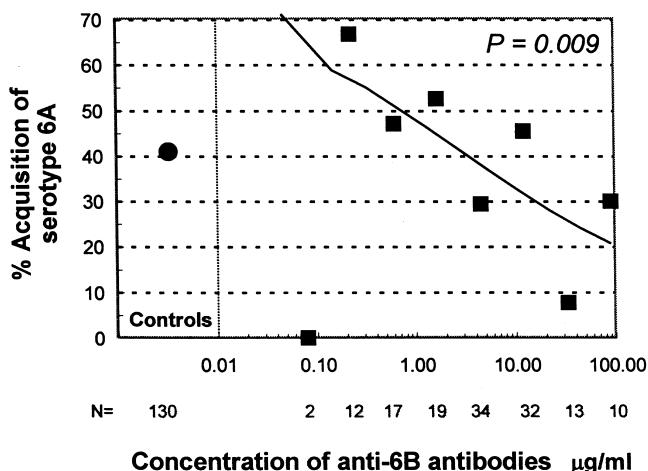
Follow-up was for 2 years, with monthly visits during the first year and visits every two months during the second year. At each visit, a nasopharyngeal sample was obtained for culturing and serotyping. Serology samples were obtained after the final dose.

The probability of acquisition as a function of IgG antibody concentration was modeled using logistic regression. Only vaccine serotypes 9V, 14, 19F, and 23F were modeled because of the limited number of acquisition events for the other serotypes. In a separate analysis, serotype 6A was modeled using the immune responses to 6B.

The serotype results are presented in Table 4 and Fig. 4 and 5. In a preliminary analysis, it was found that there was no effect of age at vaccination nor was there a significant difference ( $P = 0.40$ ) among serotypes in the



**Figure 4** Percentages of new acquisition events (in 1-log increments) and percentages of new acquisition events predicted (curve) by the logistic regression model by *Streptococcus pneumoniae* serotype (9V, 14, 19F, and 23F) among subjects who received PCV9-CRM (black squares) and subjects who received the control vaccine (black circles). Data are from Dagan et al. (5).



**Figure 5** Percentages of new acquisition events (in 1-log increments) and percentages of new acquisition events predicted (curve) by the logistic regression model for *Streptococcus pneumoniae* serotype 6A (using serotype 6B serologic values) among subjects who received PCV9-CRM (black squares) and subjects who received the control vaccine (black circles). Data are from Dagan et al. (5).

logistic regression coefficient. There was an overall significant relationship between IgG antibody concentration and the probability of acquisition, which indicates that higher levels of antibody reduce the probability of acquisition. In addition, there was a significant effect of serotype, which indicates that for a given IgG antibody concentration, serotypes have differing probabilities of acquisition. Table 4 gives the results for separate fits of the serotypes. A significant relationship was found for 14, 19F, and 6A.

Millar et al. (15) assessed colonization in American Indian infants immunized with PCV7-CRM and also noted reduced acquisition of 23F in children with increased type-specific IgG but no such relationship for type 19F.

The results indicate that IgG anticapsular antibody is a correlate of protection against colonization in immunized children. The relationship may differ among serotypes, but because of small sample sizes, this difference could not be statistically demonstrated.

In a related study, Goldblatt et al. (9) examined acquisition as a function of IgG antibody acquired through natural exposure. While serotype-specific results are limited because of small sample sizes, they did find a significant relationship for type 14 and concluded that a level of 5.0 µg/ml had a good correlation with protection. As shown in Fig. 4 for type 14, a level of 5.0 predicts a probability of acquisition of approximately 0.10.

Considering the variability in these estimates, they agree reasonably well.

Studying colonization in a mouse model of "naturally" acquired immunity, Trzcinski et al. demonstrated that CD4 T cells are responsible for protection from colonization (18). Concentrations of antibodies to several pneumococcal antigens, including PspA, PsaA, and cell wall polysaccharide, were also correlated with protection, presumably because they were correlated with the CD4 response. Notably, the live pneumococcal exposure used to induce natural immunity in this model did not induce significant anticapsular antibody responses. The model, therefore, does not contradict studies of humans showing colonization reductions by vaccine-induced anticapsular antibodies. Rather, it suggests that multiple immune mechanisms may be operative in preventing colonization and that correlating a particular measurement with disease rates does not necessarily prove that the mechanism was responsible for the protection.

## DISCUSSION

These results indicate that IgG ELISA antibody concentrations can be considered as a correlate of protection against pneumococcal AOM and colonization. Because of the available data, a correlate for IPD cannot be statistically proven, but most likely, IgG antibody is also correlated with protection against IPD. No difference among serotypes in the relationship of antibody level and protection could be demonstrated for IPD. This is probably due to the limited sample sizes in the IPD studies, since protection does differ among serotypes for AOM. The antibody levels needed to protect against IPD, AOM, and colonization apparently differ. Higher levels are required for protection against AOM and colonization than for protection against IPD. Levels for AOM and colonization are approximately the same. For IPD, a level of 0.35 µg/ml appears to be protective, while for AOM and colonization, a level of ~5.0 µg/ml may achieve a low probability of the event's occurring.

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VII

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Cynthia G. Whitney  
Matthew R. Moore

24

# Direct and Indirect Effectiveness and Safety of Pneumococcal Conjugate Vaccine in Practice

The United States introduced pneumococcal conjugate vaccine into its recommended vaccination schedule for all children <2 years of age in 2000, shortly after the vaccine was licensed (17). Demand for vaccine quickly outpaced supply, with vaccine shortages occurring intermittently between 2001 and 2004 (15, 16). Use of the vaccine increased after the shortages; according to the 2005 National Immunization Survey, 83% of children 19 to 35 months of age had received three or more doses of pneumococcal conjugate and 54% had received all four recommended doses (14). Other countries among the first to introduce conjugate vaccine included Australia, whose public health leadership recommended conjugate vaccine for all Aboriginal children in 2001, and Canada, which began routine vaccination in most provinces in 2002 (Table 1).

The number of countries routinely using pneumococcal conjugate vaccine as part of their childhood immunization program is growing. As of January 2007, approximately 14 countries recommended the vaccine routinely for all children (Wyeth Vaccines, unpublished data) (41). Other countries, such as Spain and Portugal, do not provide conjugate vaccine for all children, but the majority of children in these countries receive conju-

gate vaccine either through the private market or through membership in high-risk groups designated to receive government-purchased vaccine. For 2007, at least seven other countries are considering adopting pneumococcal conjugate vaccine.

Vaccine schedules adopted in the various countries differ. In the United States and most Canadian provinces, the vaccine is given to infants on a four-dose schedule, with doses at 2, 4, 6, and 12 to 15 months (17, 50), identical to the schedule used in the U.S. vaccine trials (4, 55). In some other settings, three doses are used, and either all three doses are given during the first 6 months of life (e.g., Australia) or two doses are given during that period, followed by a booster dose at around 1 year of age (e.g., Quebec province in Canada and the United Kingdom) (51, 65). While most countries began their program by vaccinating newborns as they turned 2 or 3 months of age, others have used catch-up schedules for children up to 23 months of age or for children up through 4 years of age who have high-risk medical conditions.

A growing body of evidence suggests that the routine use of pneumococcal conjugate vaccine is having a major impact on pneumococcal carriage, as well as disease.

**Table 1** Introduction of seven-valent pneumococcal conjugate vaccine into national immunization programs, as of January 2007<sup>a</sup>

Country	Schedule(s)	Yr of introduction	Children recommended to receive vaccine
United States	2, 4, 6, 12–15 mos	2000	Universal vaccination, nationwide
Australia	2, 4, 6 mos; polysaccharide vaccine also given at 18–24 mos for Aboriginal and Torres Strait Islander children	2001	Aboriginal and Torres Strait Islander children only
		2005	All Australian children
Canada	2, 4, 6, 12–15 mos; 2, 4, 12 mos (Quebec)	2002	Children in all Canadian provinces and territories (except Northwest Territories)
France	2, 3, 4, 12–15 mos	2003	Broadly expanded risk group recommendations
		2006	Universal vaccination, nationwide
Italy	3, 5, 11–13 mos	2003	Children in Sicilia province
		2004	Children in Puglia and Liguria provinces
		2005	Children in Calabria, Basilicata, and Molise provinces
		2006	Children in Emilia Romagna, Veneto, and Val D'Aosta provinces
Luxembourg	2, 3, 4, 12–15 mos	2005	Universal vaccination, nationwide
Qatar	2, 4, 6, 18 mos	2005	Universal vaccination, nationwide
Mexico	2, 4, 12 mos	2006	Children in low-income regions and high-risk children
Kuwait	2, 4, 6–18 mos	2006	Universal vaccination, nationwide
Germany	2, 3, 4, 11–14 mos	2006	Universal vaccination, nationwide
Greece	2, 4, 6, 15–18 mos	2006	Universal vaccination, nationwide
Norway	3, 5, 11–12 mos	2006	Universal vaccination, nationwide
Switzerland	2, 4, 6, 15–24 mos	2006	Universal vaccination, nationwide
The Netherlands	2, 3, 4, 11 mos	2006	Universal vaccination, nationwide
United Kingdom	2, 4, 13 mos	2006	Universal vaccination, nationwide
Belgium	2, 4, 12 mos	2007	Universal vaccination, nationwide

<sup>a</sup>Data are courtesy of Wyeth Vaccines and Eurosurveillance (41).

While a substantial reduction in disease among vaccinated children would have been predicted based on results from the earlier clinical trials, the observed impact has been much greater than expected because of indirect (herd) effects. In this chapter, we will review both the

direct and indirect effects of the introduction of pneumococcal conjugate vaccine into routine use. At this time, available data on vaccine impact are primarily from the United States, reflecting the timing of vaccine introduction.

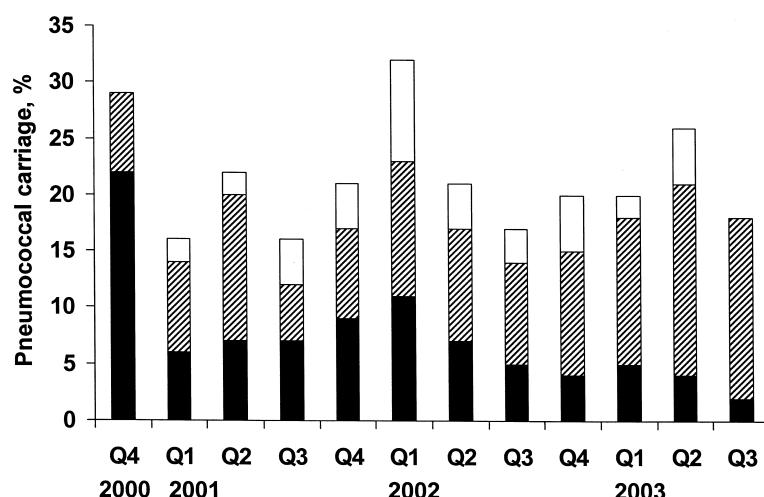
## EFFECT OF ROUTINE VACCINATION ON CARRIAGE AND DISEASE IN YOUNG CHILDREN

### Carriage

As expected from earlier reports of clinical trials (19, 43, 54), the routine use of pneumococcal conjugate vaccine as part of the U.S. vaccination schedule has reduced nasopharyngeal carriage of vaccine serotypes among children, although the overall prevalence of pneumococcal carriage has remained unchanged. An early study that evaluated carriage following routine introduction enrolled children from two medical practices in Texas during 2000 and 2001 and measured pneumococcal carriage at routine well-child visits, which occurred at 2, 4, 6, 9, 12 to 15, and 18 months of age (24). The investigators found that the proportion of vaccine type pneumococci dropped between the 12- to 15-month visit and the 18-month visit, corresponding to the timing of the fourth dose; at the same time, carriage of nonvaccine type pneumococci increased such that the overall prevalence of pneumococcal carriage remained between 24 and 30% between the 6- and 18-month visits. The trends were linked to changes in new acquisitions of pneumococci of vaccine and nonvaccine types, rather than a change in the duration of carriage. Another early study that took place in Boston evaluated carriage among 275 children 2 to 24 months old at well-child visits and visits for otitis media (57). Over the 3-year study period, the prevalence of pneumococcal carriage was generally unchanged, although there was a large decrease in the carriage of vaccine type pneumococci accompanied by

an increase in the proportion of carried isolates that were nonvaccine types (Fig. 1). In Kentucky, researchers evaluating carriage during a time of vaccine shortage noted that the carriage of vaccine serotypes decreased following the third and fourth doses and that children with prolonged intervals between the second and third doses ( $>3$  months) and the third and fourth doses ( $>8$  months) were more likely to carry vaccine type strains than children with shorter intervals between doses (33).

Cross-sectional studies evaluating carriage have also found a decrease in the carriage of vaccine type strains. In Massachusetts, a study of nasopharyngeal carriage among children  $<7$  years of age visiting primary care practices found that vaccine serotype pneumococci accounted for only 14% of colonizing strains in 2004, a decrease from 36% in 2001; at the same time, nonvaccine serotypes increased from 34 to 55% of strains, such that the overall prevalence of carriage did not change substantially (from 26 to 23% of all children) (32). In Alaska, a series of studies have evaluated carriage among Alaskan Natives living in rural villages and in urban settings (27, 29). Pneumococcal conjugate vaccine was included in the vaccination schedule for all children in Alaska in January 2001, after which coverage increased quickly; by the end of September 2003, 88.4% of Alaskan Native children 19 to 35 months old had received  $\geq 3$  doses (29). Among children under 5 years of age, *Streptococcus pneumoniae* colonization levels did not differ substantially from 1998 to 2003 in either rural (range, 51 to 60%;  $P = 0.26$ ) or urban (35 to 48%;  $P = 0.06$ ) settings. In both groups, vaccine



**Figure 1** Pneumococcal carriage among children  $<7$  years of age in Massachusetts by serotype and quarter Q1 to Q4, 2000 to 2003 (57). Solid bars, vaccine type; hatched bars, nonvaccine type; open bars, vaccine related, including serotype 19A.

type colonization decreased over time (from 55% of pneumococci at baseline to 11% in 2003 among residents of villages), while the carriage of nonvaccine type pneumococci increased; vaccine type carriage was significantly more common among children who were incompletely vaccinated or unvaccinated. At the same time, Alaskan Native adults 18 years of age or older living in the villages saw an increase in overall carriage from 13% at baseline to 26% in 2004, although the carriage of vaccine serotypes dropped from 28 to 5% over this time period (27). Adults living with a child of <5 years old were more likely to carry vaccine type strains, and among those living with children, the likelihood of vaccine type carriage fell if at least one of the children was appropriately vaccinated with pneumococcal conjugate vaccine.

### Invasive Disease

Reports from several sources indicate that the routine use of pneumococcal conjugate vaccine has had a profound effect on invasive pneumococcal disease (such as bacteremia, bactemic pneumonia, and meningitis) in children and that the effect occurred quickly following introduction (Table 2). In a multicenter U.S. study of children requiring hospitalization for invasive pneumococcal disease, investigators noted 77% fewer cases in children <2 years caused by vaccine serotypes in 2002 than the average number of cases in this group during 1994 to 2000 (34). The Northern California Kaiser Permanente health system, which conducted the first clinical trial to evaluate seven-valent pneumococcal conjugate vaccine (PCV7), has been tracking invasive disease among infants and young children in the system's large population (annual birth cohort, 38,000) (4). Between April 2002 and March 2003, Kaiser investigators found no cases of invasive disease caused by vaccine serotypes among children <1 year old, compared to between 51 and 98 cases per 100,000 person-years before vaccine licensure (3, 5). According to the Active Bacterial Core Surveillance (ABCs) program of the Centers for Disease Control and Prevention (CDC), a population-based system measuring invasive pneumococcal disease in approximately 20 million people in eight states, the total incidence of invasive pneumococcal disease (both vaccine type and nonvaccine type) among children <5 years declined by 75% from prevaccine levels by 2003. Rates of disease caused by all serotypes fell from 96.7 cases per population of 100,000 during 1998 and 1999 to 23.9 cases per population of 100,000 in 2003; the prevalence of disease caused by vaccine type strains fell 94%, from 80.0 cases per population of 100,000 to 4.6 (Fig. 2) (13, 69).

Surveillance programs evaluating invasive pneumococcal disease in single geographic areas in the United States have generally reported findings similar to those of the multisite studies (26, 31, 48, 67). However, among children who received medical care from Intermountain Health Care in Utah, the overall reduction in invasive disease was smaller than that seen elsewhere, with only a 27% reduction in disease rates by 2003 (11). In this population, an increase in disease caused by nonvaccine types accompanied a reduction in disease caused by vaccine serotypes, reducing the overall effect of conjugate vaccine on disease burden. The distribution of serotypes causing invasive disease before vaccine introduction and the age of children evaluated in this analysis differed from those typically reported in other studies, so the data may not be directly comparable.

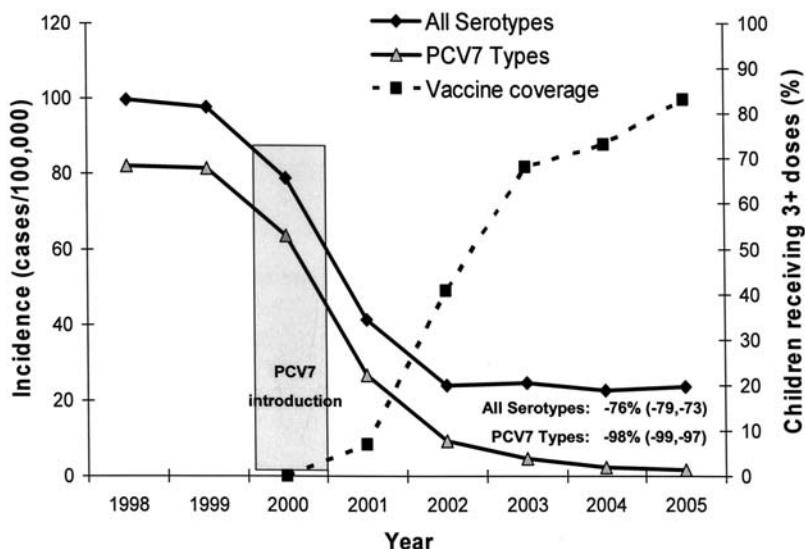
The use of conjugate vaccine in children has also reduced the differences in risk of invasive pneumococcal disease between certain racial and ethnic groups. According to ABCs data, rates of invasive disease in white children of <2 years of age fell 77% overall between 1998 and 2002 and those in black children fell 89% over the same interval; the incidence among black children under 2 years of age went from 3.3 times the rate among white children in the prevaccine period to 1.6 times the rate in 2002 (22). In Alaska, surveillance by the CDC's Arctic Investigations Program identified a large drop in disease caused by vaccine serotypes among the Alaska Native population (29). After vaccine introduction, vaccine type invasive disease fell 91% among Alaska Native children of <2 years old (from 275 cases per 100,000 children to 25 cases per 100,000) and by 80% among non-Natives of the same age (from 101 cases per 100,000 to 20 cases per 100,000), eliminating the disparity in disease caused by vaccine serotypes.

Only a few reports of vaccine impact from outside the United States are currently available, but those that have shown reductions in invasive disease rates similar to that seen in the United States. In Canada, the provinces of Alberta and Nunavut were the first jurisdictions to implement routine pneumococcal conjugate vaccination programs, in September 2002. By December 2004, an estimated 73.7% of children born between July and December 2002 had received four doses of conjugate vaccine, and another 14.6% had received three doses (35). The effect on vaccine burden was similar to if not more rapid than that seen in the United States. Compared with the combined rates in these provinces between 1998 and 2001, the rate among children 23 months of age and younger in 2004 decreased by 82%, to 11.7 cases per 100,000, for all serotypes and by 93%, to 3.9 cases per 100,000, for vaccine serotypes.

**Table 2** Impact of routine pneumococcal conjugate vaccine use on incidence of invasive disease in children, by age group and serotype

Population and/or location and baseline vs postvaccine periods (reference)	Serotype(s)	Age group yrs	Baseline incidence (cases/100,000)	Postvaccine incidence (cases/100,000)	% Change (95% CI or P value)
Northern California Kaiser Permanente subjects, 1996–1997 vs 2002–2003 (3)	All	<1	77.3	6.2	-93.7 (-78.7 to -99.0)
		<2	104.5	10.3	-90.9 (-81.8 to -96.0)
		<5	56.7	9.5	-84.1 (-74.7 to -90.5)
	Vaccine	<1	51.5	0.0	-100 (-87.3 to -100)
		<2	81.7	0.0	-100 (-95.2 to -100)
		<5	42.2	0.6	-98.8 (-94.2 to -99.9)
		Cross-reactive	16.1	0.0	-100 (-14.6 to -100)
		<2	14.7	0.0	-100 (-58.9 to -100)
		<5	8.2	1.7	-69.7 (-12.0 to -92.6)
	Nonvaccine	<1	9.7	6.2	-40.7 (-90.9 to 128.7)
		<2	8.2	8.8	17.4 (-56.9 to 182.7)
		<5	6.3	6.2	20.8 (-41.2 to 133.1)
Eight states in United States, 1998–1999 vs 2005 (47)	All	<5	98.7	23.6	-76 (-79 to -93)
	Vaccine	<5	81.9	1.5	-98 (-97 to -99)
	Cross-reactive <sup>a</sup>	<5	7.3	0.8	-89 (-79 to -84)
	19A	<5	5.3	9.3	255 (160 to 385)
	Nonvaccine	<5	6.8	11.9	74 (39 to 118)
Utah (Intermountain West), 1996–2000 vs 2001–2003 (11)	All	≤2	119	77	-36 (-47 to -23)
		>2–5	68	59	-17 (-34 to 0.03)
		>5–<18	33	25	-26 (-38 to -12)
Calgary, Alberta, Canada, 1998–2001 vs 2004 (35)	All	<2	63.6	11.7	-81.6 (0.02)
	Vaccine	<2	53.0	3.9	-92.6 (<0.001)
	Vaccine and cross-reactive	<2	59.4	3.9	-93.4 (<0.001)
	Nonvaccine	<2	4.2	7.8	84.4 (0.61)
Massachusetts, 1990–1991 vs 2001–2003 (31)	All	<5	56.9	17.4	-69 (-85 to -55)
	Vaccine and cross-reactive	<5	50.2	6.0	-88 (-103 to -74)
	Nonvaccine	<5	2.9	5.8	99 (-1 to 207)
Alaska, 1995–2000 vs 2001–2003 (29)	All	<2	204.5	75.1	-63 (<0.001)
		2–4	33.0	15.6	-53 (0.008)
	Vaccine	<2	147.3	21.2	-91, -80 <sup>b</sup> , (<0.001)
		2–4	22.4	5.6	-75 (<0.001)
	Nonvaccine	<2	42.5	49.0	(0.536)
		2–4	6.5	7.8	(0.701)
Australian Aboriginals in all states and territories, 2001 vs 2004 (64)	All	1–<2	294	84	-71 <sup>c</sup>
		<2	219.2	91.5	-58 <sup>c</sup>
2001–2002 vs 2003–2004	Vaccine	<2	192.4	49.6	-74 (<0.001)
	Nonvaccine	<2	124.0	140.4	+11.6 (NS <sup>d</sup> )

<sup>a</sup>Excluding serotype 19A.<sup>b</sup>Rates among Alaska Natives declined by 91%, while rates among non-Native Alaskans declined by 80%.<sup>c</sup>Percent change in rates estimated from published rates.<sup>d</sup>Not significant.



**Figure 2** Coverage with three or more doses of pneumococcal conjugate vaccine among children 19 to 35 months old according to the National Immunization Survey (NIS) and incidence of invasive pneumococcal disease among children <5 years of age by serotype in the CDC's ABCs areas, 1998 to 2005 (CDC, unpublished data; 13, 14, 47).

Among Australian Aboriginal children aged <2 years for whom pneumococcal conjugate vaccine became available in mid-2001, rates of invasive disease caused by vaccine types decreased by 74% between the baseline period of 2001 to 2002 and 2003 to 2004 (64).

### Evaluations of Vaccine Effectiveness against Invasive Disease

While surveillance data have evaluated pneumococcal conjugate vaccine impact on populations of young children, epidemiological studies have been used to evaluate effectiveness on an individual level. The shortages of pneumococcal conjugate vaccine that occurred between 2001 and 2004 (15, 16) meant that many children received an abbreviated two-dose series, received the primary three-dose series without the fourth (booster) dose, or experienced delays in the scheduled administration of doses. The shortages provided an opportunity to evaluate the effectiveness of incomplete vaccination with pneumococcal conjugate vaccine.

Two surveillance programs within the United States—the U.S. Pediatric Multicenter Pneumococcal Surveillance Group and the Massachusetts Department of Public Health—combined their data and used a case-only method to estimate the effectiveness of abbreviated or delayed dosing regimens against invasive pneumococcal disease (42). Among children not at high risk for invasive disease, the effectiveness of the vaccine against vaccine serotypes was estimated to be 91% for the full four-

dose series, 77% for three doses given before 7 months of age, and 71% for two doses given before 5 months of age, adjusting for study year (Table 3). A single dose given before 3 months of age did not provide statistically significant protection against vaccine serotypes, and none of the vaccine regimens provided significant protection against vaccine-related serotypes. Thus, while the full four-dose series was shown to be most effective, two or three doses may provide protection during times of limited vaccine availability.

Effectiveness was somewhat higher when measured in a large case-control study that used cases of invasive disease identified through the CDC's multisite ABCs program and age-matched controls. This study found that one or more doses of conjugate vaccine were 96% effective against invasive disease in healthy children, 81% effective in children with comorbid medical conditions (Table 3), and 76% effective overall against disease caused by strains resistant to penicillin (70). Vaccination was shown to be significantly protective against all seven individual vaccine serotypes and vaccine-related serotype 6A but not against vaccine-related serotype 19A. An examination of multiple partial and complete schedules found that nearly all provided some protection compared to no vaccine, although a single dose at <7 months was less protective than two or three doses at <7 months of age. A direct comparison of schedules of three doses before 7 months of age plus a booster dose at 12 to 15 months and of three doses before 7 months

**Table 3** Observational (retrospective) studies evaluating effectiveness of pneumococcal conjugate vaccine against invasive disease caused by vaccine serotypes in young children

Population (reference)	Study design	No. of subjects	No. of doses and age(s) at vaccination	Vaccine effectiveness % (95% CI)
U.S. children 3–59 mos old; multisite (70)	Case-control	3,294 (782 vaccinees, 2,512 controls)	≥1 dose, any age 1 dose, ≤7 mos 2 doses, ≤7 mos 3 doses, ≤7 mos 3 doses, ≤7 mos and 1 dose, 12–16 mos <sup>a</sup> 1 dose, 12–23 mos 2 doses, 12–23 mos <sup>a</sup> 1 doses, ≥24 mos <sup>a</sup>	Healthy children, 96 (93–98); children with comorbid conditions, 81 (57–92) 73 (43–87) 96 (88–98) 95 (88–98) 100 (94–100) 93 (68–98) 96 (68–98) 94 (49–99)
Healthy children <5 yrs old identified through 8 pediatric hospitals in Massachusetts (42)	Case only (indirect cohort)	400	1 dose, <3 mos 2 doses, <5 mos 3 doses, <7 mos 3 doses, <7 mos and 1 dose, 12–15 mos <sup>a</sup> 1 dose, 12–23 mos 2 doses, 12–23 mos <sup>a</sup>	39 (–80–80) 70 (28–88) 77 (50–89) 91 (18–99) 55 (–241–94) 68 (–219–97)
Children <5 yrs old in Navarra, Spain (1)	Case-control	510 (85 vaccinees, 425 controls)	≥1 dose, any age Incomplete Complete (1–3 doses depending on age)	88 (9–98) 100 (indeterminate) 81 (–54–97)

<sup>a</sup>Children receiving this regimen were considered to be fully vaccinated according to the recommended U.S. schedule.

without a booster dose suggested that the booster dose provided additional protection ( $P = 0.03$ ) (70).

A recent publication describing a case-control study from Spain also reported high effectiveness (88%) (Table 3) against invasive disease caused by vaccine serotypes (1). The study found a higher likelihood of nonvaccine type disease among vaccinated children (matched odds ratio, 6.2; 95% confidence interval [CI], 1.6 to 23.3), however, than among controls. Methods for case identification and verification of vaccination histories differed from those used in other studies, and the study occurred in a setting of low vaccine coverage (27% among controls), which may in part explain the findings.

A mathematical model of vaccination using surveillance and immunogenicity data suggested that a single dose of conjugate vaccine may be effective if the timing of administration is chosen carefully (2). Although a single dose was not predicted to be as effective as a three- or four-dose regimen, the model suggested that a single dose given between 5 and 7 months of age could prevent up to one-third of invasive pneumococcal disease. This might have the most impact in developing coun-

tries, where the cost of a full regimen of conjugate vaccine may be prohibitive.

### Pneumonia

Although pneumonia may be the single most important manifestation of pneumococcal disease, surveillance for pneumonia as a syndrome is not routinely performed in the United States or in most other industrialized nations. Nonetheless, investigators have evaluated the effect of pneumococcal conjugate vaccine introduction on pneumonia by using databases of discharge diagnosis codes and have found results as good as or better than those seen in the initial Northern California Kaiser Permanente trial. In the Kaiser trial, children receiving pneumococcal conjugate vaccine had 4.3% fewer episodes of clinically diagnosed pneumonia and 20.5% fewer chest X-ray-confirmed pneumonia episodes than children in the control arm in the per-protocol analysis (6). When the data from this trial were reanalyzed using World Health Organization criteria for chest radiograph interpretation, the estimated efficacy of PCV7 against first episodes of radiographic pneumonia increased to 30% (28).

In a study examining administrative databases from Tennessee and upstate New York, researchers found fewer visits coded for pneumonia than expected based on rates before conjugate vaccine introduction. During 2001 and 2002, there were 20 fewer ambulatory-patient visits for pneumonia per 1,000 children in Tennessee and 33 fewer ambulatory-patient visits per 1,000 children in New York, down from rates of 89 and 79 per 1,000 children, respectively, during 2000 to 2001. A recent analysis by some of the same authors using the Nationwide Inpatient Sample, the largest available in-patient database in the United States, and an interrupted time series analysis found reductions in hospitalizations coded for all-cause and pneumococcal pneumonia by comparing the period 1997 to 1999 (pre-PCV7 years) to that from 2001 to 2004 (post-PCV7 years) (25). By the end of 2004, all-cause pneumonia hospitalization rates had declined 39% in children aged <2 years, a drop of 506 hospitalizations per 100,000 children of <2 years of age, or about 41,000 pneumonia hospitalizations prevented per year. Rates of pneumococcal pneumonia (2% of all episodes of pneumonia) declined in children aged <2 years by 65%, or approximately 17 fewer hospitalizations per 100,000 children per year.

In Washington state, researchers at Group Health Cooperative, a large health care provider, evaluated pneumonia diagnoses based on ICD-9 code (presumed pneumonia), as well as the subset of patients who also had a new infiltrate noted on their chest X-ray reports (confirmed pneumonia) (52). Preliminary results indicated no consistent reductions in rates of presumed pneumonia in 2003 and 2004 compared to the prevaccine years 1998 to 2000. Significant decreases of 57 and 36% were seen for hospitalizations for confirmed pneumonia among children <1 year of age (4.7 cases per 1,000 person-years in 1998 to 2000 compared to 2.0 cases per 1,000 person years in 2003 to 2004) and 1 to 2 years of age (4.0 per 1,000 person-years in 1998 to 2000 compared to 2.5 per 1,000 person-years in 2003 to 2004).

### Otitis Media

About 15 million office visits occur in the United States annually as a result of acute otitis media (66), and costs due to otitis are estimated to be \$5 billion per year (9). *S. pneumoniae* is the most commonly reported bacterial cause of acute otitis media, with reports of the proportion of pneumococci among isolates recovered from middle ear fluid varying from 18 to 55% in published materials (7, 8, 36). Clinical trials evaluating pneumococcal conjugate vaccine found only modest decreases in otitis media episodes (4, 21), although given the high disease

burden and related costs of otitis media, any decrease observed following vaccination may be important.

Postmarketing studies indicate that routine use of conjugate vaccine is having a substantial effect on both the number and type of otitis media episodes. In a study examining administrative databases from Tennessee and upstate New York, researchers found that otitis media visits dropped 118 and 430 per 1,000 children in Tennessee and New York, respectively, from rates of 1,775 to 2,019 and 2,125 to 2,247 per 1,000, respectively, between the period from 1995 to 2000 and that from 2001 to 2002 (58). An updated analysis by the same group using the same data sets but analyzed by birth cohort found that among cohorts born in 2000 to 2001 or 2001 to 2002, after conjugate vaccine was licensed, 8 to 33% fewer children met criteria for frequent otitis media by age 2 years and up to 23% fewer had pressure-equalizing tube placement, depending on the birth year or state (59). In a separate study from New York, diagnoses of acute otitis media episodes that were treatment failures or resulted in persistent otitis media decreased to 12% of 1,232 diagnoses during the period from 2001 to 2003 from approximately 16% of 2,485 otitis diagnoses between 1995 and 2000 (12). Over this time period, the proportion of pneumococci among isolates from children with otitis media cases that had not responded to therapy fell while the proportion of *Haemophilus influenzae* among these isolates increased. Similarly, in Kentucky, the proportion of pneumococci among isolates from acute otitis media episodes decreased from 48% in 1992 to 1998 to 31% in 2000 to 2003, led by a decrease in vaccine type pneumococci; at the same time, the proportion of episodes caused by nontypeable *H. influenzae* increased (7). Finally, investigators in Texas evaluated bacterial carriage among children with acute otitis media who had been vaccinated with PCV7. Compared to historical controls before PCV7 introduction, vaccinated children had similar rates of pneumococcal carriage, but vaccinees had higher rates of carriage of *Moraxella catarrhalis* than the historical controls (74% among those vaccinated compared with 56% among the unvaccinated historical controls) (62).

### Safety

Results of postmarketing studies of the safety of pneumococcal conjugate vaccine suggest that the vaccine appears to be as safe as other routinely used vaccines. According to data from the Vaccine Adverse Event Reporting System (VAERS), a collection of passive reports of adverse events that are possibly related to vaccinations given in the United States, the majority of reports

of such events in children of <18 years of age during the first 2 years after licensure described minor adverse events previously identified in the clinical trials (71). During this time, approximately 31.5 million doses were distributed and VAERS received 4,154 reports of events that had occurred within 3 months of receiving pneumococcal conjugate vaccine, or 13 reports per 100,000 doses distributed. For the majority of reports (74%), the child had received other vaccines concurrently with pneumococcal conjugate vaccine. Serious events were described in 14.6% ( $n = 608$ ) of reports, including 117 deaths and 14 anaphylactic reactions; this proportion is consistent with the proportion of serious adverse events, 14.2%, reported from VAERS for other vaccines (72). Of the 117 deaths, 73 (62.4%) did not have an identified cause, although 59 of these were classified as sudden infant death syndrome or possible sudden infant death syndrome. Of the 44 deaths in which a cause was identified, 7 were due to pneumococcal infections (6 in partially vaccinated children). All 14 patients with anaphylactic or anaphylactoid reactions survived.

Seizures were described in 393 reports; 159 of these were considered serious (including 3 leading to death). Fifty-seven (14.5%) of the total seizures and 15 of the serious seizures (9.4%) occurred in children who had received pneumococcal conjugate vaccine without other vaccinations at the same time. Additional data were obtained for the first 98 reports of seizures; of these, 79 occurred in children who had either a history of seizures or fever at the time of the seizure.

The majority of VAERS reports involved minor adverse events similar to those that had been observed in clinical trials. In 23 cases, rechallenge with a subsequent dose of vaccine produced the same event encountered after a previous dose, suggesting a causal relationship. These included fever, irritability, and other nonspecific symptoms ( $n = 7$ ); respiratory symptoms ( $n = 4$ ); prolonged or abnormal crying ( $n = 4$ ); gastrointestinal disturbance ( $n = 3$ ); possible allergic reactions ( $n = 2$ ); seizures ( $n = 2$ ); and alopecia ( $n = 1$ ).

## INDIRECT, OR HERD, EFFECTS

Pneumococci are generally transmitted from persons carrying pneumococci in the nasopharynx to others who become carriers; a small percentage of the new carriers will go on to develop disease. The reduction in carriage of vaccine serotypes in children who have received pneumococcal conjugate vaccine means that fewer vaccine serotype pneumococci are circulating among families, in day care centers, and in the community. The reduced circulation of vaccine types from vaccinated

persons should in turn reduce carriage and disease caused by vaccine serotypes in those that have not received vaccine. This concept—protection of unvaccinated persons from disease by vaccinating a subset of the population—is known as herd, or indirect, effects. Several studies are now showing that herd effects are an important part of the public health benefit of pneumococcal conjugate vaccine use.

### Evidence for Herd Effects in Unvaccinated Children

A report from the Northern California Kaiser Permanente population early after vaccine licensure indicated that the reduction in disease was greater than the percentage of children who had been vaccinated (5). By early 2001, only 34% of children less than 5 years of age had received one or more doses of conjugate vaccine, and only 14% were fully vaccinated; in spite of this low coverage, disease caused by vaccine serotypes had dropped to 18 cases per 100,000 children from a baseline incidence of 42 to 60 cases per 100,000 in this age group. Data from the CDC indicated a similar reduction in disease greater than that expected from direct effects of vaccination: from baseline (1998 and 1999) to 2003, rates of vaccine type disease in children <5 years had dropped 94%, even though vaccine coverage with three or more doses in children 19 to 35 months of age in the United States was only 68% (Fig. 2) (13).

Children too young to have received pneumococcal conjugate vaccine also may be protected through herd effects. An analysis of invasive disease occurring in infants 0 to 90 days identified through ABCs found that rates decreased 40%, from 11.8 to 7.2 episodes per 100,000 live births, following conjugate vaccine introduction. Among black infants, rates of invasive pneumococcal disease declined significantly from 17.1 to 5.3 per 100,000. Rates of disease caused by vaccine serotype isolates dropped from 7.1 to 2.4 per 100,000 live births, while rates of disease caused by nonvaccine serotypes remained stable (60).

### Older Children and Adults

A report from Northern California Kaiser Permanente tracking disease rates through March 2003 noted that disease caused by vaccine serotypes was dropping among persons too old to have received pneumococcal conjugate vaccine (3). Among persons 5 to 19 years of age, the incidence fell from 2.6 to 1.6 cases per 100,000 person-years; in those 20 to 39 years of age, it fell from 5.7 to 2.7; in those 40 to 59 years of age, it fell from 10.2 to 8.6; and in those 60 years of age and older, the incidence fell from 35 to 26 cases per 100,000 person-years.

The reduction for all ages greater than 5 years was 25%, with the greatest reduction (52%) in 20- to 39-year-olds.

Changes in the invasive disease burden among person 5 years of age and older reported by ABCs have been generally similar to those seen in the Northern California Kaiser Permanente population (13, 40). Analyses of ABCs data show that invasive disease caused by vaccine serotypes had dropped 62% among persons 5 years and older between 1998 to 1999 and 2003, with the largest absolute rate reduction occurring in person 65 years and over (33.6 cases/100,000 in 1998 and 1999 and 11.9 during 2003) (13). An examination of older adults in the conjugate vaccine era indicated that disease was more likely than it was before conjugate vaccine introduction to occur in persons with comorbid conditions and to result in death, indicating that adults benefiting from herd effects were somewhat healthier than those who continued to get invasive disease (40). In addition, herd effects resulted in reductions among adults of  $\geq 50$  years for some invasive syndromes, like bacteremia and pneumonia with bacteremia, but less so for meningitis (40).

Studies from other surveillance programs have also shown herd effects in New York (67) and Alaska, although in the latter setting, indirect effects were significant in the non-Alaskan Native population but not among Alaskan Natives (29). In Canada, vaccine was introduced for infants in the province of Alberta in 2002. By December 2004, approximately 74% of children born in the second half of 2002 had received four doses of conjugate vaccine. The rate of invasive disease caused by vaccine serotypes among persons 65 years and older decreased by 63%, to 8.5 cases per 100,000, in 2004 compared to a baseline of the combined rates for 1998 to 2001 (35). Overall rates fell 24%, but the reduction in disease caused by all serotypes was not statistically significant. In addition, a report using administrative data from Medicare, the U.S. government system providing health care for older adults, found a 41% reduction among adults 65 years and older in hospitalization for invasive pneumococcal disease in 2002 to 2003 compared to the prevaccine baseline years (44).

While results of U.S. studies of indirect effects of pneumococcal conjugate vaccine on invasive disease are generally consistent, data on the indirect effects on pneumonia in adults are not as clear. No reduction was seen in pneumonia diagnoses among adult members of Group Health Cooperative, a large health care provider, in Washington state (52). In this study, researchers evaluated pneumonia diagnoses based on ICD-9 codes (presumed pneumonia), as well as the subset of patients

who also had a new infiltrate noted on a chest X-ray report (confirmed pneumonia). No decrease was seen in pneumonia rates by comparing postvaccine rates (2003 and 2004) to those from either 1998 to 2000 or 2001 to 2002. Conversely, another analysis using the large Nationwide Inpatient Sample database and an interrupted time series approach found lower-than-expected rates of hospitalizations coded for all-cause and pneumococcal pneumonia in 2004 for adults 18 to 39 years (25); reductions were also noted in the other adult age groups but were not statistically significant.

## REPLACEMENT DISEASE

Conjugate vaccines as currently designed can protect against only a limited number of the 90 pneumococcal serotypes. Carriage studies have shown that vaccination has reduced carriage of vaccine type pneumococci among children in the community (29, 32, 33, 46). The concomitant increase in the carriage of pneumococcal serotypes not covered by the vaccine means that non-vaccine serotypes may be transmitted and potentially cause disease more often than they did before the routine use of conjugate vaccine. An increase in disease caused by nonvaccine serotypes in the wake of a reduction in vaccine type disease is known as replacement disease. Prelicensure clinical trials of vaccine efficacy against otitis media noted significant increases in otitis caused by nonvaccine serotypes among children who had received PCV7 conjugate vaccine (21); in contrast, clinical trials evaluating invasive disease found no increase in invasive disease caused by nonvaccine serotypes (4, 18, 38). Available postmarketing data suggest that replacement invasive disease is occurring, but to date the amount of replacement disease has been minimal, except among selected high-risk populations.

### Children

Early reports of the impact of routine use of pneumococcal conjugate vaccine suggested that replacement was not occurring with invasive disease (69). The first report of statistically significant replacement invasive disease among children came from an eight-center study monitoring hospitalizations for invasive disease among children  $<2$  years of age in the United States (34). In this study, investigators found a 66% increase in non-vaccine serotype disease, although the absolute magnitude of increase in nonvaccine serotype disease was small relative to the decrease in vaccine type disease. Isolates of nonvaccine serogroups 15 and 33 had the largest increases. Data from the CDC's ABCs program have shown that the number of cases of invasive disease

caused by nonvaccine types in children <5 years of age increased 21% from the 1998–1999 average to the number in 2003 (13). In this large sample, the main serotype causing replacement disease in children has been serotype 19A, a type somewhat structurally similar to vaccine serotype 19F but for which the vaccine provides no cross protection (70).

An analysis comparing pneumococcal genotypes before and after conjugate vaccine introduction noted that the increase in 19A was due to the 19A clone most prevalent in the United States becoming more common as a cause of disease and also to the emergence of new clones with capsular serotype 19A that were previously identified as having only vaccine serotypes (56). This analysis suggests that pneumococci may be adapting to vaccine use by acquiring genetic material that allows a switch in expressed capsular type, thereby maintaining the genes associated with the virulence of the vaccine serotype while evading the immune response by expressing the polysaccharide coat of a nonvaccine serotype. Despite these data, it is not clear that all increases in disease caused by nonvaccine serotypes represent vaccine effects. In Seoul, Korea, where only a small proportion of the population has received conjugate vaccine, the proportion of invasive disease cases caused by serotype 19A increased between 1991 and 2005. Of note, the predominant 19A clone that emerged in that population was the same highly resistant clone as that which has emerged in the United States, suggesting that capsular switching may have occurred in Korea as well (37).

Data from single-site surveillance systems have noted more significant increases in disease caused by nonvaccine serotypes. In Utah, an increase in cases caused by serogroup 3 was noted among child members of a large health care system, along with an increase in the incidence of empyema and severe invasive disease between 2001 and 2003 compared to that in prevaccine years (11). In Alaska, recent data indicate that an increase in nonvaccine type disease among Alaska Native children aged <2 years began as early as 2003, 2 years after the universal introduction of PCV7. Between the baseline period (1995 to 2000) and 2004 to 2006, rates of invasive disease among children aged <2 years caused by nonvaccine serotypes increased from 95 to 229 cases per 100,000, substantially reducing the overall benefit of PCV7 (68). In contrast, replacement disease has not been as evident among non-Native Alaskans, suggesting that environmental, socioeconomic, or genetic factors may explain some of the differences seen in the two groups. Replacement disease has not been problematic among other indigenous populations, such as Australian Aboriginals (64). While disease rates among Alaska

Native children are among the highest reported, the overall size of the population is small, such that rate calculations are made based on relatively few cases; therefore, more data are needed to better understand the trends in this population.

To what extent replacement disease occurs likely depends in part on the ability of the nonvaccine serotypes to cause disease. Studies of invasiveness that have analyzed distributions of serotypes from carriage and invasive disease have shown that certain serotypes, including several of those contained in the seven-valent vaccine, are more likely to cause invasive disease than others (10). Other factors that may be important predictors of a strain's ability to cause replacement disease include whether the strain is antibiotic resistant, which lends a survival advantage over other strains, as well as its ability to be carried in the nasopharynx, which facilitates transmission. Serotypes 19A and 6A were the two most common nonvaccine serotypes causing invasive disease in children before conjugate vaccine introduction (63); both are antibiotic resistant and frequently carried. Why the incidence of disease caused by serotype 19A is increasing while that of serotype 6A disease is not may be explained by a difference in conjugate vaccine effectiveness for the two serotypes. While the seven-valent conjugate vaccine has been shown to provide protection against serotype 6A disease, no protection against serotype 19A has been shown (70).

Some evidence for replacement disease has also been seen with otitis media in postmarketing studies. A shift in pneumococci causing otitis media was also seen in Pennsylvania, where in 2001 children receiving at least two doses of pneumococcal conjugate vaccine were more likely to have acute otitis media caused by nonvaccine types (47%) than children who had not received conjugate vaccine (21%) (45).

## Adults

Infection with *S. pneumoniae* occurs more than 100 times more frequently in human immunodeficiency virus (HIV)-infected individuals than among the general population (20, 53). Some of the most dramatic increases in nonvaccine serotypes documented to date have been among adults 18 to 64 years of age with HIV or AIDS (23). In this very-high-risk population, overall rates of invasive pneumococcal disease (all serotypes) declined 19% between the 1998–1999 period and 2003. The overall decline was the result of a 62% reduction in disease caused by the seven serotypes in the vaccine and a 44% increase in disease caused by nonvaccine serotypes. A comparison group of adults 18 to 64 years

without HIV or AIDS demonstrated a very similar percent reduction in vaccine serotype disease (61%), but this group had no concomitant increase in nonvaccine type disease. These findings suggest that nonvaccine serotypes are able to cause invasive disease in immunosuppressed hosts but are not as capable as the vaccine serotypes of causing disease in normal hosts.

Replacement invasive disease has also been documented among older adults, but the magnitude has been smaller; among those 65 years and older, the estimated number of cases of invasive disease caused by nonvaccine serotypes in the United States in 2003 was 16% higher than the 1998–1999 average (Fig. 3) (13). The serotypes causing replacement invasive disease in older adults are somewhat more diverse than those causing replacement disease in children. While 19A is the most common serotype in both populations, statistically significant increases have also been noted for serotypes 3, 15B, 15C, and 33F in the elderly (30). Among Alaska Natives, serotype replacement was actually detected earlier in the adult population than in the pediatric population, with rates of invasive disease caused by nonvaccine types increasing slightly among adults aged >45 years while vaccine type disease dropped, resulting in no change in rates from prevaccine-era levels (27). Among Alaska Native adults, seven-valent vaccine serotypes accounted for a small proportion of invasive disease cases before conjugate vaccine introduction.

While replacement disease is concerning and bears watching, it is important to note that to date the magni-

tude of the increase in disease caused by nonvaccine serotypes in most populations has been small overall compared to the drop in disease caused by vaccine serotypes. Furthermore, even a severalfold increase in disease caused by a relatively rare serotype may still represent a small number of cases. According to estimates generated from the CDC's ABCs, approximately 65,000 cases of invasive disease occurred in the United States annually in 1998 and 1999, the years before vaccine licensure (13). In 2003, there were nearly 24,900 fewer cases, which included a drop in cases caused by vaccine serotypes of 29,600 and an increase in cases caused by nonvaccine serotypes of 4,700. The majority (2,700) of the increase in nonvaccine serotype cases was among adults aged 40 to 64 years, the age group which includes the most patients with later-stage HIV or AIDS (Fig. 4).

## CONCLUSIONS

Data on vaccine impact on carriage and disease following routine introduction have added a wealth of information on top of that learned from clinical trials. Not only has routine use of conjugate vaccine dramatically reduced disease in young children who have received it, but vaccination has also reduced disease in children and adults who have not. The direct effects, in general, reflect what would have been predicted from earlier clinical trials. The magnitude of the indirect effects has been important, contributing substantially to the cost-

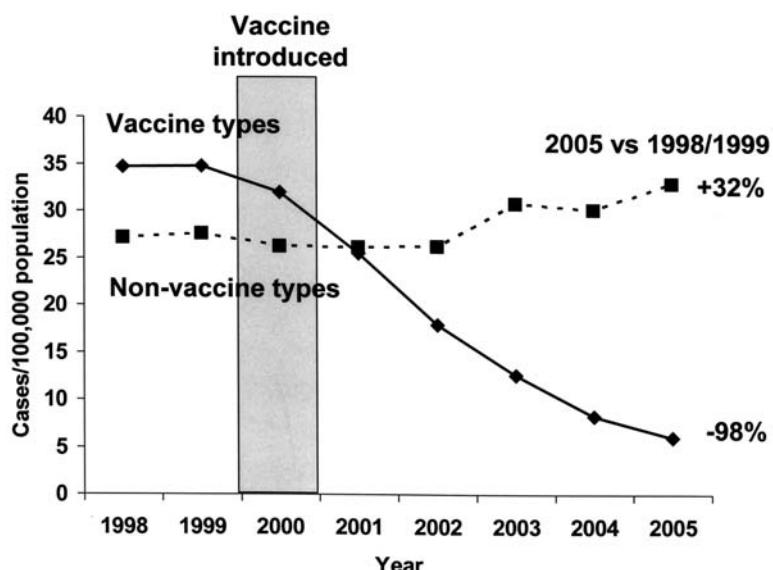
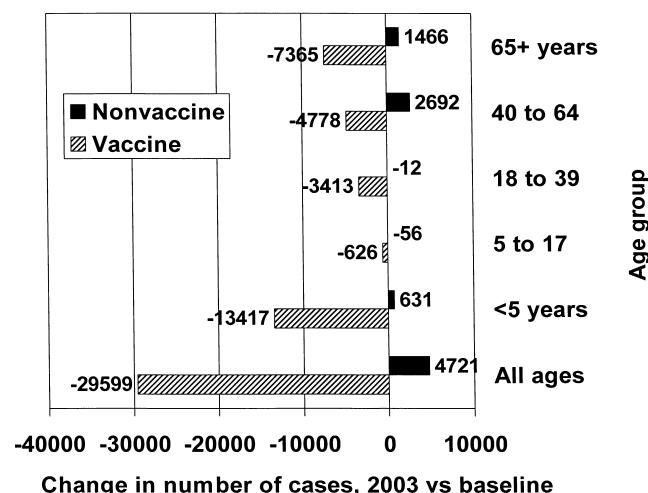


Figure 3 Incidence of invasive pneumococcal disease among adults 65 years of age and older by serotype in the CDC's ABCs areas, 1998 to 2005 (CDC, unpublished data; 13, 47).



**Figure 4** Change in projected number of episodes of invasive pneumococcal disease in the United States by serotype and age group, 1998/1999 versus 2003. Data are from the CDC's ABCs (13).

effectiveness of conjugate vaccine in the United States (61). An increase in nonvaccine type disease has been seen in some populations, such as adults with HIV and AIDS and Alaska Natives, but so far has been limited in magnitude in other populations. While we have learned a great deal in the few years that PCV7 has been used, several questions remain about the ultimate impact that pneumococcal conjugate vaccines will have on disease worldwide.

To date, pneumococcal conjugate vaccine has been used primarily in wealthier populations, who may have more or less similar patterns of transmission and prevalence of conditions that may predispose to pneumococcal disease. Whether the effects of routine conjugate vaccine use will differ in populations at higher risk for pneumococcal disease, such as those in developing-country settings, is unknown. One lesson from the observational data gathered to date may be that there is no way to predict for sure what the impact of conjugate vaccine introduction will be without trying it. Given what we have learned, the time has come to introduce conjugate vaccine in more places, a statement that has been supported by experts in pneumococcal disease from around the world (39). A critical next step is to begin using pneumococcal conjugate vaccines in developing countries. An estimated 1 million children younger than 5 years of age die of pneumococcal pneumonia in the world each year (49), and nearly all of these deaths occur in developing countries. Because of their availability and the large disease burden they could potentially prevent, pneumococcal conjugate vaccines, along

with rotavirus and meningococcal A/C vaccines, have been identified by the Global Alliance for Vaccines and Immunization as key new vaccines to promote for introduction into developing countries. Surveillance and observational research remain important to understanding vaccine effects as pneumococcal conjugate vaccines are introduced into new settings or as new vaccine formulations are introduced into countries already using the existing seven-valent vaccine.

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Ron Dagan  
Keith P. Klugman

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# Impact of Conjugate Pneumococcal Vaccine on Antibiotic Resistance

## INTRODUCTION

### History

The pneumococcus was considered for the first 20 years of the antibiotic era (the late 1940s to the late 1960s) to be universally susceptible to penicillin. In 1967, the first intermediately penicillin-resistant pneumococcus was described in Australia (47). Penicillin remained the drug of choice for treating pneumococcal infections as resistance to tetracyclines, chloramphenicol, macrolides, and trimethoprim-sulfamethoxazole (TMP-SMX) emerged (63). It was the emergence in 1978 of pneumococcal strains in Africa not only resistant to all these classes but also fully resistant to penicillin that ushered in the current era of the multidrug-resistant pneumococcus (56). During the 1980s, multidrug-resistant strains (resistant to three or more classes of drugs) were found in Spain and spread globally (63). During the 1990s, there was an inexorable increase in the prevalence of multidrug-resistant pneumococci in the United States (98) such that at the time of the introduction of the first

pneumococcal conjugate vaccine (PCV) in the year 2000, nearly half of all invasive pneumococcal disease (IPD) isolates were resistant to penicillin and/or macrolides (98).

### Mechanisms of Resistance

Most of the known bacterial mechanisms of resistance have been found in *Streptococcus pneumoniae*, with the exception of plasmid-mediated resistance, which has yet to be identified in this pathogen. Resistance to penicillin is mediated through sequential changes in penicillin binding proteins, which are altered by the acquisition through homologous recombination of fragments of foreign DNA from other streptococci, leading to a mosaic structure of the penicillin binding proteins (85). Macrolide resistance is mediated largely by the acquisition of the methylating enzyme ErmA, encoded by the gene *ermAM*, which methylates the target site of the macrolides on the rRNA (101). Occasionally, ErmA is encoded in *S. pneumoniae* by the *Streptococcus pyogenes*

Ron Dagan, Pediatric Infectious Disease Unit, Soroka University Medical Center, and Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel. Keith P. Klugman, Respiratory and Meningeal Pathogens Research Unit, National Institute for Communicable Diseases, Medical Research Council, and University of the Witwatersrand, Johannesburg, South Africa, and Hubert Department of Global Health, Rollins School of Public Health, and Division of Infectious Diseases, School of Medicine, Emory University, Atlanta, GA 30322.

resistance gene *ermA* (90). In the United States, macrolide resistance has more often been due to the efflux gene *mefE*, which is carried on a defective transposon-like element called MEGA (87). This gene is associated with 20 to 30% of macrolide resistance in most other parts of the world. In Italy, efflux is mediated by the closely related *mefA* gene (29). A small percentage of macrolide resistance is mediated by mutations at the target site in all four copies of the rRNA genes and/or mutations in the ribosomal protein genes encoding L4 and L22 (27). Fluoroquinolone resistance is mediated by two-step mutations in topoisomerase genes (27). Resistance to TMP-SMX is mediated by mutations in the dihydrofolate reductase (1) and dihydropteroate synthase (67) chromosomal genes, tetracycline resistance is mediated largely by *tetM* and occasionally *tetO* (102), and chloramphenicol resistance is mediated by the acquisition of a chloramphenicol acetyltransferase gene, probably acquired by the linearization within a transposon of the gene from a staphylococcal plasmid (100).

## RATIONALE FOR AN EFFECT OF PCVs ON ANTIBIOTIC RESISTANCE

### Vicious Cycle of Antibiotic Use and Selection of Antibiotic-Resistant *S. pneumoniae* Strains

There are many risk factors associated with antibiotic resistance in the pneumococcus, but the common risk in most cases is antibiotic exposure itself. There is significant global diversity in the proportion of pneumococci resistant to the two common classes of drugs used to treat pneumococcal infections, namely, the  $\beta$ -lactams and macrolides (55). Frequencies of resistance to both classes are low in countries such as The Netherlands where the level of antibiotic prescribing is low, while the highest frequencies of global resistance tend to be in wealthy countries with easy access to antibiotics, wealthily populations able to purchase them, and over-crowded institutions facilitating the amplification of selected resistant strains, e.g., Hong Kong, South Korea, and Japan (55, 84). Within the European Union, among countries with similar gross domestic products, there are still significant differences in rates of resistance (11, 43). Some of these differences may be explained by sociological differences, such as differences in patient expectations regarding antibiotics; e.g., some patients expect to receive antibiotics when presenting to the emergency room with a common cold (48). The selection of antibiotic resistance in the pneumococcus has occurred predominantly in strains that colonize or infect infants (2, 5, 9). This may be a reflection of increased opportunity for selection, as children carry pneumococci for longer

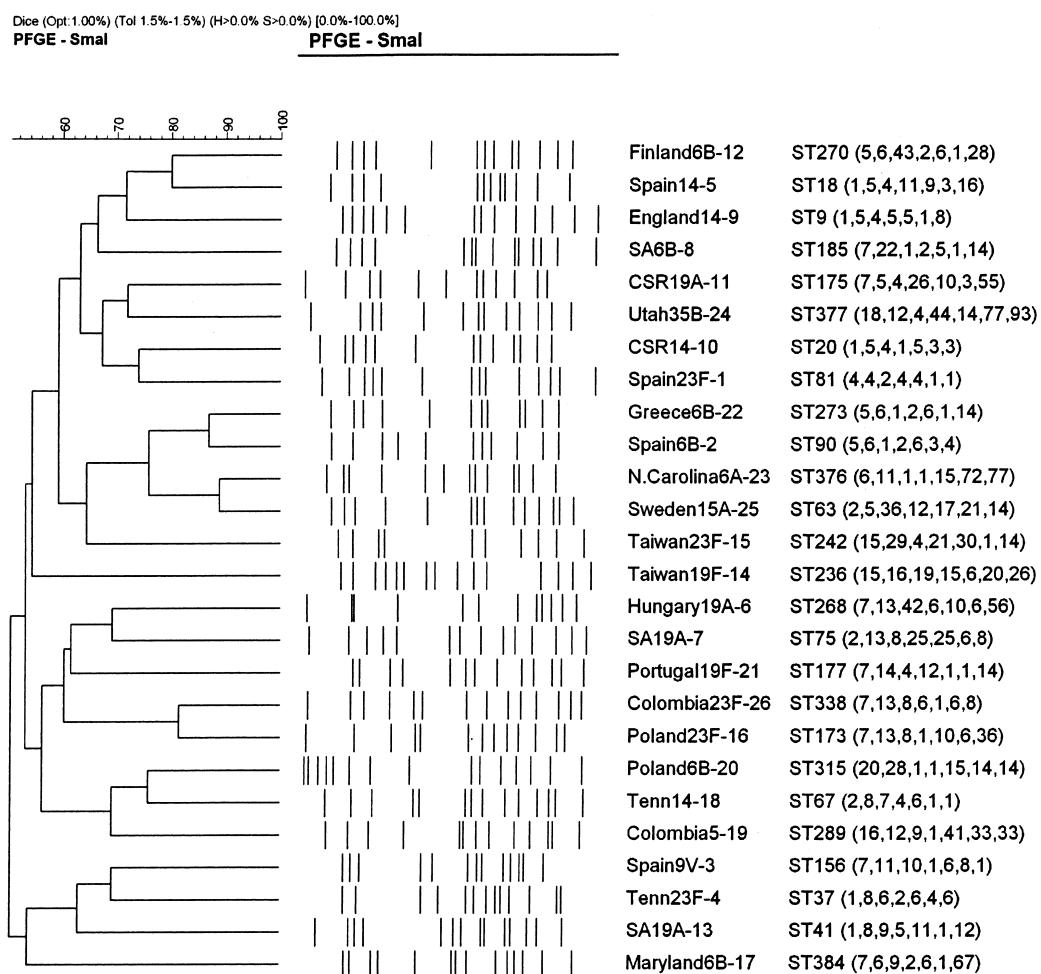
times than adults (52) and their exposure to antibiotics is greater. The only class of resistance to emerge in adults is resistance to fluoroquinolones (16, 51), a class of drugs developed for use almost exclusively in adults. The propensities of individual drugs within classes to select resistant pneumococci is vary. Studies have shown, in particular, an association between azithromycin use and macrolide resistance (30) and have demonstrated azithromycin exposure within the past 3 months to be a risk factor, not only for IPD due to macrolide-resistant pneumococci, but also for the development of multidrug-resistant strains (97).

### Serotypes and Clones and Resistance

The emergence of resistance in *S. pneumoniae* is not a diffuse introduction of resistance genes into the population of all pneumococci. Given the predilection for the emergence of resistance in strains carried in the nasopharynges of children, there is a clear association of resistance with the serotypes commonly carried in children. Thus, resistance, particularly high-level penicillin resistance and multidrug resistance, is associated with the seven serotypes 6A, 6B, 9V, 14, 19A, 19F, and 23F. The global pandemic of resistance within these seven serotypes is dominated by a relatively small number of resistant and multidrug-resistant clones that are characterized by an international network of investigators called the Pneumococcal Molecular Epidemiology Network (72) (Fig. 1). The contribution of global clones to high-level-penicillin-resistant populations of pneumococci is such that up to 93% of penicillin-resistant isolates from across the United States have been shown to belong to just eight clones (39). Populations other than children at risk for IPD due to pediatric serotypes, such as immunocompromised populations, tend to have increased numbers of resistant infections compared to the general population; one example is human immunodeficiency virus (HIV)-infected women (12). Finally, pneumococcal infections of the upper respiratory tract, too, are more often due to the colonizing serotypes than other types of pneumococcal infections and, therefore, are more likely to be antibiotic resistant (5). Day care centers (DCCs) are efficient sites for the expansion of resistant pneumococcal clones (103) and are therefore an important reservoir of resistant pneumococci in the community.

### Flow of Nonsusceptible *S. pneumoniae* from Children to the Community

Asymptomatic nasopharyngeal carriage of pneumococci is widely prevalent in both developed and developing countries, especially among young children. Carriage results in the spread of the pathogens, including nonsus-



**Figure 1.** Pulsed-field gel electrophoresis fingerprint patterns and dendrogram of SmaI restriction digests of representative isolates of 26 Pneumococcal Molecular Epidemiology Network clones. Reprinted from [http://www.sph.emory.edu/PMEN/pmen\\_clone\\_collection.html](http://www.sph.emory.edu/PMEN/pmen_clone_collection.html) with permission. (Courtesy Leslie McGee, Emory University.)

ceptible ones (60, 66, 74). As a result, young children play an important role in the transmission of pneumococcal infections within the community (15). For example, the presence of children in the household greatly influences pneumococcal carriage rates in adults, with an adult carriage rate of 18 to 29% in households with children less than 6 years of age compared with a carriage rate of only 6% in households without children (49). Therefore, the carriage of drug-resistant *S. pneumoniae* (DRSP) should be responsible for the spread of these organisms to both families and other children. Most of the reported outbreaks of DRSP disease in the United States have involved child care facilities, usually DCCs (10, 40, 53, 80, 83). In DCCs, three conditions exist that favor the development and transmission of resistant pneumococci: a large number of children, frequent close person-

to-person contacts, and intensive antimicrobial use, which seems to be particularly important in the selection of antibiotic-resistant strains. Thus, attendance at DCCs has been clearly identified as a risk factor for DRSP carriage (28, 61, 82, 89, 96). In a series of prospective studies conducted in southern Israel, the rapid and intensive spread of DRSP within DCCs and from DCCs to families was clearly demonstrated. Children aged 12 to 24 months attending DCCs with  $\geq 6$  children had a high risk of carrying DRSP compared to those attending DCCs with  $< 6$  children or not attending DCCs (relative risk [RR], 1.5; 95% confidence interval [CI], 1.2 to 1.8;  $P < 0.005$ ). For children  $< 6$  months of age with older siblings (usually attending DCCs), the risk of carrying penicillin-nonsusceptible *S. pneumoniae* (PNSP) was 4.3 (95% CI, 1.0 to 18.1;  $P < 0.05$ ). Further investigations

revealed that DCCs served as microenvironments, augmenting the spread of DRSP clones within each DCC (40, 103). Furthermore, these studies demonstrated that DRSP clones spread widely from DCCs to younger siblings of DCC attendees (42). Huang et al. developed a transmission model based on data from 16 Massachusetts communities, which explained marked differences in pneumococcal carriage across communities by variation in DCC attendance (53). A study from the United States showed that in the elderly, serotypes that are drug resistant (serotypes 6B, 9V, 14, 19F, and 23) are highly prevalent and that prevalence increases with age (33). The authors speculated that one plausible explanation is that this age group may have acquired these serotypes through contact with children, since contacts with children were shown to increase the risk of DRSP carriage in women compared with that in men in South Africa (12).

The fact that vaccinating infants and young children reduced DRSP disease and the carriage of pediatric DRSP serotypes in adults, as will be discussed in the following section, serves as the most compelling and definitive proof that children are responsible in a large part for the transmission of DRSP in the community.

## DIRECT EFFECT OF PCVs ON DRSP DISEASE AND CARRIAGE OF DRSP

### Direct Effect of PCVs on Invasive Disease Caused by DRSP in Children

Four randomized controlled studies were conducted to evaluate the efficacy of conjugate pneumococcal vaccines against pneumococcal invasive disease: two with the seven-valent PCV conjugated to a mutant, nontoxic form of diphtheria toxin, CRM (PCV7) (7, 75), and two with the CRM conjugate nine-valent vaccine (PCV9) (18, 64). However, the efficacy of the vaccine against disease caused by DRSP was studied in only one of these, using PCV9 (64). In this study, 19,922 infants received PCV9 and 19,914 received a placebo. DRSP was more common in HIV-positive than in HIV-negative children. The overall reduction of first episodes of invasive infections caused by PNSP was 67% (95% CI, 19 to 88%;  $P = 0.01$ ), episodes caused by TMP-SMX-resistant *S. pneumoniae* were reduced by 56% (95% CI, 16 to 78%;  $P = 0.01$ ), and episodes caused by any DRSP were reduced by 56% (95% CI, 21 to 77%;  $P = 0.005$ ). Efficacy against DRSP in HIV-negative children tended to be of a higher magnitude, but the difference was not statistically significant.

Available data on invasive disease caused by DRSP after the introduction of vaccines are limited to the United States, where PCV7 was introduced in the year

2000. It is not feasible to measure the real direct effect after the universal introduction of vaccines, since herd immunity and the number of doses (an issue complicated by the vaccine shortage that occurred in the United States after the initiation of vaccination [99]) play a role in reducing disease. Therefore, in this section, invasive infections caused by DRSP pre- versus postvaccination in children aged <5 years will be discussed as a surrogate for direct vaccine effectiveness.

The first published data suggesting an effect of the universal immunization with PCV7 on the rate of invasive DRSP disease in children were presented by Kaplan et al. (59), who monitored pediatric cases of invasive disease in patients admitted to eight pediatric hospitals during a 9-year period (1994 through 2002). They noted that the proportion of PNSP among pneumococcal isolates increased yearly from 1994 to 2000, reaching a plateau in 2001 at 45%, but that the proportion declined to 33% in 2002. A decrease in nonsusceptibility to penicillin in that study occurred entirely among isolates for which MICs were  $\geq 2.0 \mu\text{g/ml}$ . However, the exact patient age distribution was not specified. Talbot et al. (91) evaluated the effect of PCV7 from 2000 through 2002 in Tennessee by comparing data from the years 1995 through 1999. Among children aged <2 years, the proportion of invasive diseases caused by PNSP decreased from 59.8% in 1999 to 30.4% in 2002 ( $P < 0.01$ ), and trends for nonsusceptibility to cephalosporins and erythromycin were similar ( $P < 0.05$ ). However, in this study too, age was not specified (although all cases were in pediatric patients in studies both by Kaplan et al. and Talbot et al.) and the reductions noted were expressed in proportions rather than incidence.

Stephens et al. (87) conducted prospective, population-based surveillance in Atlanta and obtained pneumococcal isolates and demographic data from patients with IPD to determine the effect of PCV7 introduction since 2000 on invasive diseases caused by macrolide-resistant *S. pneumoniae*. They calculated cumulative incidence rates for invasive disease for 1999 to 2002 using population estimates and census data from the U.S. Census Bureau. In children aged <2 years, the incidence of disease cases caused by macrolide-resistant *S. pneumoniae* (per 100,000 children) increased steadily from 55.6 in 1994 to 134.1 in 1999, but after the introduction of PCV7, it fell to 98.4, 24.2, and 20.2 in 2000, 2001, and 2002, respectively (a reduction of 85% in 2002 compared to 1999). In general, in children aged 2 to 4 years, the trend was similar to that in children <2 years, but as expected, this reduction was less dramatic. The reduction was mainly in cases caused by serotypes

6B, 9V, 19F, 23F, and 6A but not serotype 19A, which was also often macrolide resistant.

Hennessy et al. (50) conducted a population-based study in Alaska to evaluate, among other outcomes, the incidence of invasive infections caused by DRSP. In children aged <5 years, the incidence (per 100,000) for PNSP (intermediate and resistant) fell from 26.9 in 1998 to 2000 to 9.9 in 2001 to 2003 ( $P < 0.001$ ). The respective figures for penicillin-resistant, TMP-SMX-resistant, erythromycin-resistant, and tetracycline-resistant pneumococci and those resistant to  $\geq 2$  drugs were 14.8 and 2.64 ( $P < 0.001$ ); 38.9 and 11.2 ( $P < 0.001$ ); 33.9 and 5.3 ( $P < 0.001$ ); 2.12 and 0 ( $P = 0.11$ ); and 38.9 and 9.9 ( $P < 0.001$ ).

The most comprehensive study reported so far has been that by Kyaw et al. (65). This study, conducted by the Centers for Disease Control and Prevention (CDC), used laboratory-based active surveillance in multiple representative areas in the United States (the Active Bacterial Core Surveillance) to measure invasive diseases caused by DRSP from 1996 through 2004. From 1999 to 2004, among children younger than 2 years of age, the incidence (per 100,000) of invasive diseases caused by PNSP decreased from 70.3 to 13.1 (a decline of 81%; 95% CI, 80 to 82%). The incidence of invasive disease caused by erythromycin-nonsusceptible strains was reduced from 58.6 to 12.0 (a decrease of 80%; 95% CI, 78 to 81%), and the incidence of disease caused by strains resistant to both erythromycin and penicillin was reduced from 51.5 to 8.6 (a decrease of 83%; 95% CI, 82 to 85%). For children aged 2 to 4 years, the incidence of PNSP invasive disease decreased by 60%. For disease caused by multidrug-resistant *S. pneumoniae*, the decrease in children aged <2 years was 84% (95% CI, 82 to 85%), and that in children aged 2 to 4 years was 64% (95% CI, 58 to 68%).

At the same time that the decreases in total IPD in general and in DRSP IPD in particular were observed, an increase in disease caused by nonvaccine serotype *S. pneumoniae* (the so-called replacement phenomenon) was observed. Some of the nonvaccine serotypes that cause invasive infections are DRSP. Byington et al. studied invasive pneumococcal infections among children <18 years of age admitted to medical centers in Salt Lake City, Utah, from 1997 through 2003 (13). A decrease in the number of cases caused by vaccine groups was observed during the period from 2001 to 2003 compared with the period from 1997 to 2000 (52 cases versus 94 cases, respectively). However, the reverse trend was found for cases caused by nonvaccine groups (51 cases versus 25 cases, respectively). The authors also looked at the proportion of PNSP isolates in the two pe-

riods and found that the proportion of these isolates decreased from 44 of 129 pneumococcal isolates (34%) from 1997 to 2001 to 23 of 105 isolates (22%) from 2001 to 2003 ( $P = 0.04$ ). However, almost the entire reduction in the proportion of PNSP was due to a reduction in the proportion of penicillin-resistant *S. pneumoniae* (for which the MIC of penicillin was  $\geq 2.0 \mu\text{g/ml}$ ) from 16% in 1997 to 2000 to 3.7% in 2001 to 2003 ( $P = 0.018$ ). The respective proportions of *S. pneumoniae* isolates with intermediate resistance to penicillin (PISP) were 22 and 18% ( $P$ , not significant). These findings are in accordance with the observation from other investigators that intermediate resistance to penicillin (but not penicillin resistance) is relatively prevalent among nonvaccine serotypes (37, 73, 78, 79, 94). A parallel, but more accentuated, trend in replacement with nonvaccine serotype PISP has been found in carriage and will be discussed in "Direct Effect of PCVs on Carriage of DRSP" below.

The study by Byington et al. suggests that invasive pneumococcal infections with nonvaccine serotypes that are nonsusceptible to penicillin are increasing. It was hoped that IPD caused by vaccine-related serotypes 6A and 19A would be reduced by cross protection with serotypes 6B and 19F, which are included in PCV7. Although this reduction has been observed with serotype 6A, an increase rather than a decrease in serotype 19A IPD has been observed (4, 50, 76). Steenhoff et al. recorded episodes of pneumococcal bacteremia in children aged <18 years in a hospital in Philadelphia from July 1999 to May 2005 (86). The proportion of PNSP bacteremia among all cases increased from 25% in 1999 to 2000 to 39% in 2001 to 2005 ( $P < 0.05$ ). At the same time, the proportion of cases caused by vaccine-related serotypes increased from 6 to 35% ( $P < 0.01$ ), rates of disease caused by vaccine serotypes decreased by 57%, and those of infections with nonvaccine serotypes did not change. When the figures were expressed as rates per 100,000 emergency department visits, the RR for infections with vaccine serotypes was 0.44 (95% CI, 0.34 to 0.58) for 2001 to 2005 versus 1999 to 2000, the RR for infections with nonvaccine serotypes was 1.05 (95% CI, 0.87 to 1.25), and that for infections with vaccine-related serotypes was 2.21 (95% CI, 1.08 to 1.37).

Pai et al. (76) observed that not only did serotype 19A become the most important cause of IPD in children in the United States after the introduction of PCV7, it is increasing in incidence and, not less importantly, has become increasingly drug resistant. The rate of invasive infections caused by serotype 19A in children <5 years old increased significantly from 2.6 cases

per population of 100,000 in 1999 to 2000 to 6.5 cases/100,000 in 2003 to 2004 ( $P < 0.001$ ). This increase was accompanied by a 61% increase in the frequency of penicillin nonsusceptibility ( $P = 0.008$ ), a 303% increase in the frequency of penicillin-resistant strains ( $P = 0.001$ ), and a 159% increase in the frequency of strains nonsusceptible to  $\geq 3$  classes of antibiotics ( $P = 0.002$ ). As observed during the pre-PCV7 era, clonal complex 199, predominant within serotype 19A, accounted for ~70% of invasive serotype 19A strains during 2003 to 2004. New genotypes of 19A were, however, also observed, as was evidence of the selection of 19A clones that had previously been more common among vaccine serotypes.

Thus, the reduction in invasive diseases caused by antibiotic-resistant pneumococci is tempered to a certain extent by the increase in drug resistance (mainly intermediate resistance to penicillin) among nonvaccine serotypes and vaccine-related serotypes (mainly 19A).

#### Direct Effect of PCVs on Mucosal Infections: Otitis Media Caused by DRSP as a Paradigm

*S. pneumoniae* is an important pathogen in various mucosal infections, such as otitis media, sinusitis, pneumonia, and conjunctivitis. However, data on the effect of conjugate pneumococcal vaccines on mucosal infections caused by DRSP are limited to the effect of these vaccines on otitis media.

The overall reduction of acute otitis media (AOM) cases in developed populations is not expected to be of high magnitude (7, 31, 62, 81). Because *S. pneumoniae* is usually found in less than half of all AOM cases and only ~70% of isolates in these cases are of a serotype included in PCV7 (81), and because replacement with nonvaccine *S. pneumoniae* serotypes and nonpneumococcal organisms occurs after vaccination with PCV7 (31, 62), the most prominent effect of PCVs on AOM may not be the reduction in rates of AOM in vaccinated individuals but rather a change in proportions of the various pathogens. The expected spectrum of AOM pathogens in vaccinated compared to nonvaccinated individuals includes an increased proportion of *Haemophilus influenzae*, *Moraxella catarrhalis*, and non-vaccine type *S. pneumoniae* isolates and a reduction in vaccine type *S. pneumoniae* (81). For AOM, like other entities, the majority of antibiotic resistance, especially high-level penicillin and multidrug resistance, among *S. pneumoniae* strains occurs among the five serotypes included in all vaccines (6B, 9V, 14, 19F, and 23F) (20, 58). Thus, an overall reduction in AOM caused by DRSP in vaccinated children is expected. However, data

obtained after the introduction of PCV7 into the United States are very sparse in this regard.

Block et al. documented changes in proportions of pathogens isolated in cases of severe and refractory AOM before (1992 to 1998) and after (2000 to 2003) the introduction of universal vaccination in rural Kentucky (8). In this study, the proportion of *S. pneumoniae* isolates decreased from 160 of 336 (48%) to 26 of 83 (31%) ( $P < 0.01$ ) and the proportion of *H. influenzae* isolates increased from 137 of 336 (41%) to 46 of 83 (56%) ( $P = 0.02$ ). A reduction of the proportion of PNSP and penicillin-resistant *S. pneumoniae* isolates among all isolates was suggested (53 of 336 [16%] versus 11 of 83 [13%] and 30 of 336 [9%] versus 5 of 83 [6%], respectively), but this reduction was not statistically significant. In a similar study in a suburban private practice in Rochester, NY, Casey and Pichichero studied children with persistent and nonresponsive AOM before (1995 to 2000) and after (2001 to 2003) the introduction of universal pneumococcal vaccination (14). The proportions of AOM cases caused by *S. pneumoniae* decreased from 48 and 44% in 1995 to 1997 and 1998 to 2000, respectively, to 31% in 2001 to 2003 ( $P = 0.05$ ), while the proportions of *H. influenzae* AOM increased from 38 and 43% in 1994 to 1997 and 1998 to 2000, respectively, to 57% in 2001 to 2003 ( $P = 0.02$ ). The respective figures for AOM caused by penicillin-resistant *S. pneumoniae* were 22, 6, and 0% ( $P < 0.001$ ), but no change in the proportion of AOM caused by intermediately susceptible *S. pneumoniae* was seen (12, 18, and 14%, respectively).

McEllistrem et al. (70, 71) collected 505 AOM middle-ear fluid isolates from five hospitals in the United States during the period from 1999 through 2002. As expected, the proportion of nonvaccine serotypes increased over time (from 12% in 1999 to 32% in 2002;  $P < 0.01$ ) and according to the number of PCV7 doses received (18% in those receiving  $\leq 1$  dose versus 35% in those receiving 2 to 4 doses;  $P < 0.01$ ). The proportion of cases due to vaccine-related serotypes (including serotype 19A) increased according to the number of doses received (10% in those receiving  $\leq 1$  dose versus 19% in those receiving 2 to 4 doses;  $P < 0.01$ ). The proportion of pneumococci recovered at the time of myringotomy and/or other tympanostomy tube placement that were PNSP was reduced (from 73% in 1999 to 53% in 2002;  $P = 0.03$ ). However, the overall proportion of PNSP among children with spontaneous drainage remained unchanged. This was mainly due to an increase in the proportion of serotype 19F, which was often antibiotic resistant. Most of the 19F strains isolated in these stud-

ies were of two international clones, Spain<sup>23F</sup>-1 and Taiwan<sup>19F</sup>-14.

The studies by Block et al., Casey and Pichichero, and McEllistrem et al. suffer from the same limitation—they all report proportions of pathogens and antibiotic-resistant strains causing AOM, rather than incidence. A second common limitation is that tympanocentesis was performed mainly in nonresponsive, severe cases of otitis or in cases of tympanostomy tube placement. In these cases, antibiotic resistance is influenced by previous or current antibiotic treatment, the choice of which depends to a large extent on the current practices in the given community. Thus, the true influence of conjugate pneumococcal vaccines on the occurrence of AOM caused by DRSP could not be comprehensively evaluated.

A recent study by Cohen et al. from France (17) helps in sorting out the effect of conjugate vaccines from that of antibiotic treatment on the occurrence of AOM caused by DRSP. This prospective study was conducted during the period from 2001 to 2004 by 89 pediatricians distributed throughout France. A total of 1,906 nasopharyngeal swabs for pneumococcal cultures were obtained from children with AOM aged 6 to 24 months. In addition, to increase the likelihood of the AOM's being caused by *S. pneumoniae*, children who specifically had the following findings that have previously been associated with pneumococcal AOM were enrolled: (i) a temperature of  $\geq 38.5^{\circ}\text{C}$  and/or otalgia and (ii) lack of conjunctivitis (since the presence of conjunctivitis often points to AOM caused by *H. influenzae*). The frequency of antibiotic use, as well as pneumococcal immunization, was recorded. The proportion of PCV7-vaccinated children ( $\geq 1$  dose) increased from 8.2% in year 1 to 61.4% in year 3. Overall, pneumococcal carriage and the carriage of PCV7 serotypes during AOM decreased during the three study years by 16% ( $P < 0.001$ ) and 35% ( $P < 0.01$ ), respectively. Rates of highly penicillin-resistant *S. pneumoniae* strains among all isolates were 15.4, 10.6, and 6.7% in first, second, and third years, respectively ( $P < 0.001$ ). The risk of AOM caused by penicillin-resistant *S. pneumoniae* was 4.2% for immunized children who had not received antibiotics, 8.6% for those vaccinated who also received antibiotics in the last 3 months, 10.3% for unimmunized children who had not received antibiotics, and 16.2% for unimmunized children who had received antibiotics in the last 3 months ( $P < 0.001$ ). This study shows that both vaccination and antibiotic use have an effect on the occurrence of AOM caused by DRSP.

*S. pneumoniae* strains that are not included in the PCVs and PNSP strains causing AOM were reported

even before the initiation of universal pneumococcal conjugate immunization programs (78, 79). These strains are often clonal, and some of these clones originated in vaccine serotypes that were subject to capsular switch (78). Thus, replacement with antibiotic-resistant serotypes can, at least to some extent, be expected after the introduction of pneumococcal vaccination. The increasing rate of AOM caused by DRSP serotype 19A observed in the United States after the introduction of vaccines can be explained, at least in part, by replacement due to vaccination.

Toltzis et al. conducted a study to estimate the frequency of colonization by *S. pneumoniae* strains containing both *mef* and *erm* in children treated for AOM from November 1999 to April 2002 (95). Of 221 children colonized by pneumococci, 17 (7.7%) were colonized with an organism containing both determinants. Azithromycin MICs for all *mef*- and *erm*-positive organisms were demonstrated to be  $\geq 256 \mu\text{g/ml}$ , and all these organisms were coresistant to all other agents tested. All were of serotype 19A or 19F, and all but one were the Taiwan<sup>19F</sup>-14 macrolide-resistant strain, which has a global distribution. A higher proportion of children colonized with *mef*- and *erm*-containing organisms had received  $\geq 1$  dose of PCV7 than those without these organisms ( $P < 0.01$ ). The odds ratio for carriage of *mef*- and *erm*-positive *S. pneumoniae* in those immunized with  $\geq 1$  PCV dose was 3.78 (95% CI, 1.34 to 10.64) compared with those not immunized, after adjustment for age, breastfeeding, DCC attendance, a diagnosis of AOM within the 30 days or 6 months before enrollment, the administration of oral and intravenous antibiotics within 12 months before enrollment, and hospitalization within 12 months before enrollment. A limitation of the analysis by Toltzis et al. is that the analysis of resistance relative to vaccination did not take into account that levels of both vaccination and resistance increased with time (during the winter of 1999 to 2000, children were practically not vaccinated). The authors did not adjust the analysis by year. However, it is plausible to assume that multidrug-resistant serotype 19A strains may possess an ecologic advantage in a largely immunized population, not only over serotypes included in the vaccine, such as 19F, but also over homologous antibiotic-susceptible strains of serotype 19A, as long as antibiotic use is excessive. Since the introduction of PCV7 into the United States, there has been evidence from the PROTEKT surveillance project to support this idea (32). The proportion of isolates from children expressing the *erm*(B) + *mef*(A) macrolide-resistant genotype that are serotype 19A have increased

relative to the proportion of such isolates that are vaccine serotype 19F. Among strains with this genotype isolated in 2000 to 2001, 7.8% were serotype 19A versus 86.7% that were serotype 19F, but by 2003 to 2004, 45.5% were of serotype 19A versus 51.7% of serotype 19F (32).

## DIRECT EFFECT OF PCVs ON CARRIAGE OF DRSP

As reviewed in chapter 19, of this volume, PCVs reduce the carriage of *S. pneumoniae* strains belonging to the serotypes included in the vaccine (vaccine serotypes) and even, at times, the carriage of strains related to those serotypes (vaccine-related serotypes). Replacement with nonvaccine serotypes is clearly observed in most studies (22, 74). The reduction in the carriage of vaccine serotypes has been associated with the reduction in DRSP carriage in all studies conducted with young infants. The first study demonstrating such an effect was conducted by Dagan et al. in Israel with toddlers aged 12 to 18 months immunized with a seven-valent pneumococcal vaccine conjugated to meningococcal outer membrane protein complex (23). This effect was demonstrated in more-detailed studies of toddlers attending DCCs (21) and of infants immunized with a four-valent diphtheria toxoid conjugate (24), a four-valent tetanus toxoid conjugate (24), an 11-valent vaccine with serotypes conjugated to diphtheria or tetanus toxoid (26), and a nine-valent CRM conjugate (69).

In principle, since one expects to see most resistance among serotypes 6B, 9V, 14, 19F, and 23F, the reduction of vaccine serotypes and vaccination with PCV7 should result in a net reduction in the carriage of DRSP. However, the increasing resistance in nonvaccine serotypes and in vaccine-related serotypes (especially serotype 6A, the carriage of which is not always successfully affected by PCV, and serotype 19A, the carriage of which is not affected by vaccination) raised the potential of a compensatory increase in the carriage of antibiotic-resistant *S. pneumoniae* following vaccination due to the replacement phenomenon. Furthermore, although conjugate vaccines reduced the carriage of vaccine-type pneumococcal serotypes, they did not eliminate the carriage, allowing the continuing spread, although at reduced rates, of antibiotic-resistant and multidrug-resistant vaccine type serotypes. The overall result is that so far in vaccinated communities, the carriage of antibiotic-resistant *S. pneumoniae* continues to be prevalent despite vaccination.

Frazao et al. administered PCV7 to 238 children aged 6 months to 6 years attending DCCs in the Lisbon

area in Portugal (37). Unvaccinated children of the same age from these same DCCs served as controls. Samples were obtained during six consecutive periods within 10 months. As expected, due to replacement, no decrease in the overall pneumococcal carriage rate was observed in vaccinees compared to controls (average, 68%). However, less expectedly, the frequencies of carriage of DRSP were also similar between groups (average, 38%), including the frequencies of PNSP carriage (average, 24%). The rate of carriage of DRSP of vaccine serotypes decreased significantly, but this decrease was compensated for by a gradual increase in the frequency of DRSP of nonvaccine serotypes (mainly 6A, 10A, 15A, 15C, 19A, 23A, and 33F and untypeable strains). The drug-resistant strains that were nonvaccine serotypes had different pulsed-field gel electrophoresis patterns from strains of vaccine serotypes.

After the introduction of PCV7 in the United States, dynamics similar to those observed in the Lisbon-area DCCs could be observed for pneumococcal carriage in the United States. Huang et al. (54) obtained nasopharyngeal specimens from children who were younger than 7 years during well-child or sick visits in primary care practices in 16 Massachusetts communities during 2001 and 2004. Among colonizing pneumococcal isolates, the proportion of PCV7 serotypes decreased from 36 to 14% and that of non-PCV7 serotypes increased from 34 to 55%. The overall prevalence of carriage did not change (26 to 23%); neither did that of the carriage of potentially cross-reactive serotypes (30 to 31%). There was a substantial increase in the incidence of penicillin nonsusceptibility, from 8 to 25%, in non-PCV7 serotypes; 35% were highly resistant to penicillin. The rate of penicillin nonsusceptibility increased from 45 to 56% among PCV7 serotypes while remaining stable among strains of serotypes potentially cross-reactive with PCV7 (51 versus 54%). In 2004, the most commonly carried PNSP serotypes were found to be vaccine serotype 19F; vaccine-related serotypes 6A, 23A, and 19A (the latter showed the highest increase in carriage and the highest increase in resistance); and nonvaccine serogroups 15 and 29. In an analysis performed by the same group (34), nonsusceptibility to penicillin was, as expected, more common in vaccine serotypes (45%) and vaccine-related serotypes (51%) than in nonvaccine serotypes (8%). Risk factors for PNSP colonization included child care center attendance, current respiratory tract infection, and recent antibiotic use. PCV7 immunization was associated with decreased carriage of vaccine serotypes but not with an overall decrease in *S. pneumoniae* colonization nor with a decrease in PNSP colonization.

Similar findings were observed by Moore et al. (73), who obtained 1,350 nasopharyngeal swabs from 1,275 children aged 3 to 59 months in Anchorage, Alaska, during the winters of 2000, 2001, and 2002. The carriage of TMP-SMX-nonsusceptible strains was reduced in 2002 compared to that in 2000, but the carriage of PNSP strains was not. The authors analyzed the risk of carriage of vaccine serotypes and TMP-SMX-nonsusceptible and penicillin-nonsusceptible strains by a multivariate analysis including factors such as vaccination status, age, antibiotic use, DCC attendance, clinic, and year of enrollment. The multivariate analysis revealed that children who were up to date for their age in terms of PCV7 vaccination were 40% less likely to carry vaccine serotypes. For the carriage of TMP-SMX- and penicillin-nonsusceptible strains, the only independent personal risk factor was previous antibiotic use, but not vaccine status. The findings are due to the increase in the carriage of nonvaccine serotypes that were nonsusceptible to the drugs.

Hanage et al. investigated potential mechanisms for the increase in asymptomatic carriage of DRSP strains that are of nonvaccine serotypes since the introduction of universal vaccination in the United States (46). Possible mechanisms include de novo acquisition of resistance, serotype switching, the introduction of new clones, and the expansion of existing clones. The investigators applied multilocus sequence typing of samples of 126 and 122 pneumococci collected in 2001 and 2004, respectively, from the nasopharynges of children <7 years of age in 16 Massachusetts communities. They found no evidence of penicillin resistance due to either serotype switching or de novo acquisition. Resistance increased through the expansion of previously recognized clones of nonvaccine serotypes, particularly serotypes 19A, 15A, and 35B. In serotype 19A, several unrelated clones increased in frequency, whereas in the other two serotypes, a single resistant lineage was responsible for the increased prevalence of resistant strains. These findings are in accordance with the findings by Pai et al., who studied isolates of serotype 19A causing invasive infections after the introduction of PCV7 in the United States (76).

Thus, the direct effects of PCVs on carriage can be summarized as a reduction in the carriage of vaccine serotypes, a mixed effect on nonvaccine serotypes (some reduction of serotype 6A carriage but an increase in serotype 19A carriage), and the definite replacement of the carriage of vaccine serotypes by that of nonvaccine serotype strains. The overall prevalence of *S. pneumoniae* carriage is only minimally changed. The overall prevalence of DRSP carriage has been significantly af-

fected by replacement with antibiotic-nonsusceptible nonvaccine serotypes.

### INDIRECT EFFECT OF PCVs ON DRSP DISEASE AND CARRIAGE OF DRSP (HERD IMMUNITY)

As discussed in “Flow of Nonsusceptible *S. pneumoniae* from Children to the Community” above, most of the flow of DRSP is from infants and toddlers to older children and adults. Thus, a reduction DRSP carriage in infants and toddlers by PCV should result in a reduction of the spread of antibiotic-resistant *S. pneumoniae*. The first demonstration came from a study conducted in Israel (41). In this double-blind randomized controlled study, 262 DCC attendees aged 12 to 35 months were randomized to receive PCV9 ( $n = 132$ ) or control vaccine ( $n = 130$ ). The children were monitored for 2 years. Cultures of samples from PCV9 recipients were significantly less frequently positive for vaccine type *S. pneumoniae* than those of samples from the controls (13% versus 21%, respectively;  $P < 0.001$ ). The same pattern was seen in the younger siblings of PCV9 recipients versus the siblings of controls (21% versus 34%, respectively;  $P = 0.017$ ). This difference was associated with a significant decrease in the rate of carriage of DRSP in both the PCV9 recipients and their younger siblings: the RRs (and 95% CIs) for carrying PNSP and *S. pneumoniae* strains resistant to  $\geq 1$ ,  $\geq 2$  and  $\geq 3$  antibiotic categories among younger siblings of recipients versus siblings of controls were 0.47 (0.31 to 0.70), 0.49 (0.33 to 0.71), 0.46 (0.30 to 0.73), and 0.49 (0.21 to 1.17), respectively. When acquired, vaccine serotype DRSP strains were carried for a significantly shorter period of time among siblings of PCV9 recipients than among siblings of controls. The results of this study show clearly that the flow of DRSP from young children can be reduced by PCVs. It is thus not surprising that following the introduction of PCVs for infants and toddlers in the United States, a reduction in carriage of and disease caused by DRSP occurred not only in vaccine recipients (as described in “Direct Effect of PCVs on DRSP Disease and Carriage of DRSP” above) but also in contacts (herd immunity).

The effect of universal toddler immunization with PCV7 on DRSP nasopharyngeal carriage in unvaccinated children and in adults was studied extensively in Alaska (45, 73). Moore et al. (73) demonstrated that the risk of carriage of vaccine type pneumococci was lower in 2002 than in 2000, independent of vaccination status, suggesting an indirect effect of vaccination. Carriage of cotrimoxazole-nonsusceptible pneumococci

was also significantly reduced, not only in vaccinated children, but also among nonvaccinated children without recent use of antibiotics. Carriage of PNSP showed a similar trend, but this was not significant. Hammitt et al. (45) analyzed trends in serotype distribution, and antibiotic resistance and factors associated with adult pneumococcal carriage in Alaska before and after the introduction of PCV7. The authors collected 15,598 nasopharyngeal swabs, from ~50% of all adults living in eight villages. The proportion of adults that carried PCV7 type pneumococci decreased from 28% of carriers in 1998 to 2000 to 4.5% of carriers in 2004 ( $P < 0.0001$ ). Among adults, the proportion of colonizing pneumococcal isolates that were PRSP decreased from 13% in 1998 to 2000 to 6% in 2004 ( $P = 0.05$ ), whereas the percentage of isolates that were PISP increased from 12% in 1998 to 2000 to 19% in 2004 ( $P < 0.01$ ). The reduction of PRSP carriage in the presence of an increase in PISP carriage was in agreement with the finding of a reduction in the carriage of vaccine type *S. pneumoniae* strains (which are often fully resistant to penicillin) and an increase in the carriage of vaccine-related and nonvaccine serotypes (which often carry intermediate penicillin resistance). Similarly, a significant reduction in the carriage of erythromycin-resistant *S. pneumoniae* and a trend toward a reduction in the carriage of TMP-SMX-nonsusceptible *S. pneumoniae* were observed in parallel in vaccinated children, who are believed to be responsible for the changes observed in adults. The fact that adults were more likely to carry vaccine serotypes if they lived with a child  $<5$  years old or if they lived with a child who had not been age-appropriately vaccinated with PCV7 supports this explanation.

Talbot et al., from Nashville, TN (91), observed that after the initiation of universal vaccination, pneumococcal disease rates decreased and the proportion of antibiotic-nonsusceptible isolates decreased among persons aged 2 years and older ( $P < 0.01$ ). The proportion of IPD in all ages due to fully penicillin-resistant pneumococci in Northern California increased from 3% in 1994 to 15% in 2001. Thereafter, the rates declined back to 5% by the second quarter of 2003 (6). In a prospective population-based survey, Stephens et al. (87) obtained pneumococcal isolates and demographic data from patients with IPD in Atlanta before and after PCV7 licensure (1994 to 2002). In parallel to an 85% decline in invasive infections caused by macrolide-resistant *S. pneumoniae* in children from 1999 to 2002 (rates declined from 71 to 3 cases per 100,000 children;  $P < 0.001$ ), rates of invasive infections with macrolide-resistant *S. pneumoniae* in the elderly ( $\geq 65$  years old)

declined by 76% from 1999 to 2002 (from 17 cases per 100,000 persons in 1999 to 1, 7, and 4 cases in 2000, 2001, and 2002;  $P < 0.001$ ). A similar trend, although less impressive, was also found for all other age groups. The decrease was seen in both *S. pneumoniae* strains with *mefE*-mediated macrolide resistance and those with *ermAM*-mediated macrolide resistance.

In a laboratory-based study from the Active Bacterial Core Surveillance System conducted by the CDC, a similar phenomenon was observed for DRSP in general and in relation to various antibiotics (65). Kyaw et al. reported that the rates of invasive PNSP disease in adults aged  $\geq 65$  years fell from 16.4 cases per 100,000 persons in 1999 to 8.4 per 100,000 in 2004—a reduction of 49% (95% CI, 46 to 51%). Similarly, the respective figures for those ages 5 to 19 years were 0.9 and 0.5 per 100,000—a 41% reduction (95% CI, 32 to 48%); for those aged 20 to 39 years, the figures were 1.6 and 0.8—a 51% reduction (95% CI, 46 to 51%); and for those aged 40 to 64, the respective figures were 4.3 and 2.7 per 100,000—a 36% reduction (95% CI, 33 to 40%). The proportion of isolates that were nonsusceptible to penicillin fell among adults  $\geq 65$  years old. A similar reduction in TMP-SMX and multidrug resistance was also seen in those aged  $\geq 65$  years. The same trend was seen in other age groups beyond the age of vaccinees, but the reduction was not consistently statistically significant.

Flannery et al. used the Active Bacterial Core Surveillance System to compare IPD cases among HIV-infected adults before and after the introduction of PCV7 in the United States (36). The incidence fell in 2003 compared with that in 1998 to 1999 by 19% (95% CI, 7 to 29%), and the incidence of IPD caused by vaccine serotypes fell by 62% (95% CI, 53 to 70%). However, replacement by nonvaccine serotypes and vaccine-related serotypes was more accentuated in this population than in the rest of the adult population: an increase of 44% (95% CI, 16 to 79%) was seen for the nonvaccine serotypes and an increase of 45% (95% CI, 71 to 92%) was seen for the vaccine-related serotypes. This was accompanied by a reduction of 27% in disease caused by TMP-SMX-resistant *S. pneumoniae* ( $P = 0.023$ ). A similar trend, although not statistically significant, was seen in disease caused by PNSP (20% reduction in 2003 compared to 1998 to 1999;  $P = 0.155$ ).

The data presented in this section provide compelling evidence for a strong indirect protection effect (or herd immunity) against carriage and invasive disease caused by DRSP in contact unvaccinated children and adults as a result of the introduction of routine immunization of infants and toddlers in the United States. There are few

data as yet to confirm that this phenomenon will occur in developing countries, particularly those countries where HIV infection is endemic.

## EFFECT OF PCVs ON ANTIBIOTIC USE

Since antibiotic use selects for and promotes antibiotic resistance among *S. pneumoniae* strains, an important question is whether the use of PCVs can reduce antibiotic use. In principle, if febrile illness, invasive infections, and respiratory infections are reduced by PCVs, then children should be submitted less often to antibiotic prescriptions. Evidence that such an effect is indeed occurring is difficult to obtain in regions where antibiotics are introduced universally, since secular trends in antibiotic use can be influenced by a multitude of events which may mask the vaccine effect. For example, a significant trend of reduction in antibiotic use was already observed in the United States before the introduction of PCVs (3, 34, 44, 77). Some evidence, however exists for the effect of PCV on the reduction of antibiotic use during controlled randomized clinical trials.

The northern California PCV7 efficacy study conducted from 1995 to 1998, in which 37,868 children were randomized to receive PCV7 or a control vaccine, showed a clear effect on antibiotic use (35). PCV reduced antibiotic prescriptions by 5.4% (95% CI, 4.0 to 6.7%) over all the follow-up period, starting at dose 1, and by 5.7% (95% CI, 4.2 to 7.2%) after the primary series in children monitored per protocol. PCV reduced the subset of second-line antibiotics by 12.6% (95% CI, 9.6 to 15.6%) over all the follow-up period and by 13.3% (95% CI, 9.9 to 16.5%) in the per-protocol follow-up. From dose 1 to age 3.5 years, PCV prevented a total of 35 antibiotic prescriptions per 100 children vaccinated per protocol. The authors suggested that these figures may correspond to a reduction in the prescription of antibiotics in the United States by 1.4 million prescriptions annually.

Dagan et al. conducted a double-blind randomized controlled study with PCV9 given to toddlers attending DCCs in southern Israel (25). One of the outcomes measured was antibiotic use. This was measured as the number of days during which children were on antibiotics. A total of 131 evaluable children were vaccinated with PCV9, and 130 children received control vaccine. A total of 755 illness episodes resulted in antibiotic treatment: 350 in the vaccine recipients and 405 in the controls (RR, 0.85; 95% CI, 0.75 to 0.97%;  $P = 0.02$ ). A total of 3,913 antibiotic days were reported for the 130 children in the control group, monitored for 2,759 child-months. The proportion of time during which the

children in the control group received antibiotics decreased from 9.6% for the age window of 15 to 23 months to 2.8% for that of  $\geq 48$  months. The total number of antibiotic days for the pneumococcal vaccine recipients was 3,358, representing an RR of 0.83 (95% CI, 0.79 to 0.87%;  $P < 0.001$ ). The greatest observed effect of the pneumococcal vaccine was on antibiotic treatment for lower-respiratory-tract problems (47% risk reduction), followed by otitis media (20% risk reduction) and upper-respiratory-tract infections (10% risk reduction). All non-respiratory-tract illness episodes together (grouped as others) accounted for only 10.3% of the antibiotic days, and no overall clear vaccine effect could be demonstrated for the group.

The two studies in northern California and in Israel demonstrate that PCV use can result in a reduction in antibiotic use. In addition, once a reduction in the incidence of pneumococcal disease is established, physicians may be less tempted to administer antibiotics for suspected pneumococcal disease. As an example, Gabriel et al. conducted a study to evaluate physicians' attitudes toward the management of young febrile children since the introduction of PCV7 in the United States (38). A total of 7,500 pediatricians and 7,500 emergency department physicians were surveyed with regard to their management of a hypothetical febrile 7-month-child and a hypothetical 20-month-old child without an apparent focus of fever. Specifically, physicians were asked how they would manage a febrile child who had and one who had not been vaccinated with PCV7. For treating the vaccinated 7-month-old child, pediatricians would use 11% less ceftriaxone and emergency department physicians would use 20% less than they would for the unvaccinated child ( $P < 0.0001$ ). Twelve percent fewer pediatricians and 19% fewer emergency department physicians would administer ceftriaxone ( $P < 0.0001$ ) to the 20-month-old vaccinated child than to the unvaccinated child.

Stoll et al. evaluated the incidence of occult bacteremia in pediatric emergency departments in the era of PCV7 in the United States (88). Of 329 blood cultures obtained from children age 2 to 36 months with temperatures of  $\geq 39^{\circ}\text{C}$ , only 3 (0.91%; 95% CI, 0 to 1.9%) showed bacteremia, all 3 with *S. pneumoniae*. The positive predictive value of white blood cell counts of  $\geq 15,000/\text{mm}^3$  was 3.2%. The authors concluded that (i) in the PCV7 era, occult bacteremia is uncommon in highly febrile children 2 to 36 months old; (ii) with the continued use of PCV7, the routine practice of obtaining counts and cultures may no longer be indicated; (iii) a high fever may not justify expectant treatment with broad-spectrum antibiotics.

The reduction in antibiotic use is important if one does not desire to replace existing vaccine type DRSP with nonvaccine type DRSP. Using a series of mathematical models, Temime et al. from France (92, 93) suggested that because of serotype replacement, the effects of vaccination on the reduction of antibiotic use and resistance observed today may not be sustained in the long term. As a consequence, vaccination alone may not be successful in controlling the selection of DRSP and the reduction of antibiotic exposure will remain of primary importance for controlling disease caused by DRSP.

## FUTURE TRENDS

It is predicted that as long as PCV7 is used in the presence of unmodified approaches to antibiotic treatment, replacement with nonvaccine type DRSP may hamper efforts to reduce antibiotic resistance in *S. pneumoniae* by vaccination (46, 92, 93). Thus, efforts in the future should be made in several directions.

1. *Ensure appropriate vaccination with the current vaccines.* New acquisition of *S. pneumoniae* is inversely associated with concentrations of circulating specific anticapsular immunoglobulin G (19). Thus, choosing an appropriate vaccination schedule that allows the achievement of the highest antibody concentration is important to reduce the carriage and transmission of vaccine-serotype *S. pneumoniae* and, thus, of vaccine type DRSP. Furthermore, it is suggested that the concentration of circulating immunoglobulin G may also determine to a certain extent the rate of AOM caused by some vaccine serotype *S. pneumoniae* strains (57), and thus, high antibody concentrations may reduce, to a certain extent, the rate of otitis caused by DRSP.
2. *Reduce diseases and spread of vaccine-associated serotypes, including serotypes 6A and 19A.* As discussed above, the prevalence of these two serotypes that cause antibiotic resistance is only partially reduced by vaccination with PCV7. For serotype 6A, invasive and mucosal disease is markedly reduced by PCV7, but carriage is less so, and the spread of serotype 6A DRSP still takes place; serotype 19A, as seen above, is still expanding through promotion by antibiotic treatment in the community, probably facilitated by the reduction of vaccine serotypes through vaccination. The expansion of future vaccines to contain conjugates of serotypes 6A and 19A may en-

able the reduction of the prevalence of these two serotypes, which are the most important DRSP serotypes remaining after vaccination with PCV7. However, the future emergence of new highly resistant and multidrug-resistant serotypes cannot be ruled out.

3. *Potential role of future protein vaccines.* The PCVs are limited by serotype specificity. The next generation of pneumococcal vaccines may be protein vaccines that are not serotype specific, or pneumococcal proteins that reduce carriage may be used as conjugates. In theory, coverage against all or most pneumococci by protein vaccines could reduce the circulation of DRSP.
4. *Reduce antibiotic use.* Without doubt, the overuse of antibiotics will continue to promote DRSP. Thus, all efforts should be made to substantially reduce antibiotic use in the community. Using antibiotics that may exert less selection pressure may be one solution, in addition to a total reduction in antibiotic use (68).

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Anushua Sinha  
G. Thomas Ray

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# Pharmacoconomics of Pneumococcal Conjugate Vaccines

Investment in pneumococcal conjugate vaccine (PCV) comes at considerable cost. At the time of PCV's incorporation into the U.S. childhood immunization program in mid-2000, it was the single most expensive vaccine ever introduced. The question of whether such investment is an efficient use of the resources available to the health care system, or whether other investments would provide better value, can be answered using pharmacoconomic analysis.

Pharmacoconomics is that branch of health economics that assesses health benefits gained relative to the economic costs of investments in pharmaceutical and biologic agents, including vaccines. By identifying which investments provide the greatest health benefit for the investment, pharmacoconomic evaluation provides a tool to assist decision makers in the rational allocation of limited resources. However, pharmacoeconomic evaluations may not directly address health care system capacity, equity, social justice, medical suitability, or ethical considerations, all of which also enter vaccine policy decision making.

In this chapter, we review key concepts in pharmacoconomics and the pharmacoeconomic literature pertaining to PCV, with an emphasis on recent work and future advances.

## KEY CONCEPTS IN PHARMACOECONOMIC EVALUATION

### Approaches to Pharmacoconomic Evaluation

Pharmacoeconomic evaluations integrate demographic, epidemiologic, and economic information into simple metrics that summarize the net health benefits and net costs of incorporating a novel vaccine into an immunization program. Pharmacoconomic evaluations integrate burden-of-disease information with economic costs and need to be considered alongside financing and supply issues, prior to decision making regarding vaccine introduction. The process may be iterative, as epidemiologic and/or economic data emerge and financial and supply circumstances evolve.

Four economic study designs are commonly used (16). A cost minimization analysis assumes that all interventions being analyzed result in equal health outcomes and that the primary consideration is which alternative results in lower net costs. It is generally not applicable to the economic evaluation of vaccine introduction. Cost-effectiveness analyses, cost-utility analyses, and cost-benefit analyses all evaluate the net costs and net health benefits associated with an intervention. They differ (i) in the unit used to measure health benefits and (ii) in the summary metric used to present results, as illustrated in Table 1.

Cost-effectiveness analyses and cost-utility analyses are used predominantly in vaccine-related pharmaco-economic evaluations. Cost-utility analyses are a subset of cost-effectiveness analyses in which utility-based preferences are used to estimate quality-adjusted life years (QALYs) and QALYs are used as the primary measure of health benefit. The summary metric for these two study types is the incremental cost-effectiveness ratio. The incremental cost-effectiveness ratio is summarized in Fig. 1.

Interventions with net costs (numerators) less than zero are termed cost saving, as opposed to cost-effective. While some older vaccines were found to be cost saving, newer vaccines with higher vaccine dose costs are usually associated with positive net costs (45). In these cases, a measure of cost per unit of health gained, such as the incremental cost-effectiveness ratio, can be used to allow policy makers to weigh the value for money represented by these interventions. Cost-effective interventions are those judged to represent good value for money. Various criteria for cost-effectiveness have been proposed and reviewed elsewhere (16, 20, 49).

Standard methods of cost-effectiveness and cost-utility analyses have been developed (16, 20), and the use of these standard methods facilitates the comparability

and utility of such studies as tools for policy makers to use in decision making regarding the introduction of new vaccines. Among these standard recommendations is that both costs and health benefits be discounted at a rate between 3 and 6% to reflect the greater relative value of present costs and benefits than of future costs and benefits. In addition, costs should be inflation adjusted to the same currency year by using standard methods.

### Disease Burden of Pneumococcal Infection in Pharmaco-economic Evaluation

Pharmaco-economic evaluations of pneumococcal vaccine's value are built upon models of pneumococcal disease burden. In order to estimate the value for money represented by the introduction of PCV, epidemiologic data on pneumococcal disease incidence need to be integrated with demographic data from the population of interest to develop a proper understanding of the numbers of cases of disease, death, and disability resulting from pneumococcal infection. The epidemiology of pneumococcal disease has been reviewed in detail in chapters 8 to 10. Globally, 1.6 million deaths due to *Streptococcus pneumoniae* are estimated to have occurred in 2002, with 716,000 of these deaths occurring in children under the age of 5 years. This represents 29% of the 2.5 million potentially vaccine-preventable child deaths that occurred in 2002 (50). This estimate does not capture nonfatal cases of pneumococcal illness, nor does it capture those cases of pneumococcal disease that resulted in permanent disability.

In general, pharmaco-economic evaluations of PCV can be said to take either a "top-down" or "bottom-up" approach to modeling the burden of pneumococcal disease. In the top-down approach, models are developed from an overall population health perspective, focusing on one or a few epidemiological indicators, such

**Table 1** Types of pharmaco-economic evaluations<sup>a</sup>

Study type	Units of measurement for health outcome or benefit	Summary metric
Cost minimization analysis	Outcomes assumed to be equal across all interventions	Incremental costs of intervention
Cost-effectiveness analysis	Natural units of health benefit (e.g., cases averted, deaths averted, life years saved)	Incremental cost-effectiveness ratio (e.g., dollars per death averted)
Cost utility analysis	Standardized units of health benefit incorporating individual preferences (e.g., QALYs, DALYs)	Incremental cost-effectiveness ratio (e.g., dollars per QALY gained or dollars per DALY averted)
Cost-benefit analysis	Health outcomes are monetized (e.g., willingness to pay to prevent a death)	Net benefits (monetized health benefits minus net costs)

<sup>a</sup>QALYs, quality-adjusted life years; DALYs, disability-adjusted life years.

$$\frac{(\text{Net costs})}{(\text{Net health benefits})} = \frac{(\text{Vaccination-related costs} - \text{Costs averted through prevented disease})}{(\text{Health benefits gained by vaccination})}$$

Figure 1 Incremental cost-effectiveness ratio.

as child mortality risk or the overall incidence of acute lower-respiratory-tract infection. The risk of death or disease is parsed into vaccine-preventable and nonpreventable proportions, and the burden of pneumococcal disease and the vaccine-preventable proportion of this burden are estimated on this basis. In contrast, bottom-up models aggregate individual health outcomes up to the population level in order to estimate the burden of disease. This form of modeling predominates in published pharmacoeconomic analyses of PCV (3, 9, 11, 13, 15, 18, 24, 26, 28, 29, 31, 33, 35, 42, 46, 47).

Both bottom-up and top-down models use a variety of metrics to summarize the total disease burden placed on society by pneumococcal disease. These measures include the number of cases, the number of deaths, and the number of life years lost. In addition, cost-utility studies use utility-based preferences to weight life lived either with temporary or permanent disability in QALYs. By using QALYs rather than life years in the model, these studies account for nonfatal pneumococcal disease burden and also account for disease burden posed by disabling conditions such as deafness, and seizure or motor disorders. In low- and middle-income settings, disability-adjusted life years (DALYs) are often used instead of QALYs. DALYs are measures of disease burden that give additional weight to life lived during the most economical productive ages (young and middle adulthood) and also account for productivity losses due to disability (19, 34).

### Economic Burden of Pneumococcal Infection and Vaccine-Related Costs in Pharmacoeconomic Evaluation

Pharmacoeconomic evaluations integrate the costs of vaccination and the avertable costs from prevented disease with disease burden models in order to estimate cost-effectiveness (or net benefits). These costs fall into three general categories: (i) direct health care costs; (ii) direct non-health care costs; and (iii) productivity costs, also called indirect costs in the health economic literature. Productivity costs are costs associated with decreased ability to work or engage in leisure activities by the patient or lost economic productivity due to the patient's death (20).

In pharmacoeconomic studies of PCV, potential costs of interest can be described as follows: (i) direct health care costs of the vaccine itself and its administration costs; (ii) direct health care costs of acute pneumococcal disease and associated long-term sequelae; (iii) direct non-health care costs, including transportation costs and costs of special education and long-term care for persons with disabilities; (iv) direct non-health care costs due to caregiver work loss or time loss; and (v) productivity costs due to patient work loss or time loss associated with long-term sequelae and death. Additional cost categories which have not been modeled in published evaluations, but could be incorporated, include direct health care costs of antibiotic resistance associated with pneumococcal disease and direct health care costs associated with testing and management of febrile illness without focus.

The choice of which of these cost categories to incorporate into a pharmacoeconomic evaluation depends on the analytic perspective taken, as summarized in Table 2. From the societal perspective, total societal resources are viewed as constrained and the choice of which investments to be made considers all outcomes and all costs borne by members of society. Comparisons among potential investments need not be limited to health care but could potentially include investments in education, the environment, public safety, or other fields of possible investment. In the health care system perspective, only health care-related outcomes and costs borne by the health care system are considered. Costs borne by the patient or patient's family are disregarded. Other potential perspectives include the patient and patient family perspective, the employer perspective, and the health payer perspective (16, 20).

### PHARMACOECONOMIC STUDIES OF PNEUMOCOCCAL CONJUGATE VACCINATION

#### Overview

Table 3 summarizes the main assumptions and results of published economic studies of PCV in high-income countries. All of the models in Table 3 used bottom-up

**Table 2** Costs in pharmaco-economic evaluations of pneumococcal conjugate vaccine<sup>c</sup>

Cost	Perspective	
	Societal	Health care system
<i>Direct health care-related costs</i>		
Vaccine dose cost	✓	✓
Vaccine administration costs	✓	✓
Medical care associated with pneumococcal disease	✓	✓
Costs of medical care for long-term sequelae	✓	✓
Out-of-pocket expenses for over-the-counter medicines, etc.	✓	✗
<i>Direct non-health care-related costs</i>		
Costs of long-term sequelae involving special education and other disability-related services	✓	✗
Family or other caregiver time	✓	✗
Transportation and other nonmedical out-of-pocket expenses	✓	✗
<i>Productivity costs (indirect costs)</i>		
Patient time cost related to morbidity	✓ <sup>a</sup>	✗
Patient time cost related to mortality	✓ <sup>a</sup>	✗
Patient time cost related to vaccination	✓ <sup>b</sup>	✗

<sup>a</sup>Patient time costs related to morbidity and mortality are fully captured in quality-adjusted measures, such as quality-adjusted life years, used to measure health benefits and are often not enumerated in the costs to avoid double counting.

<sup>b</sup>Patient time costs related to vaccination could be included but have most often been disregarded, as pneumococcal conjugate vaccination is often modeled as taking place during otherwise-scheduled child health visits.

✓, included; ✗, not included.

approaches, in which the experiences of hypothetical cohorts of infants were simulated over a period of time. Health outcomes and costs were estimated for the cohort by assuming that they were or were not vaccinated with PCV. The difference between these two scenarios was the estimated effect of the vaccine. All analyses used cost-effectiveness or cost-utility study designs.

## Model Assumptions

### Vaccine Efficacy and Duration of Protection

All analyses based their models' vaccine efficacy assumptions on the results of two randomized trials of PCV (7, 17). These trials were not designed to provide direct evidence of vaccine efficacy in children older than 3 years. Nevertheless, all economic analyses extrapolated from the trials' results and assumed that vaccine remained effective beyond the ages studied. Many studies assumed declining efficacy with age (3, 9, 15, 24, 26, 28, 29, 46). Two studies analyzed the impact on cost-effectiveness of assuming different durations of vaccine

protection. In one study, the cost per life year saved from the societal perspective was €134,986 assuming 5 years of protection, and €37,312 assuming 10 years (42). In another study, the cost per life year saved was €32,694 assuming 5 years of protection and €26,449 assuming 14 years (28).

### Number of Vaccine Doses

Most of the economic analyses assumed that vaccinated children received four doses of the vaccine, according to the schedule used in clinical trials (7, 17). Because additional doses have additional costs, assuming fewer doses improved cost-effectiveness when vaccine efficacy assumptions remained the same (21). Melegaro and Edmunds (31) assumed that an accelerated immunization schedule was used, in which children received three doses by 4 months of age, and that vaccine efficacy was the same as that in a four-dose schedule. In a sensitivity analysis, they compared the cost per life year saved assuming a three-dose schedule (€130,071) versus a two-dose schedule (€84,335). Marchetti and Colombo (28)

**Table 3** Summary of pharmacoeconomic evaluations of heptavalent PCV in high-income countries in Australia, North America, and Europe<sup>a</sup> (part 1)

	Lieu et al. (26)	Weycker et al. (46)	De Wals et al. (15)	Ess et al. (18)	Moore et al. (low est.) (33)	Moore et al. (high est.) (33)	Claes et al. (13)	McIntosh et al. (29)
<b>General information</b>								
Publication yr	2000	2000	2003	2003	2003	2003	2003	2003
Study population: country	USA	USA	Canada	Switzerland	Canada	Canada	Germany	UK
Currency	US\$	US\$	CAN\$	CHF	CAN\$	CAN\$	€	£
Currency yr	1997	1999	2000	NA	2000	2000	2000	2001
Discount rate: costs; benefits	3%; 3%	3%; 3%	3%; 3%	3%; 0%	0%; 0%	0%; 0%	5%; 5%	6%; 0%
No. of vaccine doses per child	4	4	4	4	4	4	4	4
Yrs of vaccine protection	5	10	9	5	5	5	10	10
Main types of costs included	MED, NMD, WL, LTS, PROD	MED, WL	MED, NMD, WL, PROD	MED, LTS	MED	MED	MED, WL, LTS, PROD	MED, NMD, WL, LTS
<b>No. of disease episodes prevented per 1,000 children vaccinated<sup>b</sup></b>								
Pneumococcal meningitis	0.16	NA	0.14	0.15	NA	NA	0.26	0.54
Pneumococcal bacteremia	3.08	NA	1.64	0.68	NA	NA	0.79	0.48
All IPD	3.24	NA	1.78	0.82	1.87	4.01	1.05	1.02
Pneumonia	13.95	30.00	20.31	2.66	12.10	23.21	61.76	9.74
Otitis media	270.26	301.00	304.62	62.41	149.79	366.80	584.52	70.45
Ventilatory tube placement	7.89	21.00	39.90	NA	NA	NA	NA	NA
Deaths	0.03	NA	0.03	0.06	0.04	0.09	0.09	0.04
Life years <sup>c</sup>	0.87	NA	0.99	8.51 <sup>g</sup>	3.17	6.75	1.77	3.27
<b>Total disease costs averted per child vaccinated, original currency (2006 US\$)</b>								
Medical costs	90	71	49 (33)	41 (30)	28 (19)	28 (19) <sup>j</sup>	145 (136)	64 (104)
Other costs (e.g., long-term sequelae-related, work loss, and productivity)	109	67	102 (69)	72 (53)	NA	NA	147 (138)	28 (45)
Total vaccine-related costs (all doses) per child vaccinated, original currency (2006 US\$)	244 <sup>k</sup>	228	263 (178)	417 (306)	315 (213) <sup>i</sup>	315 (213) <sup>i</sup>	285 (268) <sup>k</sup>	167 (271)
Break-even price HCP perspective	18	13	6 (4)	5 (4)	Negative	Negative	33 (31)	14 (23)
<b>Base case cost-effectiveness results, original currency, thousands (2006 US\$)</b>								
Cost per LYS, HCP	176	NA	212 (143)	NA	91 (62)	43 (29)	73 (69)	32 (52)
Cost per LYS, society	80	NA	125 (85)	NA	NA	NA	Cost saving	28 (45)
Cost per QALYS, HCP	NA	NA	NA	39 (29) <sup>b</sup>	NA	NA	NA	NA
Cost per QALYS, society	4.0 <sup>d</sup>	NA	116 (78) <sup>f</sup>	36 (26) <sup>b</sup>	NA	NA	NA	NA
Cost per LYS, HCP, w/herd effects	18 <sup>e</sup>	NA	NA	NA	NA	NA	NA	4.4 (7.1) <sup>l</sup>
Cost per LYS, society, w/herd effects	7.5 <sup>e</sup>	NA	NA	NA	NA	NA	NA	NA
Cost per QALYS, HCP, w/herd effects	NA	NA	NA	NA	NA	NA	NA	NA
Cost per QALYS, society, w/herd effects	3.5 <sup>e</sup>	NA	NA	NA	NA	NA	NA	NA

(Continued next page)

**Table 3** Summary of pharmacoeconomic evaluations of heptavalent PCV in high-income countries in Australia, North America, and Europe<sup>a</sup> (part 2)

	Lebel et al. (24)	Bos et al. (9)	Asensi et al. (3)	Butler et al. (11)	Melegaro et al. (31)	Navas et al. (35)	Salo et al. (42)	Marchetti et al. (28)	Wisloff et al. (47)
<b>General information</b>									
Publication yr	2003	2003	2004	2004	2004	2004	2005	2005	2006
Study population: country	Canada	Netherlands	Spain	Australia	England/ Wales	Spain	Finland	Italy	Norway
Currency	CAN\$	€	€	AUS\$	£	€	€	€	€
Currency yr	2000	2001	NR	1998	2002	2000	2004	2004	2004
Discount rate: costs; benefits	3%; 3%	4%; 4%	3%; 3%	5%; 5%	3.5%; 1.5%	5%; 5%	3%; 3%	3%; 0%	3%; 3%
No. of vaccine doses per child	4	4	4	3.3	3	4	4	3	4
Yrs of vaccine protection	10	10	10	5	10	10, 2, 3.5 <sup>n</sup>	5	14	4
Main types of costs included	MED, NMD, WL, PROD	MED, WL, LTS	MED, NMD, WL, PROD	MED, LTS	MED, LTS	MED, NMD, WL, LTS, PROD	MED, NMD, LTS, WL, PROD	MED, WL	MED, WL, LTS, PROD
<b>No. of disease episodes prevented per 1,000 children vaccinated<sup>b</sup></b>									
Pneumococcal meningitis	0.22	0.24	0.25	0.24	0.13	0.18	0.07	0.23	0.13
Pneumococcal bacteremia	1.79	0.44	2.08	1.97	0.48	3.68	1.00	0.83	0.09
All IPD	2.01	0.67	2.34	2.21	0.61	3.86	1.08	1.43	0.23
Pneumonia	24.08	16.89	16.18	14.22	4.22	4.62	25.09	8.70 <sup>o</sup>	0.90
Otitis media	236.47	211.36	347.62	257.26	98.93	151.32	260.22	130.00	55.69
Ventilatory tube placement	31.17	NA	NA	NR	NA	9.71	NA	NA	NA
Deaths	0.04	0.06	0.04	0.05	0.02	0.07	0.02	0.03	0.02
Life yrs <sup>c</sup>	1.03	1.63	1.26	1.07	1.01	1.45	0.47	2.46	0.43
<b>Total disease costs averted per child vaccinated, original currency (2006 US\$)</b>									
Medical costs	108 (73)	12 (10)	118 (145)	68 (42)	6 (9)	49 (46)	110 (135)	23 (28)	83 (102)
Other costs (e.g., long-term sequelae- related, work loss, and productivity)	89 (60)	35 (30)	107 (131)	NA	NA	78 (73)	35 (43)	29 (36)	80 (98)
<b>Total vaccine-related costs (all doses) per child vaccinated, original currency (2006 US\$)</b>	<b>270 (183)</b>	<b>181 (156)</b>	<b>194 (238)</b>	<b>314 (194)</b>	<b>120 (187)</b>	<b>217 (204)<sup>k</sup></b>	<b>208 (255)</b>	<b>117 (144)</b>	<b>216 (265)</b>

Break-even price HCP perspective	27 (18)	Negative	30 (37)	13 (8)	Negative	5 (5)	26 (32)	7 (9)	20 (25)
<b>Base case cost-effectiveness results, original currency, thousands (2006 US\$)</b>									
Cost per LYS, HCP	155 (105)	NA	60 (74)	230 (142)	113 (176)	112 (105)	211 (259)	38 (47)	311 (381)
Cost per LYS, society	79 (59)	83 (72)	Cost saving	NA	NA	62 (58)	135 (166)	26 (32)	124 (152)
Cost per QALYS, HCP	NA	NA	NA	NA	60 (93)	NA	45 (55)	NA	140(172)
Cost per QALYS, society	NA	71 (61) <sup>m</sup>	NA	NA	NA	NA	29 (36)	NA	56 (69) <sup>f</sup>
Cost per LYS, HCP, w/herd effects	NA	NA	NA	NA	5.3 (8.2)	NA	NA	NA	153 (188)
Cost per LYS, society, w/herd effects	NA	NA	NA	NA	NA	NA	NA	NA	58 (71)
Cost per QALYS, HCP, w/herd effects	NA	NA	NA	NA	5.0 (7.8)	NA	NA	NA	96 (118)
Cost per QALYS, society, w/herd effects	NA	NA	NA	NA	NA	NA	NA	NA	37 (45)

<sup>a</sup>Only data relating to the primary or “base case” are included. Data not explicitly reported in the papers were inferred, where possible, to allow for direct comparisons. All cost-effectiveness ratios were explicitly reported in the original papers. Except where noted, no data listed in this table include the effects on nonvaccinated individuals. Exchange rates used were from the Federal Reserve, New York, as of July of the original currency year, if reported, or publication year. No other adjustments for changes in price levels have been made. NA, not applicable, not reported, or could not be reliably inferred; MED, medical; NMD, nonmedical (such as transportation); WL, parent or caregiver work loss or time loss; LTS, medical and nonmedical costs (such as special education) associated with long-term sequelae; PROD, productivity costs associated with disability or premature death; IPD, invasive pneumococcal disease; HCP, health care payer perspective; LYS, life years saved; QALYS, quality-adjusted life years saved.

<sup>b</sup>Disease episodes prevented per child are a function of incidence rates (which typically vary with age), efficacy, and the number of years covered by the study. Episode counts in Lieu et al. and Butler et al. are discounted. Episode counts in other studies were explicitly stated as being undiscounted, or no indication was given.

<sup>c</sup>For those studies where benefits were discounted, life years in this table are discounted.

<sup>d</sup>Reported in a subsequent paper (40), based on the original model.

<sup>e</sup>Reported in a subsequent paper (41), based on the original model.

<sup>f</sup>Quality of life adjustments were made for long-term sequelae only.

<sup>g</sup>Quality-adjusted life years saved. Only life years with sequelae from meningitis were quality-adjusted.

<sup>h</sup>The “sickness fund” perspective as reported in Ess et al. is here associated with the health care payer perspective. Sickness funds charges are said to represent about half of the “real” costs of care. For the “societal” perspective, Ess et al. inflate the sickness funds charges by a factor and add special education and residential care costs associated with long-term sequelae. This definition of “societal” costs is not comparable to that of other studies.

<sup>i</sup>Includes 5% wastage.

<sup>j</sup>Although disease incidence is higher in the “high estimate” compared to the “low estimate,” medical costs averted do not appear to reflect this difference.

<sup>k</sup>Discounted.

<sup>l</sup>Reported in a subsequent paper (30), based on the original model.

<sup>m</sup>Quality of life adjustments were made for invasive pneumonia, retardation, spasticity, seizures, and hearing loss only.

<sup>n</sup>Vaccine was assumed to be effective in preventing IPD for 10 years, otitis and pneumonia for 2 years, and tympanostomy tube placement for 3.5 years.

<sup>o</sup>Only hospitalized pneumonia was included.

also assumed three doses of vaccine in their base case analysis. When four doses were assumed in a sensitivity analysis, cost per life year saved increased from €26,449 to €42,575.

### Discounting

The decision to employ discounting of costs and/or benefits and the discount rates chosen are among the most influential assumptions in pharmaco-economic models of vaccination. Most studies of the PCV discounted both costs and benefits at 3%, in accordance with recent guidelines (20). Other studies used discount rates ranging from 0 to 6%, usually citing country-specific recommendations. Four studies did not discount benefits, resulting in more favorable estimates of cost-effectiveness than would have been found if benefits were discounted (18, 28, 29, 33).

In pharmaco-economic analyses of PCV's value, cost-effectiveness is more sensitive to the discounting of health benefits than to the discounting of costs. Costs of vaccination occur early on and are subject to less discounting than life years, which accrue over the full course of the expected life span. Butler et al. (11) estimated that in a cohort of 250,000 infants, PCV would save 1,064 life years if no discounting was done but only 268 life years if benefits were discounted at 5%. Without discounting of costs or benefits, the cost per life year saved was 44,740 Australian dollars, compared to 230,130 Australian dollars when costs and benefits were discounted at 5%.

### Quality of Life Adjustments

In high-income countries, the most common health benefit of PCV is in reducing morbidity, not mortality, which is relatively rare. However, the most commonly used metric, cost per life year saved, does not take reductions in morbidity into account and captures only health benefits gained due to averting deaths. Although estimating cost per QALY saved in the base case is recommended (20), most studies of the PCV have not used this metric. The reason for this choice may be the paucity of preference values, i.e., quality of life measures, relating to pneumococcal disease in infants and young children and the cost and difficulty of obtaining such measures. Prosser et al. (40) interviewed parents to measure preference values for preventing disease associated with pneumococcal infection. When these data were incorporated into an existing model (26), the vaccine's cost per QALY saved was \$4,000, compared to \$80,000 before the incorporation of quality-of-life ad-

justments. Melegaro and Edmunds (31) and Salo et al. (42) included quality-of-life adjustments for all pneumococcal outcomes, based on published data derived from parent surveys (4, 12), physician surveys (36, 37), or authors' assumptions (39). A number of other studies estimated cost per QALY saved but included quality-of-life adjustments for only a subset of outcomes, such as long-term disability (9, 15, 18, 47).

### Indirect Effects Due to PCV

In the years following the introduction of PCV into the U.S. childhood immunization schedule, national data indicated that rates of invasive disease among adults and unvaccinated children and adolescents began to decrease sharply, presumably due to herd immunity, an indirect effect associated with vaccination. Given that most cases of pneumococcal disease and most deaths from pneumococcal disease in developed economies occur in adults, the inclusion of this herd effect significantly improves measures of the vaccine's cost-effectiveness. Two studies estimated cost-effectiveness with and without the inclusion of herd effects (31, 47), while two others (26, 29) were revised after publication to include them (30, 41).

The U.S. incidence data provided direct evidence for herd immunity effects among nonvaccinated persons, for invasive pneumococcal disease only, although it may be reasonable to assume that this effect extends to pneumonia as well. In their base case herd effect analyses, Ray et al. (41) and Wisloff et al. (47) assumed that reductions in disease among nonvaccinated persons were limited to invasive pneumococcal disease, while Melegaro and Edmunds (31) assumed reductions in invasive pneumococcal disease, pneumonia, and otitis media. McIntosh et al. (30) assumed reductions in invasive pneumococcal disease and hospitalized pneumonia. Ray et al. (41) estimated that during the first 5 years after the introduction of PCV, the vaccine saved 421 lives before taking the herd effect into account and 5,159 lives after taking it into account. Similarly, McIntosh et al. estimated that vaccination of a cohort of children would save 29 lives before the herd effect is included and 1,168 lives after it is included (30). These analyses illustrate the impact herd immunity can have on estimates of health benefit gained as a result of vaccination and, as a consequence, final estimates of cost-effectiveness.

There is evidence that PCV has caused an increase in pneumococcal disease caused by nonvaccine serotypes through serotype replacement. Only one pharmaco-economic study has explicitly modeled serotype replacement in a sensitivity analysis (31). In this analysis, because nonvaccine serotypes were assumed to cause

less-severe disease than vaccine serotypes, even 100% replacement did not eliminate the vaccine's benefits.

### Disease Outcomes and Vaccine Efficacy

#### Invasive Pneumococcal Disease

Most pharmacoeconomic evaluations assumed that PCV prevents invasive pneumococcal disease, pneumonia, and otitis media. Because they can differ substantially in their costs and outcomes, pneumococcal bacteremia and pneumococcal meningitis were usually modeled separately. Sources of invasive pneumococcal disease assumptions varied widely, depending on the data available in each country, as did the disease incidences used and the proportion of invasive pneumococcal disease that was meningitis. Bacterial surveillance systems, hospital diagnosis data, previously published studies of incidence, and author estimates were variously used to arrive at age-specific estimates of invasive pneumococcal disease incidence.

All studies assumed that the vaccine was effective only in preventing invasive pneumococcal disease caused by the seven serotypes included in the currently marketed vaccine. Most authors combined country-specific serotype distributions (if known) with the published serotype-specific vaccine efficacy to estimate overall vaccine efficacy against invasive pneumococcal disease. Because of differences in the way incidence and efficacy have been reported, the most comparable measure of the overall impact of the vaccine is the number of disease episodes prevented per 1,000 children vaccinated, which we have calculated where possible (Table 3). Estimates of invasive pneumococcal disease episodes prevented per 1,000 children vaccinated range from a low of 0.23 in the study of Wisloff et al. (47) (Norway) to a high of 3.86 in the study by Navas et al. (35) (Catalonia, Spain), with the unweighted average from all studies being 1.76. These differences reflect primarily different assumptions regarding disease incidence and, to a lesser extent, the proportion of disease caused by vaccine serotypes.

#### Pneumonia

One of the greatest challenges in modeling the economic value of PCV lies in developing estimates of pneumonia incidence that are (i) in accord with the vaccine efficacy data available from clinical trials and (ii) appropriate to the population being modeled. Preliminary reports from the randomized trial indicated that vaccine efficacy against clinical pneumonia was 11.4% (6). A number of the earlier economic models used this preliminary efficacy estimate (3, 9, 15, 18, 24, 26, 33, 46). Later pub-

lished studies reported vaccine efficacy against "all clinical pneumonia" (6% for all ages in the intent-to-treat group) and against "clinical pneumonia and positive film" (17.7%) (8). In that trial, "all clinical pneumonia" represented any episode in which a clinical diagnosis of pneumonia was made by the treating physician. "Clinical pneumonia and positive film" represented a subset of children with a clinical diagnosis of pneumonia that also got a chest radiograph that met study criteria for positivity. These cases were assumed to be more likely to be caused by the pneumococcus. The trial-based estimates of vaccine efficacy against pneumonia reflected both local pneumonia etiology and local patterns regarding the diagnosing of pneumonia. The extrapolation of these efficacy data to other settings for use in economic modeling is challenging. While efficacy against clinical pneumonia indicated by a positive film may be more generalizable than efficacy against clinical pneumonia, incidence data for this category of disease are usually less readily available.

Most authors of the cost-effectiveness analyses of PCV modeled the incidence of clinical pneumonia (3, 9, 15, 18, 24, 29, 33), while others used variations of the definition of clinical pneumonia by a positive film for disease incidence and efficacy (11, 13, 28, 31, 35, 42, 47) or estimated the incidence of and efficacy against pneumococcal pneumonia based on the trial results and assumptions regarding the proportion of disease caused by the pneumococcus (26). The estimated numbers of pneumonia cases prevented per 1,000 children vaccinated range from 2.66 in the study by Ess et al. (18) (Switzerland) to 61.76 in the study by Claes and Graf von der Schulenburg (13) (Germany), with the unweighted average across all studies being 16.98.

#### Otitis Media and Ventilatory Tube Placement

Vaccine efficacy against acute otitis media was reported in two trials (7, 17). The U.S. trial defined acute otitis media by clinical diagnosis only. The Finnish trial reported vaccine efficacy against all-cause acute otitis media and acute otitis media confirmed by culture to be pneumococcal. Because population-based data on the etiology of acute otitis media is usually not available, all but one (13) pharmacoeconomic study modeled all-cause otitis media, and most (3, 9, 11, 15, 18, 24, 26, 28, 29, 33, 35, 46) used the vaccine efficacy estimate from the U.S. trial (7).

A number of studies explicitly incorporated vaccine's enhanced efficacy against ventilatory tube placement and more complicated (thus, more costly) cases of otitis media (3, 15, 24, 26, 35, 42, 46). Other studies incorporated the higher costs associated with complicated

otitis media and tube placement but may not have varied vaccine efficacy for simple versus complicated otitis media (9, 11, 13, 18, 47). The estimated number of otitis cases prevented per 1,000 children vaccinated range from 55.69 in the study by Wisloff et al. (47) (Norway) to 584.52 in the study by Claes and Graf von der Schulenburg (13) (Germany), with the unweighted average across all studies being 226.98.

### Case Fatality and Life Years

Differences in assumptions relating to case fatality rates, along with various assumptions regarding discounting, may be the most important factors explaining differences in cost-effectiveness estimates across the studies. Nine studies assumed that pneumococcal death occurred only as a result of invasive pneumococcal disease (3, 9, 15, 18, 24, 26, 28, 42, 47), while six studies also assumed that pneumonia could result in death (11, 13, 29, 31, 33, 35). Estimated invasive pneumococcal disease case fatality rates among children ranged from 0.95% in the study by Lieu et al. (26) (U.S.) to the 10-fold-higher 9.67% in the study of Bos et al. (9) (The Netherlands).

Because the incidence of pneumonia is very high relative to that of invasive pneumococcal disease, the inclusion of deaths caused by pneumonia can have an important effect on cost-effectiveness estimates. Although in developed countries, pneumonia case fatality rates are low, Butler et al. (11), Navas et al. (35), and Claes and Graf von der Schulenburg (13) estimated the percentage of preventable deaths caused by pneumonia to be 19, 36, and 54%, respectively.

The total estimated number of deaths prevented per 1,000 children vaccinated ranged from 0.02 in the studies by Melegaro and Edmunds (31) (United Kingdom), Salo et al. (42) (Finland), and Wisloff et al. (47) (Norway) to 0.09 in the study by Claes and Graf von der Schulenburg (13) (Germany), with the unweighted average across all studies being 0.05. Four studies that did not discount benefits (18, 28, 29, 33) estimated the highest number of life years saved. The number of life years saved per 1,000 children vaccinated ranged from 0.43 in the study by Wisloff et al. (47) to 6.75 in the “high estimate” of Moore et al. (33). Ess et al. (18) estimated 8.51 life years saved per 1,000 children vaccinated, but these included quality-of-life adjustments for long-term sequelae.

### Costs

The societal perspective was the primary perspective taken by the majority of studies, although secondary analyses based on the health care system's perspective were also presented by most authors. In the analysis by

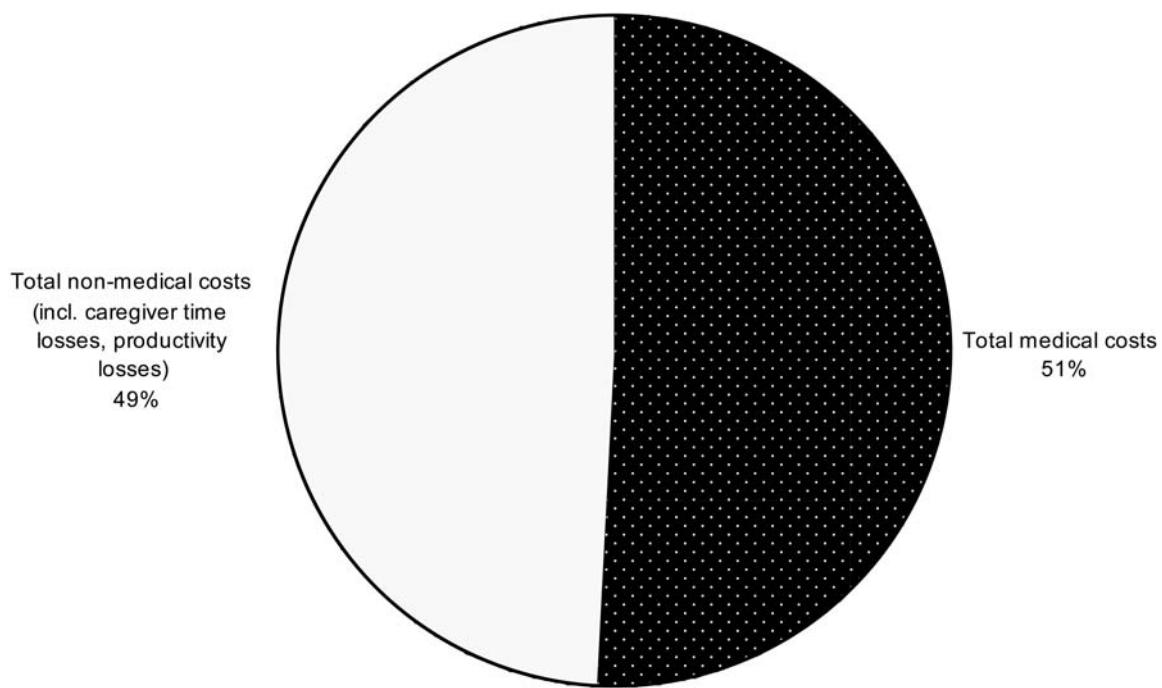
Lieu et al. (26), for example, the cost per life year saved was estimated to be \$80,000 from the societal perspective and \$176,000 from the health care system perspective, using a base case estimate of \$58 per vaccine dose.

Vaccine dose cost estimates were drawn from a variety of sources, including wholesale list price, government-negotiated public market price, or some combination of the two. The vaccine dose costs ranged from a low of \$34 per dose in the study by Bos et al. (9) (The Netherlands) to \$73 per dose in the study by Claes and Graf von der Schulenburg (13) (Germany). One study (33) assumed that a portion of purchased vaccine doses would go unused due to wastage. Most studies included a cost of administering the vaccine, usually assuming that the vaccine is given in the same visit as other childhood vaccines. Total vaccine costs (including administration) per child vaccinated range from \$144 to \$306, with an average of \$220.

Estimating direct medical costs for specific diseases, and long-term, disease-related sequelae, can be methodologically challenging. A wide variety of cost data sources have been used in economic studies of PCV. For unit costs, most studies relied on some form of published price lists or reimbursement rates. Unit costs were often for individual services such as a visit, a lab test, or a hospital day. In order to estimate the cost of the average episodes of meningitis, for example, these unit cost data need to be combined with estimates of resource usage. The technical appendix to the study by McIntosh et al. (29) provides a detailed example of these methods. Lieu et al. (26) used proprietary data from the cost-accounting system of a large health plan to estimate medical costs.

The most common direct non-health care cost included in the studies was the value of caregiver time spent tending to sick children. Most studies that included parent or caregiver costs explicitly stated that they were valuing the time of lost work (9, 13, 26, 29, 35, 42, 46, 47), while others stated or implied that they were valuing lost time, whether from leisure or work (3, 24, 28). All studies used wage rates to value lost caregiver time. Some studies included other direct costs, such as over-the-counter drug use and transportation costs (3, 15, 24, 26, 29, 35, 42), and a number of studies included special education or residential care costs associated with deafness or disability (9, 11, 13, 18, 26, 35).

In pooling across the subset of studies that clearly distinguish medical from nonmedical costs, approximately 51% of the expected savings associated with the use of the PCV is from averted medical costs, while 49% is from other costs such as lost productivity and caregiver time (Fig. 2). Most of the disease cost savings



**Figure 2** Estimated percentages of total costs averted by PCV by medical versus nonmedical costs in high-income countries. incl., including.

are associated with averted otitis media cases (52%), and a substantial percentage (30%) are associated with sequelae such as ventilatory tube placement, hearing loss, and neurological disability (Fig. 3).

Five studies included productivity costs associated with deafness or disability (13, 26, 35, 42, 47), and eight included productivity costs associated with death (3, 13, 15, 24, 26, 35, 42, 47). Estimated lost wages were usually used as the value of productivity costs. When calculating cost per life year saved, or cost per QALY saved, standard guidelines recommend that the productivity costs associated with death not be included in the cost numerator because the health benefit denominator (life years) is considered to capture the full value of that time (20). Four of the studies that provided estimates of productivity costs associated with death explicitly stated that they excluded these costs when calculating the cost per life year saved (3, 15, 24, 26). Four other studies (13, 35, 42, 47) make no mention of excluding these costs when calculating the cost per life year saved, and in some cases it appears that these costs were incorporated into the net costs and net benefits, in effect leading to double counting of these costs.

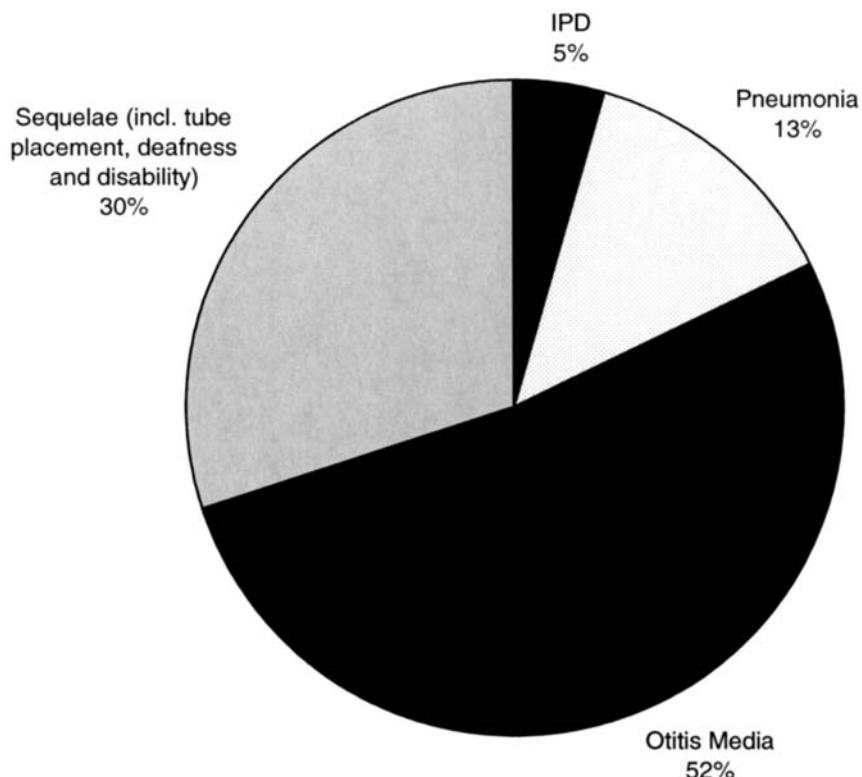
### Cost-Effectiveness Measures and Results

The “break-even” vaccine price from the health care system perspective is the vaccine dose cost at which the

savings in medical costs from averted disease exactly offset the additional costs of vaccination. Three studies imply that the savings in medical costs would not cover the vaccine’s administration costs, and thus, the vaccine’s theoretical break-even price would be less than zero (9, 31, 33). For the remaining studies, the health care system perspective break-even price ranged from \$4 (15, 18) to \$37 (3), with an average of \$17.

Cost per life year saved from the health care system perspective ranged from \$29,000 (33) to \$381,000 (47), with an average of \$130,000. Eleven studies reported costs per life year saved from the societal perspective. Two of these studies found the vaccine to be cost saving, largely because they assumed relatively high rates of otitis media and efficacy against otitis media (13) or relatively high medical and nonmedical costs of otitis media (3). Among the remaining studies, cost per life year saved from the societal perspective ranged from \$32,000 (28) to \$166,000 (42), with an average of \$83,000.

The inclusion of herd effects significantly improved estimated cost-effectiveness. In the model by Lieu et al. (26), when herd effects were included (41), the societal perspective cost per life year saved went from \$80,000 to \$7,500, and in the study by Wisloff et al. (47), the cost per life year saved went from \$152,000 to \$71,000. These analyses suggest the potential importance of pneumococcal disease burden in the elderly and other



**Figure 3** Estimated percentages of total costs averted by PCV by disease type (including medical and nonmedical costs) in high-income countries. IPD, invasive pneumococcal disease; incl., including.

nonvaccinated populations in estimating PCV's cost-effectiveness.

Where quality-of-life adjustments for all pneumococcal conditions were included, the cost-effectiveness ratio decreased, from \$80,000 per life year saved to \$4,000 per QALY saved in the study by Lieu et al. (26, 40), from \$166,000 per life year saved to \$36,000 per QALY saved in the study by Salo et al. (42), and from \$152,000 per life year saved to \$69,000 per QALY saved in the study by Wisloff et al. (47). All three analyses point to the importance of the pneumococcal disease burden associated with morbidity relative to the disease burden associated with mortality in high-income countries.

### Pharmacoeconomic Evaluations of PCV in Low- and Middle-Income Countries

Given the disproportionately high burden of pneumococcal disease in low- and middle-income countries (25), an understanding of the value for money presented by PCVs in these settings is a pressing need for donors, international organizations, and countries alike. However, while the pharmacoeconomic literature pertaining to the introduction of PCV in high-income countries in

Australia, Europe, and North America is robust, published analyses from low- and middle-income countries remain rare.

Three published international analyses of the cost-effectiveness of PCV in low- and middle-income countries are summarized in Table 4. Two earlier analyses of PCV by Miller and McCann (32) and Shepard et al. (43) were published as part of larger studies for the Children's Vaccine Initiative, in which several newer vaccines were subjected to economic evaluation simultaneously. Both analyses were completed before the availability of phase III clinical trial results and used either preliminary estimates of PCV efficacy or vaccine efficacy estimates from a polysaccharide vaccine study of children. The analysis conducted by Shepherd et al. (43) incorporated a bottom-up model that captured disease burden due to pneumococcal pneumonia alone but did not account for invasive pneumococcal disease or otitis media. Miller and McCann (32) took a top-down approach to modeling pneumococcal vaccine's pharmacoeconomics, estimating death rates attributable to *S. pneumoniae* from Gambian data for low-income countries. Neither analysis incorporated disease costs into estimates of

**Table 4** Review of pharmaco-economic evaluations for middle- and low-income countries<sup>a</sup>

Characteristic	Study (reference) by:		
	Shepard et al. (43)	Miller and McCann (32)	Sinha et al. (44)
Yr of publication	1995	2000	2007
Currency	U.S. dollars	U.S. dollars	International dollars
Currency yr	1992	1998	2000
Study population	Global birth cohort, minus infants in industrialized, market economy countries	Global birth cohort	Birth cohort in 72 countries meeting criteria for GAVI Fund eligibility
Design	CUA	CEA	CUA
Perspective	Health care system	Health care system	Societal
Geographic scope of analysis	Countries with very high, high, or middle under-5 mortality rates	Global	Countries with per capita GNI of < \$1,000
Modeling approach	Decision tree	Decision tree	Decision tree
Analytic horizon	Lifetime	Lifetime	Lifetime
Health outcome(s) measured	QALYs gained	Deaths averted, life years saved	Deaths averted, DALYs averted
Cumulative incidence of <i>S. pneumoniae</i> -attributable deaths	Lifetime incidence, 900/100,000	Incidence in children of <5 yrs, 173/100,000	Incidence in children of <5 yrs, 530/100,000
Nonfatal invasive pneumococcal disease	Not included	Not included	Not included
Nonfatal pneumonia	Not included	Not included	Not included
Acute otitis media	Not included	Not included	Not included
Herd immunity effects included?	No	No	No
Vaccine efficacy (%)	51 <sup>b</sup>	66–100 <sup>b</sup>	3–13 <sup>c</sup>
Vaccine schedule	2 dose	3 dose	3 dose
Vaccine costs, as reported			
Cost per dose	\$2.00	\$5.00–15.00	\$5.00
Administration and delivery costs	\$0.18	\$0.18–1.50	\$0.34–0.84
Disease costs, as reported			
Lives saved per 1,000 vaccinated	Not included	Not included	\$150–304
Cost-effectiveness ratio, as reported	Not reported	3–5	5
Sensitivity analyses	\$57 per QALY saved	\$56 to \$112 per life year saved	\$80 to 100 per DALY averted
Discounting (%)	3	3	3

<sup>a</sup>CUA, cost-utility analysis; CEA, cost-effectiveness analysis.<sup>b</sup>Vaccine efficacy against pneumococcal deaths.<sup>c</sup>Vaccine efficacy against all-cause mortality between 3 and 29 months of age.

cost-effectiveness. A vaccine dose cost of \$2 (1992 U.S. dollars) was used by Shepard et al. (43) in a two-dose schedule and a vaccine dose cost of \$5 to \$15 (1998 U.S. dollars) in a three-dose schedule was used by Miller and McCann (32). Both studies found the introduction

of PCV to be cost-effective, with cost-effectiveness ratios of \$79 per QALY saved to \$134 per life year saved.

In a more recent cost-effectiveness analysis, Sinha et al. (44) developed a top-down model encompassing countries having per-capita gross national incomes of

≤\$1,000 and meeting other criteria necessary for eligibility for the Global Alliance for Vaccines and Immunization Fund (<http://www.gavialliance.org>). Assuming a vaccine dose cost of 5 international dollars, a three-dose immunization schedule, and 2003 rates of coverage with diphtheria-tetanus-pertussis vaccine, pneumococcal vaccination was projected to prevent 262,000 deaths per year (7%) among children aged 3 to 29 months, in the 72 developing countries studied, thus averting 8.34 million DALYs annually. If every child could be reached, up to 407,000 deaths per year would be prevented. At a vaccine cost of \$5 per dose, vaccination would have a net cost of \$838 million, with a cost of \$80 to \$100 per averted DALY, depending on whether averted cases of nonfatal pneumococcal disease were included in the health benefit effect. By using each country's per-capita gross national product per DALY averted as a benchmark for highly cost-effective interventions, vaccination at this price was projected to be highly cost-effective in 68 out of 72 countries. When the vaccine's protective effect was extended up to age 5 years and herd immunity effects among nonvaccinated young children were included, the cost-effectiveness ratio dropped to \$22 per DALY averted.

In this analysis, the cost of each dose of vaccine was a key driver of cost-effectiveness. The authors' assumption that the price of PCV may be far lower for developing countries than industrialized countries was based on experience with other childhood vaccines. Internationally, a two-tier pricing structure for childhood vaccines has existed in public markets for many years. For example, the vaccine doses needed to fully immunize a child with eight antigens in the Expanded Program on Immunizations (EPI) cost more than \$100 at U.S. prices using U.S. formulations but only \$13 at United Nations Children's Fund prices using less costly formulations (27). The lower prices offered by manufacturers for newer products on international public markets can be achieved when manufacturers recoup their research and development costs on vaccine sold to industrialized countries and agree to sell vaccines for use in developing countries at the marginal cost of production (5).

A recent regional pharmacoeconomic analysis of primarily middle-income countries in the Latin American and Caribbean region conducted by the Sabin Vaccine Institute found that PCV, if given at 2003 diphtheria-pertussis-tetanus vaccination rates, could prevent approximately 9,500 child deaths annually (1 death prevented per 1,000 children vaccinated) and avert \$180 million in direct medical and non-medical costs across the region. Vaccine-related costs ranged from \$200 million to \$1.8 billion as the price of a dose of vaccine

ranged from \$5 to \$53. The incremental cost-effectiveness ratio from the societal perspective increased from \$62 per DALY averted to \$5,039 per DALY averted as vaccine dose cost was varied across the same range (14).

Two cost-effectiveness analyses accompanied phase III vaccine clinical trials of the nine-valent pneumococcal conjugate vaccine in South Africa and the 11-valent pneumococcal conjugate vaccine in the Philippines (48). Abstracts or published reports from these studies were not available as of this chapter's press date. The Pan American Health Organization recently developed and disseminated a simplified cost-effectiveness model of PCV for use by EPI managers and epidemiologists responsible for communicable disease surveillance in Pan American Health Organization member states (38). Country-level cost-effectiveness analyses of PCV from Chile, Uruguay, Brazil, The Gambia, and Kenya have either been completed, are under way, or are funded and soon to begin. All of these studies, as results become available, will add greatly to the understanding of the pharmacoeconomics of PCV in low- and middle-income countries.

This emerging pharmacoeconomic work is supplemented by an existing literature on the cost of illness for pneumonia and meningitis in low- and middle-income settings (1, 2, 22, 23, 48). Cost-of-illness studies provide an understanding of the economic burden imposed by the direct health care costs associated with meningitis, pneumonia, and potentially acute otitis media. However, they are limited in that they do not take into account the full costs associated with the introduction of a vaccine, nor do they fully consider potential health benefits resulting from vaccination. A large body of literature addressing the estimation of vaccine program costs for traditional EPI vaccines and new vaccine introduction costs has recently been reviewed (10). Both cost-of-illness studies and analyses of vaccine program costs provide useful data for input into more complete pharmacoeconomic evaluations in low- and middle-income settings.

## CONCLUSIONS

Policy makers often must make recommendations regarding PCV introduction before all the effects of a vaccine can be known or relevant data from their population in question can be fully developed. In this common circumstance, pharmacoeconomic evaluations afford the decision maker a consistent and rational method of integrating available information in order to arrive at an estimate of PCV's value for money.

Many new and forthcoming vaccines, because of high development and production costs, may not be cost saving in the way that many older vaccines were, but they may reduce morbidity and mortality and improve quality of life enough to make them worth the investment. We believe that the evidence indicates that, at its current price, PCV is cost-effective in high-income countries. If the herd effect is large and extends to pneumonia, the vaccine may even be cost saving in some settings.

While the pharmaco-economic literature from middle- and low-income countries is still developing, early studies suggest that vaccine may also be highly cost-effective in these settings, especially if a tiered pricing scheme for vaccine is used, making vaccine available to these countries at a lower price than to high-income countries. Moving forward, the greatest need for PCV's pharmaco-economic evaluation is the development of economic models for low- and middle-income countries. While several analyses at the global level have been completed and published, decisions regarding the introduction of the vaccine will be made at the country level. Hence, regional and country-level pharmaco-economic analyses are urgently needed to understand the value for money that the introduction of PCV affords these countries.

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Orin S. Levine  
Brian Greenwood

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# Opportunities and Challenges for Pneumococcal Conjugate Vaccines in Low- and Middle- Income Countries

Pneumonia is the leading infectious cause of death worldwide, accounting for approximately 3.9 million deaths among persons of all ages each year (World Health Organization, 2005). Pneumonia mortality is a problem particularly among infants and young children. It is estimated that approximately 2.0 of the 10.6 million deaths that occur annually among children under the age of 5 years are due to pneumonia (32). About one-half of these deaths occur among children in sub-Saharan Africa, and an additional 30% occur in southern Asian countries (Fig. 1) (6, 35). It is estimated that there are at least 150 million cases of pneumonia each year in children under the age of 5 years in developing countries and that 11 to 20 million of these cases are severe enough to require admission to a hospital (28). Thus, preventing pneumonia in children is a high priority for the developing world.

Determining the cause of pneumonia in young children is difficult, but nearly all studies undertaken in the developing world have identified *Streptococcus pneumoniae* (the pneumococcus) as the most frequent bacterial cause of severe pneumonia. In 1986, Shann re-

viewed the results of 13 lung aspirate studies conducted during the previous 50 years among children with severe pneumonia in developing countries. Bacteria were isolated from 62% of aspirates; 27% of these isolates were pneumococci (30). More recent studies that have employed both blood culture and lung aspiration in selected cases have obtained even higher pneumococcal isolation rates (e.g., reference 1).

Recent studies in Africa have shown that *S. pneumoniae* is also a major cause of bacteremia in children. At a general hospital on the coast of Kenya, acute bacterial infections were a major cause of admission (3). The pneumococcus accounted for 25% of isolates from patients with bacteremia, and pneumococcal bacteremia was associated with 9% of all child deaths in the hospital. A high incidence of bacteremia was also found among children attending the outpatient department of this hospital; once again, the pneumococcus accounted for about half of the isolates (5). Also in The Gambia, Mali, and Mozambique (7, 24, 27), the pneumococcus was the most frequent cause of bacteremic illness in children. In these studies, the observed incidence of invasive



**Figure 1** Projected number of pneumonia deaths among children less than 5 years old. Projections are for the year 2005 and are based on WHO estimates published in reference 35. The size of each circle corresponds to the projected number of child pneumonia deaths in that country.

pneumococcal disease (IPD) was many times higher than the rates from similar studies in industrialized countries. Even higher incidence rates of IPD have been found in communities with a high prevalence of human immunodeficiency virus (HIV) infection (23).

The pneumococcus is one of the three major causes of bacterial meningitis in developing countries (12). Among the leading types of bacterial meningitis, pneumococcal meningitis is notable for its severity. In developing countries, mortality from pneumococcal meningitis approaches 50%, and nearly one-half of all survivors are left with disabling sequelae (10).

The relative importance of pneumococcal meningitis is increasing. As the *Haemophilus influenzae* type b (Hib) conjugate vaccine becomes more widely used, the burden of Hib meningitis is rapidly declining, and thus, the relative importance of pneumococcal meningitis increases. Also, in the past few years, pneumococci of serotype 1 have caused large outbreaks of meningitis among persons of all ages in Ghana (20) and Burkina Faso (40).

In 2003, the World Health Organization (WHO) estimated that up to 1 million children die each year from pneumococcal disease, primarily pneumococcal pneumonia (37). Currently, the WHO provisionally estimates that pneumococcal infections are responsible for 1.6 million deaths each year, including approximately 716,000 deaths among children <5 years of age (36). To arrive at more precise global estimates and to generate country-specific estimates of the burden of pneumococcal disease in children, the WHO recently undertook a systematic review and modeling exercise. The estimates from this more rigorous approach, including global, regional, and country estimates, will be available late in 2007. The mortality estimates from this review are expected to be consistent with previous ones. The credibility of these newer estimates is expected to be much higher, though, as a result of the rigorous and systematic review process used to derive them.

Because of the enormous burden of pneumococcal disease in the developing world, some of the earliest vaccine trials were conducted in developing countries. Controlled trials of pneumococcal polysaccharide vaccine formulations were undertaken with South African gold miners in the 1970s. These trials showed that these vaccines could confer protection against IPD due to vaccine serotypes in adult miners, a population with a very high incidence of disease (2). In the 1980s, trials of 14- or 23-valent pneumococcal polysaccharide vaccines were carried out among children in the highlands of Papua New Guinea. In the largest of these trials, involving over 6,000 children aged 6 to 60 months, overall

mortality was reduced by 19% and mortality from pneumonia was reduced by 59% (26). These results illustrate the importance of pneumococcal disease as a cause of child mortality in this community. However, these impressive results were followed up with neither further trials of pneumococcal polysaccharide vaccine nor the introduction of the vaccine in Papua New Guinea as a routine immunization. The next efficacy trials of pneumococcal vaccines in developing countries came nearly 20 years later, with the advent of pneumococcal conjugate vaccines.

Three trials of pneumococcal polysaccharide conjugate vaccines have now been conducted in impoverished communities. These trials have evaluated two different vaccine formulations. One formulation was a nine-valent vaccine that included serotypes 1, 4, 5, 6B, 9V, 14, 18C, 19F, and 23F conjugated to the CRM<sub>197</sub> carrier protein, a nontoxic variant of diphtheria toxoid. The other was an 11-valent formulation that added serotypes 3 and 7F to the nine serotypes in the other vaccine. The carrier proteins in this vaccine were diphtheria toxoid and tetanus toxoid. A summary of the vaccines tested in trials and those under development is included in Table 1.

In the first trial, conducted in Soweto, a black township near Johannesburg, South Africa, a nine-valent conjugate vaccine reduced the incidence of IPD caused by vaccine serotypes in HIV-negative children by 83% and that of radiographic pneumonia by 20% (19). Corresponding figures for HIV-positive children were 65 and 13%, respectively. In The Gambia, the same nine-valent vaccine reduced vaccine type IPD by 77%, radiographically confirmed pneumonia by 37%, all-cause hospital admissions by 15%, and overall mortality by 16% (9). Most recently, in a periurban area of the Philippines, an 11-valent conjugate vaccine reduced radiographically confirmed pneumonia by 22.9% (95% confidence interval, -1.1 to 41.2%) among children 3 to 24 months of age, but with significant heterogeneity in age-specific efficacy. Among children 3 to 11 months of age, the efficacy was 34% (95% confidence interval, 4.8 to 54.3%), while among children 12 to 23 months old, the efficacy was 2.7% (95% confidence interval, -43.5 to 34.0%) (22).

The results of these trials have provided reassuring evidence that pneumococcal conjugate vaccines are as effective at the individual level in communities where the pressure from infection is high and the population is impoverished as they have proven to be in the United States (33, 34).

On the basis of the results of the trials in South Africa, The Gambia, and the Philippines and accumulating

**Table 1** Summary of tested vaccines and vaccines under development

Stage of development and vaccine (manufacturer)	Vaccine serotypes	Carrier protein(s)	Status
Licensed or on path to licensure			
7-valent (Wyeth)	4, 6B, 9V, 14, 18C, 19F, 23F	CRM <sub>197</sub>	Licensed and registered in >70 countries
10-valent (GSK)	1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F	<i>H. influenzae</i> protein D	Licensure expected in late 2008
13-valent (Wyeth)	1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F	CRM <sub>197</sub>	Licensure expected in late 2009 or early 2010
Tested in phase 3 trials but not on path to licensure			
9-valent (Wyeth)	1, 4, 5, 6B, 9V, 14, 18C, 19F, 23F	CRM <sub>197</sub>	
11-valent (Sanofi)	1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F	Diphtheria and tetanus toxoids	
11-valent (GSK)	1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F	<i>H. influenzae</i> protein D	
7-valent (Merck)	4, 6B, 9V, 14, 18C, 19F, 23F	<i>Neisseria meningitidis</i> serogroup B outer membrane protein	

epidemiologic data that pneumococcal disease is a major cause of death in children across the developing world, a strong case has been made to begin the introduction of pneumococcal conjugate vaccines into countries across the developing world (21). In 2007, the WHO's Strategic Advisory Group of Experts recommended that pneumococcal conjugate vaccination, beginning with the licensed seven-valent vaccine, be considered a high priority for introduction into developing countries. Those countries with child mortality rates of greater than 50 per 1,000 live births or more than 50,000 child deaths per year were identified as the countries where the greatest impact of the vaccine would be expected (39).

A concerted international effort will be needed to achieve this goal. In this chapter, we review some of the specific challenges to the introduction of pneumococcal conjugate vaccines into the developing world and ways in which these challenges may be met.

## FACTORS THAT WILL INFLUENCE THE UPTAKE AND IMPACT OF PNEUMOCOCCAL VACCINES IN LOW- AND MIDDLE-INCOME COUNTRIES

Many factors influence the uptake and impact of any new vaccine, and pneumococcal vaccines are no exception. While there may be several ways to organize these factors, one way to consider them is to break them down into three main types of influencing factors: epidemiologic or biologic, programmatic, and financial

(Table 2). The biggest challenge with pneumococcal vaccines is to understand the interrelationship among these factors and how changes in one may affect another. In this section of the chapter, we explore some of the challenges presented by managing these multiple factors and how they potentially affect pneumococcal vaccine uptake and health impact.

### Epidemiologic Factors, Including Infection Pressure, Serotype Distribution, and Underlying Conditions

In many developing countries, the epidemiology of pneumococcal disease differs from that in industrialized countries in ways that may influence both the uptake of the vaccine and the overall health impact of its widespread use. First, the rate of acquisition of pneumococcal infections appears to be higher in developing-country settings that are characterized by a high degree of crowding and poor sanitation. Nasopharyngeal colonization occurs earlier in life—as early as the first month of life—and the prevalence rates of pneumococcal colonization are generally higher among children in these settings than among children in industrialized countries. For example, in a recent study in The Gambia, the prevalence of nasopharyngeal colonization exceeded 90% among children under 5 years of age and exceeded 50% among adults (15). By contrast, in industrialized countries, prevalence rates are often one-half the rates observed in this Gambian study (11). Thus, in developing countries, it appears that the pressure from infection is likely to be much higher than that in industrialized countries.

**Table 2** Factors that influence the uptake and impact of pneumococcal vaccines in low- and middle-income countries

Type	Factor(s)
Epidemiologic	Infection pressure
	Serotype distribution
	Underlying conditions
Programmatic	Cold chain and logistics
	Routine use vs campaigns
	Schedules
Financial	Financing
	Pricing
	Supply

Second, the distribution of serotypes causing pneumococcal disease in developing countries may differ from that observed in industrialized countries and may correspond to a greater incidence of disease due to serotypes not included in pneumococcal conjugate vaccines (13, 14). For example, in many published studies, serotypes 1 and/or 5 are reported to account for a large proportion of pneumococcal disease in children (4, 16, 29). As a result, there is likely to be some regional variation in the amounts of disease covered by different formulations of pneumococcal conjugate vaccines (Table 3). These differences may influence both the uptake of the vaccine and the health impact.

Drawing inferences from comparisons of the published serotype distribution data from different countries, however, should be undertaken with caution. There are several reasons why these comparative epidemiology studies are difficult. First, published reports of studies of pneumococcal serotype distribution often contain only data from cerebrospinal fluid (CSF) iso-

lates or tend to overrepresent CSF isolates in relation to the role of pneumococcal meningitis as a cause of pneumococcal mortality. For example, according to WHO estimates, about 90% of the mortality from pneumococcal disease among children is due to pneumococcal pneumonia. And yet, CSF specimens were the source of more than 50% of the isolates providing serotype information in many of the studies of serotype distribution (14). If the distributions of serotypes causing meningitis and pneumonia are the same, then these are unbiased estimates and projections based on these data will accurately predict the impact on pneumonia. However, it is likely that some serotypes are more likely to cause meningitis than pneumonia, and vice versa, and hence, studies of mainly isolates from CSF would tend to bias the reported serotype distribution.

Another factor to consider in drawing inferences from serotype distribution data is that some pneumococcal serotypes, such as serotypes 1 and 5, appear to occur in episodic outbreaks while other serotypes appear to be more stable in their incidence over time. Thus, reports of only 1 or 2 years of data may over- or underrepresent the proportion of disease due to these epidemic serotypes depending on whether the serotype incidence was increasing or declining. Differences in the age groups sampled, because some serotypes (e.g., serotypes 1 and 5) are more common among older children and adults than among young children, and differences in the patients sampled (e.g., inpatients, outpatients, or both) can also contribute to variations in the observed serotype distribution.

Perhaps most importantly, however, consideration of the proportion of pneumococcal disease covered by a vaccine formulation may lead to public health decisions that might promote the inequitable use of the vaccine. A measure that would be more useful would be the incidence of vaccine-preventable disease. For example, in the United States, serotype distribution data showed that in the American Indian population of the Southwest, only about 50% of invasive disease was due to the serotypes included in the seven-valent vaccine. For the general U.S. population, the comparable figure was over 80%. By this measure, one would have concluded that the highest priority for the vaccine's use would be the general population of the United States, not the American Indians. However, when the overall incidence of pneumococcal disease is paired with the serotype distribution to calculate an incidence of vaccine-preventable disease, it becomes quite clear that the absolute rate of disease prevented is greatest in the American Indian population, not the general U.S. population. In most developing countries, the rates of pneumococcal disease

**Table 3** Proportion of IPD serotypes included in leading vaccine formulations, by region (based on data available between 1980 and 1999)<sup>a</sup>

Region	Proportion (%) of IPD serotypes included in:		
	7-valent vaccine	10-valent vaccine	13-valent vaccine
United States and Canada	86	88	92
Latin America	60	81	87
Africa	62	81	87
Europe	71	84	89
Asia	38	66	73
Oceania	73	81	86

<sup>a</sup>Data are based on figures from reference 13. Assumes that serotype 6B protects against serotype 6A disease.

and mortality are many times greater than those in industrialized countries, and hence, the incidence of vaccine-preventable disease is also many times higher, even if serotype coverage is somewhat lower.

Underlying conditions are also important factors that differ between industrialized countries and developing countries and that may influence the vaccine's effectiveness and impact. Underlying illnesses and chronic conditions such as malaria, HIV infection, undernutrition, anemia, low birth weight, and sickle-cell disease are many times more common in developing countries than in industrialized countries. These conditions may affect the risk of disease, the effectiveness of the vaccine, or both. HIV infection and sickle-cell disease are well-known risk factors for pneumococcal disease and increase the incidence of disease in affected children by 40-fold or more (23).

In the case of HIV infection, the South African trial shows that the point estimate of vaccine efficacy is lower among HIV-infected children than among non-HIV-infected children. However, because the risk of pneumococcal disease among HIV-infected children is so much higher, the absolute vaccine-attributable reduction in incidence among HIV-infected children is several times higher than that among the non-HIV-infected children. In short, in an absolute measure, the vaccine prevents many more cases among HIV-infected than non-HIV-infected children, even though the point estimate of efficacy appears to be lower. Similar information is not available from trials with patients with sickle-cell disease, but a similar relationship may be expected.

In studies with Hib conjugate vaccine, children with malaria had a diminished immune response to the vaccine as compared to children without malaria (31). Thus, a similar pattern may be expected with pneumococcal conjugate vaccines. Fortunately, the prevalence of malaria is generally low in the first few months of life, when conjugate vaccines are likely to be given to infants, so the risk of a major impact on the vaccine's effectiveness in infants is relatively low. Coincidental malaria is likely to be more of a problem for programs that aim to vaccinate older children (e.g., catch-up campaigns). In areas where malaria is seasonal, it would be prudent to conduct any catch-up campaigns outside of the period of maximum malaria transmission to avoid this potential effect. Malnutrition, low birth-weight, and anemia may be anticipated to diminish the immune response to pneumococcal conjugate vaccines. Operational and/or clinical research to assess the impact of these factors on vaccine response would be useful for setting future policies for vaccine use in populations where these conditions are prevalent.

In the context of routine immunization programs in developing countries, the epidemiologic factors outlined above may combine or interact with one another to produce vaccine effects that differ from those observed in industrialized settings. Perhaps the most important outcome of these factors may be differences in the indirect effects of vaccination. Specifically, because of the prevalence of these conditions in developing countries, the impacts of vaccination on transmission between vaccinated and unvaccinated persons may differ from those in industrialized countries and in turn produce different results in terms of herd immunity and/or serotype replacement.

When used in developing countries, pneumococcal conjugate vaccines are expected to reduce nasopharyngeal carriage of pneumococci of vaccine serotypes, contributing to a herd immunity effect (18). However, vaccine-induced reductions in vaccine serotype pneumococcal colonization are also associated with increases in colonization by nonvaccine serotypes. When nonvaccine serotypes begin to occupy a niche that has been vacated by vaccine serotypes, the phenomenon is considered serotype replacement. Thus, it is possible that increases in colonization with nonvaccine serotypes may lead to increases in the incidence of pneumococcal invasive disease due to nonvaccine serotypes, or replacement disease.

In the United States, surveillance data show that routine childhood vaccination has been associated with (i) large, rapid declines in the incidence of overall and vaccine type invasive disease among children aged 2 years, (ii) reductions in vaccine type IPD among unvaccinated children and adults, and (iii) increases in non-vaccine serotype disease that were small relative to the overall reductions in vaccine type disease (8).

In developing countries, it is not clear whether the differences in serotype distribution and the increased infection pressure will lead to more or less serotype replacement. For example, serotypes 1 and 5, which are not included in the currently licensed seven-valent formulation, are common causes of invasive disease in some developing countries. While it is clear that the incidence of serotypes 1 and 5 can vary substantially from year to year, even in the absence of vaccination, it is not clear whether the immunologic pressure exerted by use of the seven-valent vaccine would influence the rate or magnitude of outbreaks due to these serotypes. In the United States, the serotype that appears to be clearly involved in replacement disease is serotype 19A (25). This serotype is commonly carried in children and is also frequently antibiotic resistant. Fortunately, serotypes 1 and 5 are rarely found among colonization isolates, and as

selective pressure from vaccination is likely to be exerted in the nasopharynx, there are good biologic reasons to anticipate that serotype replacement, if it does occur, is more likely to come from nonvaccine serotypes that are more commonly found in the nasopharynx. Likewise, a report from Korea shows an increase of 19A invasive disease in an area when little seven-valent vaccine has been used, again highlighting the difficulties in showing a temporal relationship between vaccine use and serotype replacement due to a specific serotype (17).

Global formulations capable of preventing the vast majority of pediatric pneumococcal disease, with little regional variation, are in phase 3 testing. Pneumococcal conjugate vaccines containing serotypes 1 and 5 are expected to be licensed between 2008 and 2010 (Table 1). This is reassuring because, even if the widespread use of the seven-valent vaccine did lead to increases in serotype 1 and 5 disease, it is unlikely that this effect would be seen immediately. Thus, by the time that a vaccine would be needed, infant vaccines containing these serotypes are likely to be available.

Similarly, the impact of these factors on the occurrence of herd immunity is also unclear. The additional crowding may increase or reduce the impact of herd immunity. The high and sustained prevalence of colonization may induce long-lasting protection by providing natural boosting through immunologic challenge with pneumococci of the same or different serotypes. On the other hand, the prevalence of underlying conditions may shorten the duration of protection by diminishing the initial and subsequent responses to vaccination.

The randomized, controlled trial of a nine-valent vaccine in The Gambia showed that even in a situation of high pressure from infection, a pneumococcal conjugate vaccine can provide individual protection at a level comparable to that seen in wealthier societies (9). The trial in The Gambia, however, was not designed to determine whether routine immunization would confer the type of herd immunity that has been observed in the United States. Experience from the American Indian populations in the United States, in which the epidemiologic pattern of pneumococcal disease is quite similar to that in populations in many developing countries, provides some useful evidence to suggest that some herd immunity effect should be expected. In the end, only routine widespread use of the vaccine in developing-country sites with strong baseline surveillance will provide a full answer to this important question.

### Programmatic and Logistic Factors

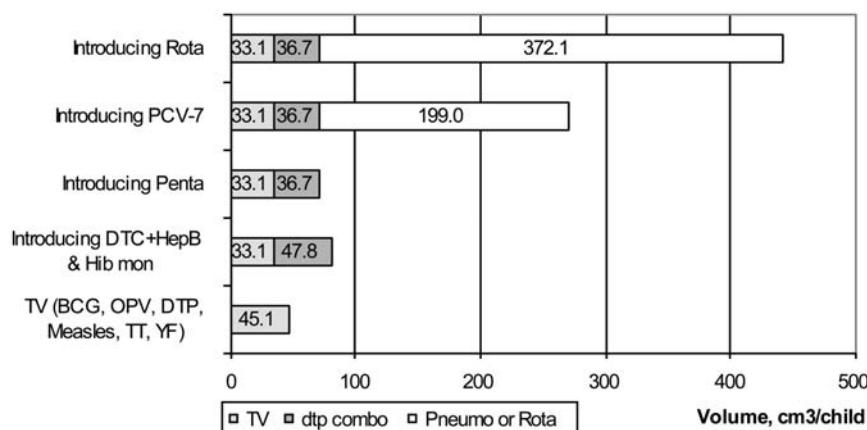
In all countries, but particularly in developing countries, there is a tight relationship between the logistical

and programmatic capacity in the country and the characteristics of the vaccine itself, which can influence significantly vaccine introduction and use. When a vaccine's characteristics are well-suited to local programs, national programs can generally ensure high levels of vaccination coverage, including populations that may lack access to routine clinical services. In these cases, the vaccine and the program work in sync to achieve disease control targets. When the vaccine and program are not optimally suited, significant investments in logistic and human resources and/or acceptance that optimal disease control approaches are not possible may be required.

In developing countries with an effective Expanded Program on Immunization, the delivery of a pneumococcal conjugate vaccine should not provide a major challenge, provided that it can be administered at the same time as other vaccines (i.e., does not require additional, different visits), fits within the existing cold-chain and transport systems, and does not require special handling or storage requirements. Vaccines that come in fully liquid preparation and do not require a reconstitution step have additional advantages for ease of administration.

The licensed seven-valent pneumococcal conjugate vaccine (Prevnar; Wyeth) is currently available in a fully liquid, single-dose prefilled syringe. This presentation is popular with private practices in middle- and high-income countries and even in public clinics in high-income countries where power supplies and refrigeration are not a problem. In developing countries, this formulation will present some challenges for introduction. Its packed volume (59.7 cm<sup>3</sup>/dose) is less than those of nearly all other vaccines available in prefilled syringes but substantially more than the packed volumes of vaccines in multidose vials. Specifically, the introduction of this vaccine will require substantial (three- to fivefold) increases in the cold-storage capacity, significant increases in the frequency of shipments from central to peripheral centers, or a combination of both (Fig. 2).

The advantages of this presentation are that it simplifies somewhat the management of immunization service supplies because it is only one item and eliminates the risk of a mismatch of separate items (e.g., not enough syringes to match the number of vaccine doses). A significant drawback of this current presentation is that the syringe is not "auto-disable," that is, it is not a single-use disposable. Current WHO-United Nations Children's Fund-United Nations Population Fund policy recommends auto-disable syringes for immunization services because auto-disable syringes eliminate the risk of unsafe injections from the reuse of contaminated syringes



**Figure 2** Net storage volumes of vaccines per child. Calculations were provided by Souleymane Kone, WHO, Geneva, Switzerland. Rota, rotavirus vaccine; PCV-7, seven-valent pneumococcal conjugate vaccine; penta, penta-valent vaccine; DTP, diphtheria-tetanus-pertussis vaccine; Hep B, hepatitis B vaccine; Hib mon, monovalent Hib; TV, tetanus vaccines; BCG, *Mycobacterium bovis* BCG; OPV, oral polio vaccine; TT, tetanus toxoid; YF, yellow fever vaccine; pneumo, pneumococcal vaccine.

([www.who.int/injection\\_safety/toolbox/en/Bundling.pdf](http://www.who.int/injection_safety/toolbox/en/Bundling.pdf)).

Next-generation pneumococcal conjugate vaccines are expected to come as fully liquid vaccines in single- and multidose vial presentations. These presentations should facilitate greatly the integration of the vaccine into the supply chain in developing countries and reduce the costs associated with the expansion of storage facilities. A major challenge will be to arrive at a vial size or sizes that balance the needs to minimize wastage rates and maximize the use of cold-chain and logistic capacities.

The introduction of new vaccines, such as pneumococcal conjugate vaccine, provides opportunities as well as challenges for the immunization and health systems. For example, in many countries, meningitis and pneumonia are widely recognized by mothers as important and severe causes of illness. Thus, in some countries with low levels of coverage with existing vaccines, the publicity and attention given to the introduction of pneumococcal conjugate vaccine may help to increase coverage with existing vaccines.

Mass campaigns are a commonly used strategy for immunizing hard-to-reach populations (e.g., nomadic populations and populations in civil-war-affected areas). The use of pneumococcal conjugate vaccines in campaigns may be an excellent way to “front load” prevention. Vaccinating children ages 1 through 4 years with a single dose, for example, would prevent a substantial amount of illness among children in this age group and increase the potential for herd immunity to prevent dis-

ease among unvaccinated adults and children. Also, using the vaccine in a campaign helps reach children who may not be reached by routine services and may have the highest risk of pneumococcal mortality. Pneumococcal conjugate vaccines may be well suited to use in mass campaigns in tropical areas because they are highly heat stable. Another advantage of this approach is that it allows the linkage of pneumococcal vaccination with the delivery of other health interventions. Recent successful examples of this approach include the provision of malaria bed nets, micronutrients, antihelminthics, and measles vaccines in a single campaign.

Another important factor that influences the uptake of a new vaccine is its dosing schedule. The best fit with an Expanded Program on Immunization is a vaccine that can be given at the same time (preferably even in the same syringe) as existing vaccines and that thus does not require any additional visits. In the United States, a schedule of four doses of pneumococcal conjugate vaccine is recommended, with three doses given during the first 6 months of infancy and a booster dose given during the second year of life. In the United Kingdom, two doses are recommended in the first year of life, followed by a booster dose in the second year of life. Few developing countries have a routine system capable of delivering a booster dose in the second year of life. Fortunately, clinical trials in developing countries have demonstrated convincing evidence of the protective efficacy of three doses of pneumococcal conjugate vaccine, given on the same schedule as diphtheria-tetanus-pertussis, hepatitis B, and oral polio vaccines. As a re-

sult, the WHO's Strategic Advisory Group of Experts has recommended a three-dose regimen in infancy for developing countries (39). Trials of alternative regimens using fewer than three doses and using different sequences of doses are under way and will provide important data for influencing policy in the future.

### Financial Constraints

Public spending on health in developing countries is generally very low. Few low-income countries spend more than \$30 per capita annually on all health care. Most vaccine programs are supported through public funding, and new vaccines are expected to cost dollars, not pennies, per dose. Therefore, financing is a major challenge for the introduction of new vaccines, including pneumococcal vaccines, in developing countries.

The reverse side of this financial challenge for countries is that the lack of credible, predictable, sustained financing—and profitable pricing—fails to provide incentives for vaccine manufacturers to invest in the development of supplies of pneumococcal vaccines for developing countries. For pneumococcal vaccines, the lack of sufficient manufacturing capacity is a useful illustration of this point. Even if the costs of developing a pneumococcal conjugate vaccine can be paid for by supplying it to richer countries, at least 3 to 4 years' lead time, and hundreds of millions of dollars, are required to build a vaccine manufacturing plant to serve the developing world.

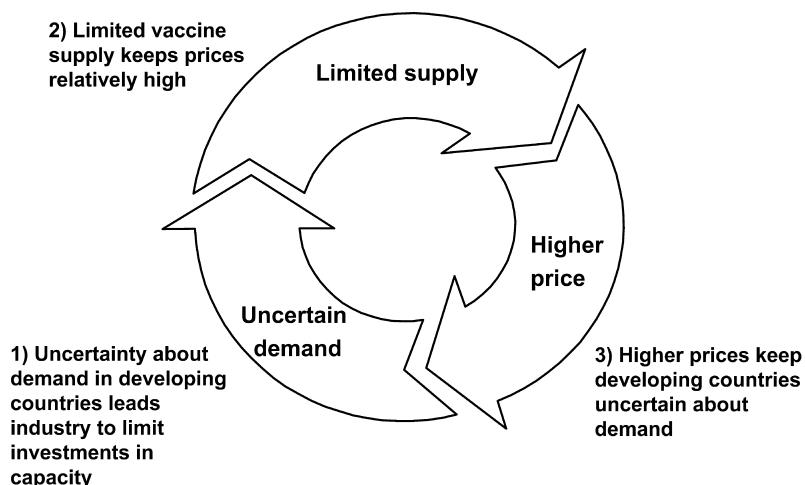
Without a credible, predictable commitment by developing countries (or donors on behalf of these countries), manufacturers will limit their supply and capacity investments to be adequate for supplying the highly

profitable markets, such as Europe, North America, and private markets of low- and middle-income countries. When supply is limited and equivalent to high-priced demand, the prices remain high and demand in developing countries remains low. Without active efforts to manage these forces, the result is a vicious cycle that perpetuates the historical delays in vaccine uptake, such as those observed with Hib conjugate vaccines and others (Fig. 3).

Achieving sustained, predictable financing is critical to the introduction of pneumococcal conjugate vaccines into underprivileged communities where they are needed most. For low-income countries, a group of international donors, led by the United Kingdom, Italy, and Canada, and the Global Alliance for Vaccines and Immunization (GAVI) and the World Bank are launching a new approach to overcoming this problem. The approach is called an Advance Market Commitment (AMC). This initiative represents an innovative, market-based approach to bring about the changes in supply and pricing needed to ensure accelerated and sustained vaccine use in low-income countries.

The AMC works by simulating the same market forces that lead to private investment in the development of vaccines and drugs for richer countries. It represents an efficient use of aid resources because it legally binds international donors and vaccine manufacturers into an agreement that facilitates vaccine demand by developing countries.

It accomplishes its goals in a few important ways. First, donors and technical agencies like the WHO set out the characteristics of the vaccine candidate that will meet the needs of developing countries in advance of licensing of the vaccines. The donors back this up with a



**Figure 3** Without active management, supply and demand forces can lead to challenges in obtaining affordable, sustainable vaccine supplies for developing countries.

legally binding commitment in advance of licensure to purchase a set quantity at a price that reimburses the companies for their risks and investments. For pneumococcal vaccines, this commitment is expected to ensure U.S. \$1.5 billion to procure the vaccine and this funding is expected to last 7 to 9 years. In this way, the donor commitment eases risks for manufacturers and developing countries by providing credible, predictable financing in advance of demand, that is, when strategic decisions on manufacturing capacity need to be made.

The AMC also binds manufacturers into the process in a way that benefits developing countries and represents a good use of international aid. Manufacturers sign on to the AMC by agreeing to supply vaccines at the relatively higher prices that the AMC promises, and when the AMC financing is depleted (because the \$1.5 billion has bought the set amount of doses), they agree to provide the vaccines over the longer term at more affordable prices. Manufacturers are paid only for doses that developing countries demand, and therefore, there is an incentive for them to develop a product that would be preferred over those of other manufacturers. In this way, this aid mechanism is results driven and does not benefit any supplier unless that supplier's product is the one that developing countries want.

Developing countries play several major roles in this process. First, they contribute to setting the "target product profile"—that is, the specifications of the vaccine that they would want to use. Second, the AMC pays manufacturers only for doses of vaccine that are demanded by developing countries. Lastly, GAVI policies require developing countries to commit some financing (i.e., a copayment) for vaccines that they demand through GAVI (including vaccines demanded through the AMC). Thus, the commitment of national funds to a copayment for pneumococcal vaccines is another of the ways that developing countries participate in the AMC and contribute to its success.

The AMC helps overcome three of the major obstacles faced by developing countries in terms of financing pneumococcal conjugate vaccine introduction. By specifying the vaccine characteristics, it helps ensure that developing countries get the vaccines they want, not just the ones that the industry offers. With the 7- to 9-year subsidy from donors, it makes early, accelerated vaccine introduction possible by bridging the difference between the prices that countries can afford (i.e., the copayment) and those that manufacturers need to make the vaccine available. By negotiating supplier agreements to include affordable long-term pricing, the AMC helps ensure developing countries that by the time that national governments need to pick up the bulk of the

costs, the vaccine's price will be within reach of national governments themselves.

Financing is critical to increasing the uptake of pneumococcal conjugate vaccines. However, experience shows that overcoming financing obstacles alone is not sufficient to ensure vaccine uptake. Efforts to coordinate the range of activities needed to establish and communicate the value of pneumococcal vaccines (and new vaccines generally) can also help reduce the demand risk for suppliers and for donors and countries. Surveillance to monitor the impact of vaccination on serotype changes and on unvaccinated populations (e.g., herd immunity) will continue to be important for sustaining the political will to finance the vaccine and for responding to epidemiologic changes.

### Middle-Income Countries

In 2005, 3 billion people—nearly one-half of the world's 6.4 billion total population—lived in middle-income countries (defined by the World Bank as those with a gross national income per capita between \$876 and \$10,725). While these countries are middle income, they also include large low-income populations in which the incidence and degree of severity of pneumococcal infections are expected to be high. The use of pneumococcal vaccines in middle-income countries has the potential to prevent a large number of pneumococcal infections, but introduction and sustained use will face substantial challenges, most importantly in terms of financing and pricing.

GAVI and its associated GAVI Fund provide an opportunity for some countries to overcome financing obstacles. However, GAVI eligibility is defined by having a gross national income of less than \$1,000 per capita, and thus, only a small fraction of the countries defined by the World Bank as middle income are eligible for financial support through GAVI. For example, populous countries like China, Brazil, and the Philippines are not covered by GAVI. In these countries, the private market is often large and highly profitable. As a consequence, new vaccines, including pneumococcal vaccines, are often registered quickly in these countries and are available on the private market at prices comparable to those charged in the United States, Europe, and elsewhere. The challenge in these countries is to ensure access for the children and communities who need the vaccine the most and who are least able to afford private market prices.

One approach to this problem is to undertake a phased introduction of the vaccine that begins with government-sponsored funding for the deployment of the vaccine in the highest-risk communities first. The government of Mexico is using this approach, for example,

to gradually increase coverage with the seven-valent pneumococcal conjugate vaccine. The vaccine was used first to vaccinate 42,000 children in 58 counties in nine states. These communities were selected because they had a disproportionately high infant mortality rate. The program is gradually being expanded to 16 states in 2007. By the end of 2007, it should include approximately 326,000 children and all states where at least 50% of the population are indigenous persons. This graduated approach to the use of the vaccine, beginning with the highest-risk populations, is likely to be an approach commonly used in the future by other countries that find the price of full, routine use of a vaccine to be beyond their financial means. Still better, more-creative approaches to ensuring vaccine access for middle-income countries, and especially for poor populations in middle-income countries, are needed.

## MEETING THE CHALLENGES

The successful prevention of pneumococcal disease in developing countries requires collaborative action by national governments, international donor governments and foundations, and vaccine suppliers (Fig. 4). In addition, support from the research community will be needed to determine how pneumococcal vaccines can be introduced most effectively into communities with limited resources and to monitor their impact.

### EXPECTED IMPACT OF EXPANDED PNEUMOCOCCAL VACCINATION PROGRAMS IN DEVELOPING COUNTRIES

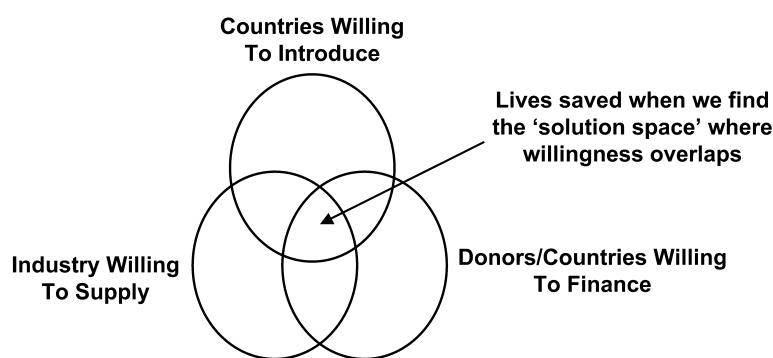
The health impact of infant and childhood pneumococcal vaccination is expected to be greatest in developing countries, especially those with high infant and child mortality rates, where >90% of global childhood pneu-

monia deaths occur (35). In these countries, the impact of pneumococcal vaccination on childhood illness and mortality will be many times greater than that in industrialized countries. For example, based on the results of the trial in The Gambia, vaccination is expected to prevent about 700 deaths per 100,000 children vaccinated, whereas in the United States, vaccination is expected to prevent about 6 deaths per 100,000 children vaccinated—a difference of over 100-fold.

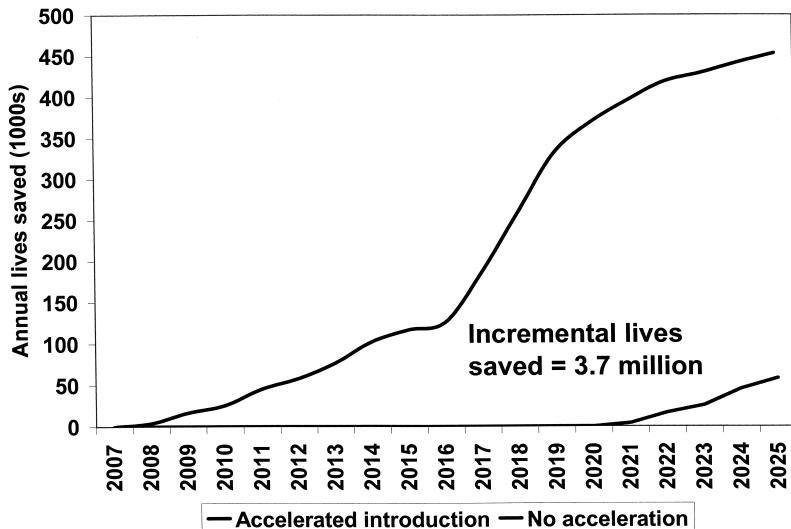
The major determinants of the health impact of pneumococcal vaccination in developing countries will be the speed with which the vaccines are taken up and applied, the coverage levels reached (and whether the vaccines reach the highest-risk children), and the indirect effects that follow widespread use. A recent, relatively simple analysis of the expected health impacts in the 72 GAVI-eligible countries (i.e., the 72 countries with the lowest gross national income per capita) illustrates the value of accelerated vaccine introduction. By comparing the accelerated introduction forecast with a forecast based on historical precedents like hepatitis B and Hib conjugate vaccines, it can be shown that accelerated pneumococcal vaccination may prevent 3.7 million child deaths that would otherwise go unpreventable (Fig. 5). This calculation does not include any prevention of deaths among older children or adults as a consequence of herd immunity.

## THE ROAD AHEAD

The accelerated introduction of conjugate vaccines in developing countries will greatly reduce the burden of pneumococcal disease and establish a market for pneumococcal vaccination. This market will drive the pneumococcal vaccine pipeline to produce the next generation of products. Currently, more than 20 different vaccine candidates are in various stages of development



**Figure 4** Prevention of pneumococcal disease occurs when willingness of donors, countries, and suppliers overlaps.



**Figure 5** Impact of accelerating pneumococcal vaccine introduction on child mortality in GAVI countries.

and testing. The next generation of products are likely to come from emerging market suppliers and multinational companies and to include both conjugates and other approaches (e.g., common-protein vaccines).

Ultimately, the successful and sustainable use of pneumococcal vaccines (and all new vaccines) in developing countries will depend on increasing health budgets, and immunization budgets specifically, and ensuring long-term predictable financing from international donors to help bridge the gap between what countries can afford and what suppliers need in order to sustain supply for the market. Innovative mechanisms of financing like the AMC show the ability of vaccines to generate this kind of change. Success will require continued efforts to increase the value assigned to vaccines by donors, developing countries, and individual citizens so that people and governments are prepared for prices of dollars, not pennies, per dose.

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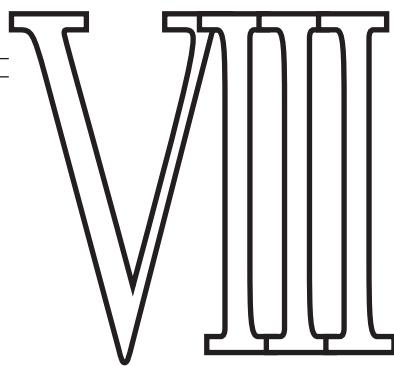
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James C. Paton  
John W. Boslego

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## Protein Vaccines

### LIMITATIONS OF CURRENTLY AVAILABLE PNEUMOCOCCAL VACCINES

The development, manufacture, and administration of pneumococcal polysaccharide vaccine (PPSV) and pneumococcal conjugate vaccine (PCV) have provided significant public health benefit, and these vaccines continue to be important tools in the battle against the pneumococcus. However, these vaccines provide protection against only the specific serotypes contained within the vaccines and have other limitations.

For 23-valent PPSV (PPSV23), a major limitation is the inability of the purified polysaccharide (PS) to induce protective immunity in children less than 2 years of age. Furthermore, some of the serotypes are weak immunogens and not very efficacious. The serotypes that are weak immunogens are also more often associated with antibiotic resistance. Vaccine-induced antibody concentrations decline within a few years of vaccination, and revaccination does not induce a booster response. Finally, efficacy in various high-risk groups, especially the elderly, has been questioned and debated.

For seven-valent PCV (PCV7) and future-generation PCV10 and PCV11, a major limitation is the relatively small numbers of serotypes contained in the vaccines.

This limitation places restrictions on the breadth of strain coverage and the amount of disease burden that can be prevented with their use. The relatively small numbers of serotypes also raise the potential threat of serotype replacement in the geographic areas where the vaccines are used, which would further reduce the impact on disease burden and may require expensive reformulations of the vaccines with different serotypes. The PCVs are complex vaccines from a development and manufacturing perspective, given that each capsular PS serotype is chemically distinct and has to be individually optimized with respect to protein/polysaccharide ratios, conjugation technology, and other characteristics. The PCVs have so far proved too costly to be afforded by developing-world countries, where the need for them is the greatest.

### PROSPECTS FOR NEW PNEUMOCOCCAL VACCINES

The established and potential limitations of the PPSV and PCV have sparked efforts to identify protein components of the pneumococcus that are common to all or the vast majority of strains worldwide. The recent

observation that age-related reduction in the incidence of invasive pneumococcal disease in children does not correlate with the natural acquisition of antibodies to PS provides support for the possibility that other immune mechanisms, perhaps including antibody responses to noncapsular antigens, may play a key role in natural resistance to infection (57).

If such common-protein vaccines are successfully developed, they could have a number of advantages over the currently available capsular PS-based vaccines. Similar to other protein vaccines, the pneumococcal common-protein vaccine should induce high concentrations of antibody and immunologic memory in infants and children, who respond well to T-cell-dependent protein antigens. Common-protein vaccines will probably be composed of several distinct protein components, which will direct the immune system to attack the organism at various stages of its pathogenesis. Indeed, if protein components associated with nasopharyngeal colonization are included and effective in blocking such colonization, the vaccine could potentially result in a significant reduction in transmission and a large herd immunity effect. An effective common-protein vaccine would reduce or eliminate the threat of serotype replacement inherent in the capsular PS-based vaccines and provide broad coverage worldwide. Finally, efficient and high-level expression of proteins can be engineered in recombinant systems, enabling large-scale production at potentially very low cost, resulting in vaccines that are more affordable in developing countries.

## PURIFIED-PROTEIN VACCINES

A number of candidate pneumococcal protein antigens have been examined for vaccine potential, including the toxin pneumolysin and a diverse array of surface proteins. The surface proteins can be divided into several distinct classes, based on the manner in which they are attached to the pneumococcal surface, which in turn affects the extent to which they protrude beyond the capsular layer. The major classes are the choline-binding proteins (CBPs), the metal-binding lipoproteins, the sortase-dependent surface proteins, and the pneumococcal histidine triad proteins. The biological properties and vaccine potential of representatives of each of these groups are described below.

### Pneumolysin

Pneumolysin (Ply) was the first pneumococcal protein to be proposed as a vaccine antigen (86), and its structure, function, and role in virulence have been reviewed re-

cently (76). Briefly, it is a potent 53-kDa thiol-activated pore-forming cytolysin produced by virtually all strains of *Streptococcus pneumoniae*. Ply belongs to a family of toxins produced by gram-positive bacteria of several genera. These toxins share a common mode of action, which involves binding to cholesterol in target cell membranes, followed by insertion into the membrane and oligomerization to form large transmembrane pores, thereby lysing the cell. Ply is a bifunctional toxin, and in addition to its cytotoxic properties, it has the capacity to directly activate the classical complement pathway in the absence of specific antibody (with a concomitant reduction in opsonic activity in serum) (75, 87). In vitro studies using purified toxin have demonstrated that Ply has a variety of detrimental effects on cells and tissues, which undoubtedly contribute to the pathogenesis of disease (89). These effects include the inhibition of the bactericidal activity of leukocytes, the blockade of proliferative responses and immunoglobulin (Ig) production by lymphocytes, the reduction of ciliary beating of the human respiratory epithelium, and direct cytotoxicity for respiratory endothelial and epithelial cells. Thus, pneumolysin may function in pathogenesis by interfering with both phagocytic and ciliary clearance of pneumococci, by blocking humoral immune responses, and by aiding the penetration of host tissues (89).

The injection of purified pneumolysin into rat lungs induces severe lobar pneumonia, indistinguishable histologically from that seen when virulent pneumococci are injected (32). It stimulates the production of interleukin-1 $\beta$ , tumor necrosis factor alpha, and nitric oxide by macrophages through the induction of the NF- $\kappa$ B pathway (15, 43). Malley et al. (65) demonstrated that the inflammatory response of macrophages to Ply involves signaling through Toll-like receptor 4 (TLR4) and that this signaling synergizes with that of pneumococcal cell wall components, which signal through TLR2. Additional insights into the role of pneumolysin in pathogenesis have been gained by studies of the behavior of defined pneumolysin-negative mutants of *S. pneumoniae* in a number of animal models. Such strains have significantly reduced virulence in mouse models of sepsis and pneumonia (8). Intranasal challenge with these mutants results in a less severe inflammatory response, a reduced rate of multiplication within the lung, a reduced capacity to injure the alveolus-capillary barrier, and a delayed onset of bacteremia compared with challenge with the wild-type strain (89). Additional site-directed mutagenesis studies have shown that both the cytotoxic and complement activation properties of the toxin contribute to the pathogenesis of pneumococcal pneumonia (10, 96).

Although native Ply is a protective immunogen in mice (86), it is not suitable as a human vaccine antigen because of its toxicity. However, structure-function studies have identified a number of specific amino acid residues that can be mutated or deleted in order to reduce cytotoxicity. For example, Ply and other members of the thiol-activated toxin family have a conserved undecapeptide Trp-rich motif in their C-terminal regions (amino acids 427 to 437 in Ply), which includes the only Cys residue in Ply. Site-directed mutagenesis studies (14, 98) showed that mutations such as Cys<sub>428</sub>→Gly and Trp<sub>433</sub>→Phe reduced the cytolytic activity of Ply by up to 99.5% (96). Other mutations in this region and elsewhere in the molecule were also capable of reducing cytolytic activity (14, 41, 60). Recently, even greater reductions in cytolytic activity have been achieved by the deletion of Ala<sub>146</sub>, which blocks oligomerization and completely prevents pore formation (52). An earlier study showed that an Asp<sub>385</sub>→Asn mutation in a separate domain of Ply abolishes the complement activation property (75).

The above-cited studies have enabled the construction of a variety of nontoxic but immunogenic "pneumolysoids," which were easily purified from recombinant *Escherichia coli* expression systems (88). Ply is a highly conserved protein, and extensive analyses of genes from a wide range of *S. pneumoniae* serotypes have detected negligible variation in deduced amino acid sequence, auguring well for broad coverage. Indeed, the immunization of mice with a pneumolysoid carrying a Trp<sub>433</sub>-Phe mutation resulting in over 99% reduction in cytotoxicity (designated PdB) provided a significant degree of protection against all nine serotypes of *S. pneumoniae* that were tested in models of sepsis and bacteremic pneumonia (2). PdB was also protective in a mouse model of localized pneumonia (18).

Humans are known to mount an antibody response to pneumolysin as a result of natural exposure to *S. pneumoniae*, and purified human anti-pneumolysin IgG also passively protects mice from challenge with virulent pneumococci (77). Thus, it is anticipated that the various pneumolysoids will be immunogenic in humans. However, pneumolysoid may not provide a sufficient degree of protection to be an effective stand-alone human vaccine antigen. Ply is not displayed on the surface of the pneumococcus. Rather, it is located in the cytoplasm and is released into the external milieu by spontaneous autolysis in some strains (7), as well as by an as-yet-uncharacterized export mechanism in others (3). Antibodies to pneumolysin are presumed to impart protection by neutralizing the biological properties of the toxin, thereby impeding the kinetics of infection, rather

than by stimulating opsonophagocytic clearance of the invading bacteria. Thus, protein-based vaccines combining pneumolysoid with pneumococcal surface proteins capable of eliciting opsonic antibodies would be expected to be more effective than vaccines consisting of pneumolysoid alone.

### CBPs

CBPs are a diverse family of proteins which are tethered to the pneumococcal surface via noncovalent interactions with phosphoryl choline moieties on cell wall teichoic acid and membrane lipoteichoic acid. This binding is mediated by a domain comprising up to 10 highly conserved 20-amino-acid repeats. For most of the CBPs, these repeats are located near their respective C termini (63, 101, 115). Up to 15 proven or putative CBPs have been identified by the examination of *S. pneumoniae* genome sequences, but some of these are not present in all strains. In spite of the similarity of the choline-binding repeat domains, the remainders of the CBP molecules are structurally and functionally dissimilar. CBPs have diverse functions, including cell wall modification, adherence to host cell surface molecules, and the modulation of complement activation (101). Those with potential as vaccine antigens are discussed below.

### Pneumococcal Surface Protein A

Pneumococcal surface protein A (PspA) is one of the best-characterized members of the CBP family and has strong credentials as a vaccine antigen. It is found on the surfaces of all pneumococci (25) and has a proven role in the pathogenesis of disease, as evidenced by the significantly reduced virulence of defined PspA-negative pneumococci in animal models (72, 109). Its principal function appears to be the inhibition of complement-dependent host defenses mediated by factor B. This inhibition results in reduced deposition of C3b onto the pneumococcal surface and the concomitant impairment of complement receptor-mediated clearance (109). PspA also binds lactoferrin (36, 39), and this may aid in the colonization of host mucosae by protecting the pneumococcus from the bactericidal effects of apolactoferrin (100). The biological properties of PspA reside in the N-terminal portion of the molecule, which forms a highly charged, largely alpha-helical antiparallel coiled-coil structure (42, 47). This region of PspA is exposed on the surface of *S. pneumoniae*, and the presence of the capsule does not impede accessibility to exogenous antibodies (26, 34), suggesting that anti-PspA should also be opsonophagocytic.

The immunization of mice with a soluble 43-kDa N-terminal PspA fragment has been shown to be highly

protective against systemic challenge (103) and pneumonia (18). This region of the molecule is variable in terms of its amino acid sequence, although this does not appear to impact upon biological function (93). PspA proteins produced by various *S. pneumoniae* strains have been grouped into three families, with 95% of isolates producing PspA belonging to families 1 or 2 (42, 110). In spite of the significant variation in amino acid sequence, the helical domain of PspA contains epitopes that elicit antibodies that are highly protective against challenge with *S. pneumoniae* strains producing heterologous PspA types (73).

Studies of the vaccine potential of PspA have extended to human trials, and immune sera from volunteers immunized with a family 1 PspA fragment reacted with 37 different *S. pneumoniae* strains belonging to diverse capsular and PspA types (78). Moreover, the sera passively protected mice against challenge with *S. pneumoniae* strains of three different capsular types expressing either family 1 or family 2 PspAs (17). Thus, it appears likely that a human vaccine may only need to include two or three different PspA types in order to provide near-species-wide protection. A theoretical concern that arose in a subset of patients during the human trials was that the PspA antigen, which has a coiled-coil structure, elicited antibodies capable of cross-reacting with human cardiac myosin. However, the significance of this finding is questionable. Both nasopharyngeal carriage and infection with *S. pneumoniae* elicit antibody to PspA and can even boost the low levels of the antibody present in 10 to 20% of human sera that is reactive with human myosin. Such natural exposure to pneumococci is of course very common, but it has never been shown to be linked to any autoimmunity.

In addition to its promise as a parenteral vaccine antigen capable of preventing systemic disease, PspA exhibits considerable promise as a mucosal vaccine antigen for the prevention of nasopharyngeal carriage. As described previously, a vaccine capable of preventing carriage is likely to impart substantial herd immunity. Intranasal immunization with full-length native PspA, using the cholera toxin B subunit (CTB) as an adjuvant, has been shown to elicit significant mucosal and serum antibody responses and to protect mice against both nasal carriage of *S. pneumoniae* and systemic disease (113, 114). However, a recombinant N-terminal PspA fragment (also administered with CTB) appeared to be less effective, reducing the level of colonization after intranasal challenge but not preventing it altogether (16). On the other hand, parenteral immunization with PspA elicits negligible levels of mucosal antibody and no detectable protection against carriage (113). The potential

efficacy of mucosal immunization with PspA for the prevention of carriage in humans is also supported by the findings of a human volunteer study, which demonstrated that preexisting (naturally acquired) antibody to PspA prevented colonization after the intranasal administration of *S. pneumoniae* (71).

#### PspC/CbpA

Several other members of the CBP family have been proposed as vaccine antigens. The best characterized of these was isolated independently in three laboratories and is referred to as either pneumococcal surface protein C (PspC) (19), choline-binding protein A (CbpA) (94), or SpsA (38) (the foremost terminology will be used here). Its choline-binding repeat region is approximately 95% identical to that of PspA, while its N-terminal helical portion, like PspA, is highly variable. The N-terminal half mediates binding to the secretory component of IgA (38, 40), as well as to C3 (24) and factor H (28). PspC has been shown to be involved in the adherence of pneumococci to cytokine-activated lung epithelial cells in vitro, as well as to glycoconjugates previously identified as pneumococcal binding ligands. Furthermore, PspC-deficient pneumococci have a reduced capacity to colonize the nasopharynges of infant rats and mice (4, 95). Interestingly, PspC appears to be expressed at greater levels in transparent-phase pneumococci, which are favored in the nasopharynx over opaque-phase variants (95). PspC may also be directly involved in the invasion of nasopharyngeal cells through interaction with the secretory component associated with the polymeric Ig receptor pIgR (116). The fact that PspC can interact with C3 and factor H is also strongly suggestive of a role in systemic disease, and significant differences in virulence between *pspC*-negative and otherwise isogenic wild-type pneumococci have been demonstrated in mouse models of lung infection and bacteremia (4). An earlier study did not detect differences in virulence between wild-type and *pspC*-negative pneumococci in an intraperitoneal challenge model, but the mutation of both *pspC* and the pneumolysin gene had an additive attenuating effect (12).

The immunization of mice with PspC is highly protective against intravenous or intraperitoneal challenge with *S. pneumoniae* (20, 80). Theoretically, the suitability of PspC as a vaccine antigen may be diminished to some extent by the fact that it is present on only about 75% of *S. pneumoniae* strains (20, 38). However, immunization with PspC was shown to provide significant protection against a strain that did not produce the protein (20). This effect can be explained by the fact that polyclonal antibodies to PspC cross-react with PspA, as

well as other protein species (20). Furthermore, in those strains that lack *pssC*, the locus is occupied by an allele, *hic*, which encodes a protein with a high degree of similarity to PspC in the N-terminal half (44, 45), and this protein may also be recognized by PspC antibodies. Interestingly, the C-terminal portion of Hic does not have a choline-binding domain but instead has a sortase-dependent cell wall anchorage domain, including an LPXTG motif, typical of gram-positive bacteria (45). Like PspC, Hic can bind to factor H and thereby interfere with complement activation (46). However, there are differences in the precise nature of this interaction, as Hic binds to short consensus repeats 8 to 11 of factor H, whereas PspC binds to repeats 13 to 5 (31).

### Other CBPs

As mentioned previously, access to the pneumococcal genome sequence has facilitated the search for additional vaccine antigens, because it enables entire families of genes encoding proteins with recognizable structural features to be targeted (91). The CBPs are good examples of this approach. Although several members of this family were previously identified by conventional techniques, such as elution from the cell surface with choline, a search of the genome sequence identified a dozen or so functional genes encoding proteins with choline-binding motifs. Site-specific mutagenesis was then used to demonstrate that five of the novel CBPs (CbpD, CbpE, CbpG, LytB, and LytC) are involved in *in vitro* adherence to epithelial cells, nasopharyngeal colonization, or sepsis, thereby identifying them as vaccine candidates (35). LytB and LytC are unusual in that their choline-binding domains are located in the N-terminal parts of the molecules, while the C-terminal portions have murein hydrolase activity (62). Purified recombinant LytB and LytC were tested for protective efficacy as part of another large-scale study. Immunization with these proteins conferred significant protection against intraperitoneal challenge in mice, although the degree of protection observed was marginally less than that observed with PspA, which was used as a control antigen (112).

Another CBP with cell wall modification (in this case, amidase) activity is the major pneumococcal autolysin LytA. Mutagenesis of the *lytA* gene prevents the autolysis of pneumococci that occurs spontaneously in stationary-phase cultures, or upon the addition of deoxycholate, and also attenuates virulence in mouse models of sepsis. It may seem paradoxical that the inactivation of what is essentially a suicide gene could have such an effect. However, LytA is largely responsible for release of intracellular Ply, inflammatory cell wall degra-

dation products and other cell-associated virulence factors, and so the prevention of autolysis may be of considerable benefit to the host (7, 9, 23). Exogenous antibody to LytA is capable of penetrating the surface layers of the pneumococcus and inhibiting autolysis and the release of pneumolysin *in vitro*. Active immunization of mice with purified LytA also elicited a degree of protection similar to that elicited by pneumolysinoid against challenge with fully virulent pneumococci, but it conferred no significant protection against challenge with high doses of a pneumolysin-negative strain. This suggested that the LytA-induced protection is mediated largely through the blockade of pneumolysin release (59).

### Lipoproteins

The pneumococcal genome includes over 30 putative lipoproteins, with prolipoprotein signal peptidase recognition sequences (105). These so-called lipobox motifs direct the covalent attachment of a diacyl glycerol moiety to the N-terminal Cys residue of the mature protein, anchoring the protein to the outer face of the plasma membrane. Thus, lipoproteins are located beneath the cell wall and the capsule in *S. pneumoniae*. These lipoproteins have diverse functions, the most common being to serve as substrate-binding components of ATP-binding cassette (ABC) transport systems, and many are important for the growth and survival of the pneumococcus *in vitro* and *in vivo*. Their cellular location suggests that they are not exposed on the cell surface to any significant extent, which in turn implies that they are unlikely to elicit opsonic antibodies. However, this does not necessarily preclude their utility as vaccine targets, since exogenous antibody may diffuse through the capsule and cell wall layers and inhibit the biological function of the lipoprotein. Indeed, several pneumococcal lipoproteins have been shown to have potential as vaccine antigens, as discussed below.

### Pneumococcal Surface Antigen A

Pneumococcal surface antigen A (PsaA) is a highly conserved 37-kDa lipoprotein produced by all pneumococci. It was initially thought to be an adhesin based on sequence homology with putative lipoprotein adhesins of oral streptococci, but it is actually the binding component of an Mn<sup>2+</sup>-specific ABC transport system (30). Defined *psaA*-negative mutants of *S. pneumoniae* are virtually avirulent for mice and exhibit markedly reduced adherence *in vitro* to human type II pneumocytes (11, 69). This defect is presumed to be a consequence of growth retardation due to an inability to scavenge Mn<sup>2+</sup> *in vivo*, as well as pleiotropic effects on the expression of a range of cellular processes or virulence

factors. Intracellular Mn<sup>2+</sup> appears to play a critical role in the regulation of the expression of oxidative stress response enzymes and intracellular redox homeostasis, and *psaA*-negative pneumococci exhibit hypersensitivity to superoxide and hydrogen peroxide (70, 108).

One study has shown that parenteral immunization of mice with purified PsaA in the presence of strong adjuvants elicits significant protection against systemic challenge with *S. pneumoniae* (104). However, in other studies, immunization with PsaA elicited only marginal protection and was less efficacious than pneumolysin in an intraperitoneal challenge model (33, 79). The dimensions of PsaA (approximately 7 nm at its longest axis) (54) are such that if it is indeed anchored to the outer face of the cell membrane via its N-terminal lipid moiety, it is unlikely to be exposed on the outer surface of the pneumococcus. This deduction is consistent with the fact that, whereas the known surface-exposed domains of PspA and PspC are variable, the amino acid sequence of PsaA is highly conserved (97). Gor et al. (34) used flow cytometry to compare the surface accessibility of PsaA and that of PspA in 12 *S. pneumoniae* strains to exogenous specific antibodies. PspA was readily detectable on the surfaces of all strains, whereas PsaA was not. This finding correlated directly with the protective efficacy of either active or passive immunization with the respective protein or antibody; significant protection against systemic challenge was achieved using PspA or anti-PspA, but not using PsaA or anti-PsaA. Given the virtual absence of surface exposure, any protection elicited by immunization with PsaA is unlikely to be a consequence of enhanced opsonophagocytic clearance. Rather, it is presumably due to the *in vivo* blockade of ion transport, which necessitates the diffusion of antibody through the capsule and cell wall layers. Such penetration of antibody is likely to be concentration dependent, and so high anti-PsaA titers may be required for protection. Moreover, the accessibility of PsaA to exogenous antibody may well be influenced by the thickness of the capsule, which may vary from strain to strain. The expression of pneumococcal capsule biosynthesis genes has also been shown to be up-regulated during invasive infection (81). In contrast, pneumococci colonizing the nasopharynx are thought to down-regulate capsule expression, thereby facilitating interaction between surface adhesins and the host mucosa. Consistent with this hypothesis, several studies have shown that the intranasal immunization of mice with PsaA in the presence of strong mucosal adjuvants such as CTB significantly reduces the level of nasopharyngeal carriage of *S. pneumoniae* (16, 29). A lesser, but still significant, reduction in susceptibility to carriage was also

achieved by the subcutaneous immunization of mice with synthetic lipidated multiantigenic PsaA peptides (48). Thus, at least in the nasopharynx, PsaA appears to be accessible to exogenous antibody. Importantly, a recent study has shown that immunization with a PsaA-CTB fusion protein significantly reduces the carriage of *S. pneumoniae* in mice without significantly disturbing the oropharyngeal microflora (92).

#### Iron Transporter Lipoproteins PiuA and PiaA

Two other metal-binding lipoproteins have been proposed as pneumococcal vaccine antigens. These proteins, designated PiuA and PiaA, are components of two separate ABC iron transport systems. At least one of these proteins is required for the optimal growth of pneumococci in iron-depleted media, and they are capable of acquiring iron from hemoglobin (21). Indeed, PiuA has been shown to be capable of directly binding both hemin and hemoglobin (102). PiuA and PiaA are produced by all pneumococci and their genes are highly conserved (49). Mutagenesis studies using both lung and intraperitoneal models of infection have shown that both proteins contribute to virulence in mice (21). They are immunologically cross-reactive, and the immunization of mice with either protein confers a degree of protection against intraperitoneal challenge similar to that elicited by the pneumolysin PdB. Moreover, immunization with a combination of PiuA and PiaA results in additive protection (22). Although a direct comparison has not been conducted, immunization with either PiuA or PiaA provides a higher degree of protection against systemic disease than that previously reported for PsaA in the same mouse model and with the same *S. pneumoniae* challenge strain (79). Like PsaA, PiuA and PiaA are predicted to be attached to the outer face of the plasma membrane (102), and so the superior protective efficacy of the latter proteins ought not to be due to a difference in accessibility to exogenous antibody. However, Jomaa et al. (49) have shown by flow cytometry that both PiaA and PiuA are accessible to exogenous antibody in intact bacteria and that these antibodies stimulate *in vitro* opsonophagocytic activity, particularly in the presence of complement. They also reported that the antibodies did not appear to interfere with iron uptake *in vitro*. Recently, mucosal immunization with PiuA and PiaA has been shown to elicit antibody responses both in serum and in respiratory secretions, which protected mice against intranasal challenge (50). The reason for the apparent difference in surface accessibility between PsaA and the two iron-binding lipoproteins is unclear, given their predicted location. One possibility is that in the latter two cases, at least a proportion of the proteins are

released from the membrane and are then able to bind to more exposed domains on the pneumococcal surface, where they can interact with exogenous antibody more freely. Regardless of the underlying mechanism, available data suggest that PiaA and PiuA have more promise than PsaA as vaccine antigens, at least for the prevention of systemic disease.

### Pneumococcal Histidine Triad Proteins

The pneumococcal histidine triad proteins are a recently recognized family of surface proteins that have an unusual polyhistidine motif, HXXHXH, repeated five or six times in their amino acid sequences. The prototype, PhtA, was discovered as part of a genome-wide screen for potential vaccine antigens (112). Over 100 proteins were expressed and tested for efficacy in a mouse model, and PhtA was one of only five that were protective. The others were the CBPs LytB and LytC (discussed previously), the cell wall-associated serine protease PrtA, and another protein of unknown function designated PvaA (112). Further examination of the pneumococcal genome sequence revealed three additional related open reading frames encoding proteins designated PhtB, PhtD, and PhtE, each with five or six copies of the histidine triad motif. The four proteins range in size from 91 to 114 kDa and are closely related at the amino acid sequence level, exhibiting 32 to 87% identity; this similarity is strongest in the N-terminal regions (1). Although their signal peptides all contain an LXxC motif, this motif does not appear to function as a true lipobox, as it is not labeled by [<sup>3</sup>H]palmitate (37). Thus, the manner in which the Pht proteins are attached to the pneumococcal cell surface and the precise site of attachment is uncertain. Nevertheless, flow cytometric analyses have shown that the C-terminal regions are more readily accessible than the N-terminal regions to exogenous antibodies (1, 37), suggesting that the proteins are tethered via their N termini. Although the histidine triads are believed to form a novel Zn<sup>2+</sup>-binding motif (94), the biological function of the Pht proteins remains unknown.

There is a high degree of protein sequence conservation among individual Pht proteins from diverse *S. pneumoniae* serotypes (37), which, combined with a degree of immunological cross-reactivity between the proteins, augers well for broad strain coverage by these candidate vaccine antigens. Immunization with purified PhtA, PhtB, or PhtD has been shown to confer significant protection against intraperitoneal challenge with type 3, 6A, 6B, and one of two type 4 *S. pneumoniae* strains (1, 112). PhtD has also been shown to protect against intranasal challenge with a type 3 strain (117),

while immunization with either PhtB or PhtE also protects against type 3 pneumococci in models of sepsis and pneumonia (37). In the latter study, immunization experiments with truncated PhtE fragments localized the protective epitopes to the more surface-exposed C-terminal region of the molecule. However, notwithstanding these promising results, the only direct comparative studies of the protective efficacy of Pht proteins with those of other well-characterized antigens indicate that the level of protection elicited is no better than that achieved by either PspA or pneumolysin (83, 112).

### Sortase-Dependent Surface Proteins and Other Vaccine Candidates

Sortase-dependent surface proteins of gram-positive bacteria are identifiable by the presence of a C-terminal anchoring motif, which consists of a conserved LPXTG sequence followed by a hydrophobic domain and usually a tail of positively charged residues. This motif is recognized by sortase, a membrane-localized cysteine protease, which cleaves between the T and G residues and covalently links the processed protein to the peptidoglycan cross bridges (99). In pneumococci, the inactivation of the sortase gene releases known sortase-dependent surface proteins, such as the major pneumococcal neuraminidase NanA, and reduces adherence to pharyngeal cells (51). NanA-deficient mutants of *S. pneumoniae* have been shown to have a reduced capacity to colonize the upper and lower respiratory tracts of mice (69, 84) and the nasopharynxes and middle ear clefts of chinchillas (106). An early study indicated that purified NanA had modest but significant protective efficacy (weaker than that of pneumolysin) in a mouse sepsis model (58). More recently, it has been shown to be protective against both carriage and otitis media in chinchillas (61, 107).

Other sortase-dependent pneumococcal surface proteins have been proposed as vaccine candidates, including hyaluronidase (Hyl) and the IgA1 protease (Iga). The latter is of interest, as its sortase motif is located in the N-terminal region of the molecule but is nevertheless essential for proper function and surface localization (5). However, although these proteins have been shown to contribute to pathogenesis using either *in vitro* or *in vivo* models (12, 111), Hyl is at best a weak protective antigen (90), while Iga is yet to be tested for protective efficacy.

A number of other apparently surface-associated pneumococcal proteins with at least theoretical vaccine potential have been identified, in most cases by using immunoproteomic approaches. This list includes proteins that lack export or anchorage signals and would

be predicted to be cytoplasmic. Examples include metabolic enzymes such as enolase (which also binds plasminogen) (6), 6-phosphogluconate dehydrogenase (a putative adhesin) (27), and fructose-biphosphate aldolase and glyceraldehyde-3-phosphate dehydrogenase (56), as well as the heat shock protease ClpP (53). Another candidate is the putative proteinase maturation protein PpmA (85), although its degrees of surface exposure and protective efficacy have been questioned (34).

## COMBINATION PROTEIN VACCINES

Virtually all of the pneumococcal proteins under consideration as vaccine antigens are directly or indirectly involved in the pathogenesis of pneumococcal disease. The mutagenesis of some combinations of virulence factor genes, for example, those encoding pneumolysin, PspA, and PspC, has been shown to synergistically attenuate pneumococcal virulence in animal models, implying that the respective proteins function independently in the pathogenic process (12, 83). This finding strongly suggests that immunization with combinations of these antigens may provide additive protection. Moreover, there may be differences in the relative protective capacities of the individual antigens against particular *S. pneumoniae* strains, particularly for surface-exposed antigens that exhibit some degree of sequence variation. Thus, a combined pneumococcal protein vaccine may elicit a higher degree of protection against a wider variety of strains than any single antigen. To date, only a limited number of combination experiments have been performed. Nevertheless, the immunization of mice with various combinations of pneumolysin, PspA, and PspC provides significantly increased protection against sepsis and pneumonia than immunization with any of the proteins on their own, whereas combinations involving alternative immunogens such as PsaA and PhtB or PhtE are less effective (18, 79, 82). On the other hand, intranasal immunization with a combination of PspA and PsaA is more effective than either antigen alone in preventing carriage (16). Clearly, additional comparative studies of the protective efficacy of the better-characterized proteins, as well as the more recently identified vaccine candidates (both singly and in combination), are required to enable informed decisions on the formulation of a protein-based pneumococcal vaccine.

Consideration should also be given to using protein antigens as alternative carriers in PS-protein conjugate vaccines, and conjugates of pneumolysin with type 19F PS have been shown to be highly immunogenic and protective in mice (55, 88). In a more recent study,

pneumolysin was shown to be a very effective carrier protein in a tetravalent conjugate vaccine formulation including PS types 6B, 14, 19F, and 23F (74). The use of pneumolysin, or other suitable pneumococcal proteins, as carriers for PS in conjugate vaccines may also minimize any problems associated with the overuse of existing carrier proteins.

## KILLED WHOLE-CELL VACCINES

A candidate pneumococcal whole-cell vaccine (WCV) is in the early stages of development. WCV is made from ethanol-inactivated mutant *S. pneumoniae* whole cells which have been genetically modified to be unencapsulated (to better expose the noncapsular antigens) and autolysin defective (to increase growth density) and to express pneumolysin, not pneumolysin. When given intranasally or orally to laboratory animals with a mucosal adjuvant, WCV reduces nasopharyngeal colonization, middle-ear infection, and fatal pneumonia caused by encapsulated pneumococci of all tested serotypes (64, 66). WCV induces immunity directed to a variety of conserved pneumococcal antigens and has the potential to be efficacious worldwide.

Research suggests that WCV may function through a novel immune mechanism of protection. Studies showed that WCV provided protection against colonization in MuMT mice (congenitally unable to make antibody) but no protection in nude mice lacking T cells. Further studies showed that immunity could be induced in the absence of CD<sup>8+</sup>, but not CD<sup>4+</sup>, T cells (67). Earlier studies showed that WCV induces serum antibody, which protected mice from invasive disease when administered passively prior to challenge (64). Thus, WCV may induce two tiers of protection: a novel T-cell-mediated reduction in colonization and antibody-mediated immunity against invasive disease.

A potentially attractive characteristic of WCV is its particulate configuration. Studies have demonstrated that bacterium-derived particles are more immunogenic at the mucosal surface than soluble antigens and less likely to be tolerogenic due to a copresentation of antigens and molecules that engage TLRs of the innate immune system (13). WCV engages TLR2 and TLR4 and appears to be far more potent in preventing colonization than another known protective antigen—the cell wall PS. The cell wall PS induces a type of protection similar to that induced by the WCV when given intranasally in purified form (68).

The development of a WCV goes against the current trend in vaccinology toward the selection of well-characterized protective components that can be standard-

ized by chemical and physical criteria. However, if the vaccine is demonstrated to be safe and effective, WCV would have very attractive attributes, especially for manufacture and use in developing-world settings. The vaccine could be made inexpensively from cells of a single strain and could readily be made by developing-world manufacturers. It is likely that WCV could be freeze-dried and stored without refrigeration, thereby avoiding cold-chain expense. Finally, WCV would be administered to nonsterile sites, avoiding costs associated with sterile needles and syringes.

### CONSIDERATIONS FOR THE CLINICAL DEVELOPMENT OF A COMMON-PROTEIN VACCINE

The major target populations for a pneumococcal common-protein vaccine are infants and young children and the elderly. Clinical development could be directed at both target populations simultaneously or sequentially. For either target population, a Phase 1 clinical study to evaluate the safety and immunogenicity of the common-protein vaccine in healthy adults would initiate the development process.

For the infant and young children indication, the second study would evaluate the vaccine in toddlers to confirm the safety and immunogenicity. Stepping down to infants, a Phase 1/2 study would be conducted to evaluate safety and immunogenicity across several vaccine dosage concentrations. The vaccine regimen would be expected to consist of three to four injections on a 6-, 10-, 14-, and 52-wk or similar schedule. The common-protein vaccine would be administered concomitantly with standard infant vaccines (e.g., the diphtheria-pertussis-tetanus vaccine), and evaluations would be needed to ensure that the common-protein vaccine does not interfere with immune responses to the standard vaccines, especially the *Haemophilus influenzae* type b vaccine. Because the incidence of pneumococcal colonization in young infants is very high, an evaluation of the impact of the vaccine on the acquisition of pneumococcal carriage could be done in early studies. While the absence of an impact on carriage should not halt the development of a common-protein vaccine, a moderate or large impact on carriage would be a very encouraging observation and suggest a higher probability of vaccine effectiveness. During early-phase studies, it would be very important to establish and validate serologic assays, including functional assays, to improve the assessment of the vaccine performance and, potentially, to identify serologic correlates of protection in Phase 3 studies.

Since the common-protein vaccine would have a different mechanism of action from the capsular PS-based vaccines, a large Phase 3 clinical end point study should be conducted. Since a Phase 3 study of a common protein vaccine is many years away, it is likely that PCVs will be in use in many locations, thereby requiring the use of a PCV as an active control vaccine. Alternatively, a PCV could be administered to both treatment groups. In either case, the main study end point would be pneumococcal disease caused by serotypes not contained in the PCV. A secondary end point would be pneumococcal disease caused by any serotype. As for the specific pneumococcal disease end point, options include radiologically confirmed pneumonia, clinical pneumonia, and invasive pneumococcal disease. Regardless of the disease end point, it will be critical to develop sensitive and specific diagnostic tools such as PCR and/or antigen detection technologies in order to confirm that *S. pneumoniae* is the etiologic agent and to determine the serotype, since the primary analysis will be conducted in cases of pneumococcal disease caused by serotypes not contained in the vaccine. The sample size for such a trial will depend upon the attack rates of pneumococcal disease caused by serotypes not contained in the vaccine in the study area, but the trial is likely to be very large and complex. Several years of effort will be needed to adequately prepare to conduct the study.

Clinical development for an elderly adult indication will be similar to that for infants, to include dose ranging and evaluations of various vaccination regimens. It is likely that an adjuvant will be necessary and that the vaccine regimen will require two or three injections in order to maximize the immune responses. A large-scale efficacy study will likely be required by regulatory agencies, even if the vaccine is shown to be effective in infants and children. That requirement will present a huge financial and logistical obstacle since the attack rates for pneumococcal disease in the elderly are lower than those in infants and young children. Furthermore, since PPSV23 would be the likely control vaccine, the main study end point would need considerable debate. It is unlikely to be pneumococcal disease caused by serotypes not in PPSV23, since so few such cases would be expected. Instead, it would likely be a comparison to PPSV23.

### SUMMARY

The ongoing high global morbidity and mortality associated with pneumococcal disease and the complications caused by increasing rates of resistance to antimicrobials have underpinned extensive efforts in recent

years to develop more effective vaccination strategies against *S. pneumoniae*. These efforts have benefited from a better understanding of the mechanisms of pathogenesis of pneumococcal disease and the advances made possible by the advent of recombinant-DNA technology and access to genome sequence data. The polyvalent PS vaccines have no doubt prevented a large number of deaths from invasive disease among recipients belonging to those patient groups for whom this vaccine is currently recommended. The newer PS-protein conjugate formulations will also confer a very high degree of protection against the included serotypes on young children and may also have an impact on the prevalence of drug-resistant strains. However, there is now general acceptance that this vaccination approach is not without its drawbacks, and as explained above, the initially substantial clinical benefits that are expected to be derived from the widespread use of conjugate vaccines may diminish with time. It will take many years for the overall impact of conjugate vaccines on disease burden and the population biology of *S. pneumoniae* to become fully apparent. At the very least, the use of the conjugate vaccines will buy time for the development of cheaper, non-serotype-specific vaccines based on combinations of protein antigens.

The studies referred to in this chapter have shown that there are a number of pneumococcal proteins that exhibit potential as vaccine antigens. However, the assessments of their protective efficacy have been carried out in different laboratories using a variety of animal models and challenge strains. Clearly, a comprehensive series of directly comparative protection studies needs to be performed in order to determine which of these proteins provides the strongest protection against the widest variety of *S. pneumoniae* strains.

It must be emphasized, however, that the success of these protein vaccines is not dependent upon real or perceived failure of the conjugates. Rather, the two approaches should be viewed as complementary, each having an important role to play in the global prevention of pneumococcal disease. Nor should the development of parenteral protein vaccines impede future research on mucosa- or DNA-based delivery systems, which may further improve the presentation of protective antigens to the immune system, thereby optimizing host responses.

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## *Conclusions*

Since its discovery in 1875, the pneumococcus has played an important role in the development of the biosciences, particularly our knowledge of microbial pathogenesis, genetics, immunology, and vaccinology (see chapters 1 and 2). Strain-specific immunity was first demonstrated in 1885 by Fraenkel (2), and agglutinating antibodies were discovered in 1891 by Metchnikoff (7). That phagocytic cells were critical for host defense was shown by Gamaleia in 1888 (3); that immune serum promotes phagocytosis was revealed by Issaeff in 1893 (5); and that immune serum could protect rabbits against challenge was demonstrated by the Klemperers in 1891 (6). The basic elements of our current knowledge of host defense against pneumococci were thus already in place more than 100 years ago.

The major protective antigens of pneumococci, termed soluble specific substances, were shown to be polysaccharides by Heidelberger and Avery in 1923 (4). The discovery of the ability of pneumococci to pass the hereditary capacity to make a specific capsular polysaccharide from one strain to another, through the process of transformation, ultimately provided the tools which enabled Avery, MacLeod, and McCarty to show in 1944 that DNA was the transforming principle (1), i.e., the chemical matter of genes. This observation laid the foundation of modern genetics.

The discovery that capsular polysaccharides are the major virulence factor and protective antigen of pneumococci was the basis for two major immunologic approaches for the treatment and prevention of disease. Serum therapy used in the 1920s and 1930s significantly improved the outcomes of serious pneumococcal infections. Vaccines, first based on the whole bacteria and later on the capsular polysaccharide, showed promise in prevention (see chapters 1 and 2). Both approaches were, however, abandoned when antibiotics became widely available in the 1940s, and it was not recognized until the 1960s that antibiotics had much less impact on invasive pneumococcal disease (IPD) than initially expected. Polysaccharide vaccines were then “rediscovered” and licensed, but only for individuals

older than 2 years of age. Until the introduction of a pneumococcal conjugate vaccine (PCV) in the first decade of the new millennium, none of these interventions had a dramatic impact on this extraordinarily adaptable microbe.

This book provides a comprehensive description of the immunology, clinical efficacy, and public health impact of the PCV since its introduction in 2000. What has become clear is that the PCV has dramatically reduced the incidence of vaccine-type IPD in the immunized population. Efficacy is most dramatic for IPD but is also significant for pneumonia and otitis media. The vaccine has been demonstrated to reduce all-cause pneumonia, hospitalization, and mortality in a rural, developing-country setting. In the developed world, the vaccine has reduced otitis media and antibiotic prescriptions for respiratory diseases. The reduction in pneumococcal colonization in the target population in the United States has also resulted in a secondary reduction of transmission of pneumococci to unimmunized individuals and high levels of protection through herd immunity. These findings have confirmed indirect epidemiologic evidence that young children are the main vectors spreading pneumococci in the population.

The efficacy studies of the PCV have also served as probes to determine the incidence of pneumococcal infections that are otherwise undiagnosable clinically. Pneumococci have been found to contribute significantly to the global burden of otitis media and pneumonia (see chapters 20 and 22).

So will PCVs eliminate the enormous morbidity and mortality caused by this organism globally and make pneumococcal infections of only historical interest? Based on the past performance of *Streptococcus pneumoniae* when put under selective pressure from factors such as antibiotics, it is difficult to feel sanguine that we have won the battle.

Already, after 7 years of widespread use of the PCV, the numbers of nonvaccine types causing IPD, otitis, and colonization are rising. The replacement phenomenon is especially marked among populations at very high risk for infection, such as Alaskan natives and those with human immunodeficiency virus infection, in whom the net impact of the seven-valent PCV conjugated to the mutant diphtheria toxin CRM has been blunted by the emergence of nonvaccine strains. For the vast majority of the population, however, replacement strains represent a very small proportion of pneumococci causing disease compared to the vaccine types that were eliminated by the PCV. However, over time, replacement could become more prominent in all populations if nonvaccine types can gradually acquire the transmission and virulence characteristics of the current vaccine types. Some researchers, including Bob Austrian, to whom this book is dedicated, believe that the virulence of pneumococci is largely dependent on the biologic properties of the capsular polysaccharides of these organisms and that, therefore, very few strains other than those currently known to be virulent in humans are likely to become major human pathogens in the future.

Given the current situation, what are some of the major challenges for the pneumococcal vaccine field?

- The first and foremost is to devise systems for introducing the PCVs more quickly into populations who will benefit the most: children in developing countries in Africa and Asia and the emerging countries in South America, China, India, and the former USSR. This challenge is addressed in chapter 27.
- The second is to broaden the spectrum of serotype coverage by the current PCV to provide a higher level of global serotype coverage and to do so at costs that will support a global vaccine program.

- A third would be to develop new vaccines which would close the gaps in coverage by PCVs. One such approach is the protein-based vaccines summarized in chapter 28, but the challenges of developing such vaccines in the face of highly effective PCVs are considerable. There is a glimmer of hope that novel avenues inducing T-cell-mediated resistance to colonization may provide another totally different approach to a pneumococcal vaccine.
- A fourth would be the development of an improved vaccine for elderly and other high-risk patients. The current 23-valent polysaccharide vaccine is effective against IPD, but efficacy wanes gradually after 5 years, and repeated immunization is not generally recommended because of concerns about hyporesponsiveness and reactogenicity (see chapter 17).

If one considers the historical perspective, which has shown that pneumococci will respond by adaptation and evolution to immune pressure, given enough time, then we may consider developing vaccines which minimize that selective pressure. This approach may mean, counterintuitively, a vaccine which does not affect nasopharyngeal carriage but protects only systemically. Obvious drawbacks are the likely loss of herd immunity (which could be compensated for by immunizing all at-risk individuals) and a likely reduced efficacy for acute otitis media and perhaps pneumonia. Whether such vaccines are even feasible is uncertain. The alternative, a vaccine directed specifically against carriage, may lead to replacement carriage of other potential human pathogens.

In any event, it will be of critical importance to continually monitor pneumococcal disease rates and the population structure of this pathogen as broader-spectrum vaccines are introduced, with particular attention to the emergence of non-vaccine-type disease, the emergence of other pathogens, and the impact on antibiotic resistance.

GEORGE R. SIBER  
KEITH P. KLUGMAN  
P. HELENA MÄKELÄ

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