

Andrew W. Artenstein  
*Editor*

# VACCINES

## A Biography



# Vaccines: A Biography

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Editor

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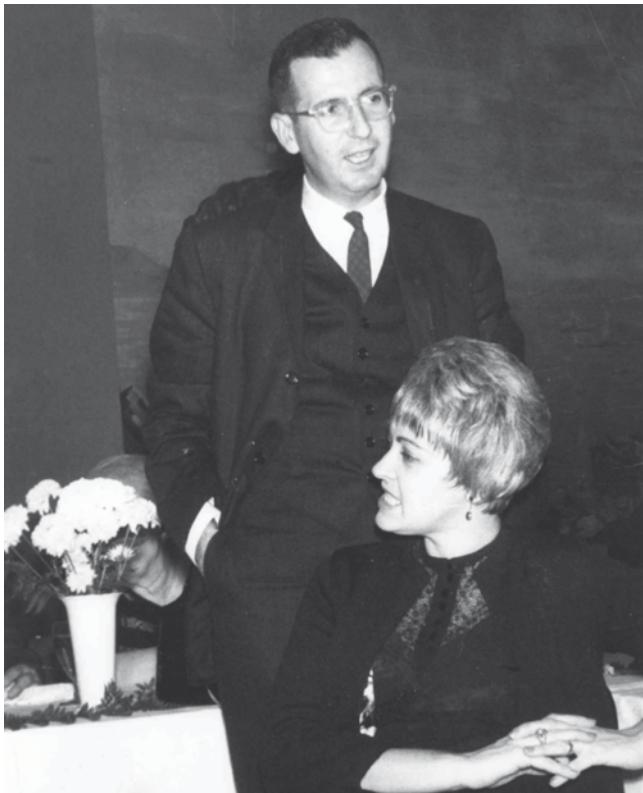
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*To my parents, Malcolm (1930–1976)  
and Sylvia (1933–2007), who inspired  
me in countless ways. They made the story  
personal.*



# Preface

Why another book about vaccines? There are already a few extremely well-written medical textbooks that provide comprehensive, state-of-the-art technical reviews regarding vaccine science. Additionally, in the past decade alone, a number of engrossing, provocative books have been published on various related issues ranging from vaccines against specific diseases to vaccine safety and policy. Yet there remains a significant gap in the literature – the history of vaccines.

*Vaccines: A Biography* seeks to fill a void in the extant literature by focusing on the history of vaccines and in so doing, recounts the social, cultural, and scientific history of vaccines; it places them within their natural, historical context. The book traces the lineage – the “biography” – of individual vaccines, originating with deeply rooted medical problems and evolving to an eventual conclusion. Nonetheless, these are not “biographies” in the traditional sense; they do not trace an individual’s growth and development. Instead, they follow an idea as it is conceived and developed, through the contributions of many. These are epic stories of discovery, of risk-takers, of individuals advancing medical science, in the words of the famous physical scientist Isaac Newton, “by standing on the shoulders of giants.” One grant reviewer described the book’s concept as “triumphant”; although meant as an indictment, this is only partially inaccurate. What in medicine could be more triumphant than conquering disease?

A prominent theme woven throughout the book is the interdependence of incremental scientific advances and investigators on one another and how these ultimately led to practical, preventive solutions to major public health problems in society. The book is nearly chronological in its approach to this history. Each chapter is written to stand independently, yet those who read it from cover to cover will discover that despite its broad scope, it is the “smallness” of the world of vaccine science and the inter-relatedness of its themes and characters that fascinates. The book is organized such that anchoring chapters are interspersed throughout; their purpose is to essentially introduce eras, reflecting the way in which I have chosen to present this biographical history. Smallpox represents a disease-specific chapter and an anchor chapter as well, because it served as the sentinel moment – the starting point – from which all vaccine science is measured. From there, vaccines developed in clusters proximate to major scientific developments. The evolution of microbiology and immunology as distinct sciences in the nineteenth century paved

the way for the first productive period of vaccines in a manner analogous to what the discovery of viruses and subsequently tissue culture methods meant for the fruitful vaccine period of the latter half of the twentieth century. The book ends with its final anchor chapter, one meant to provide a foundation for what may be the next surge in vaccine science related to molecular and genomic medicine.

Why another book about vaccines now? There are, to be fair, two forces that have acted in synergistic fashion and driven me to write this book at this time. First, it is a subject about which I am passionate; it is, literally and figuratively, in my blood. I find the histories inspirational yet humbling, fascinating yet at times tragic. They have all the trappings of fiction: strong protagonists who succeed against sometimes great odds, interpersonal conflicts, deceit, political intrigue, ethical dilemmas, and dramatic, if not staged, events. They are set in the major centers of Europe and the United States, on farms and in slums, and in exotic venues from Calcutta to French Indochina to Cairo to Panama. They occur in the halls of academia, the chambers of government, and on the battlefields of war.

The other, compelling motive to pursue this project at this juncture is that many of the vaccine biographies detailed in this book describe events that occurred in the recent past; many of those intimately involved in these histories are still with us, some are still actively contributing to the field of vaccinology; many have contributed chapters to this work. Of course, many of the pioneers are gone, although in some cases quite recently. I see *Vaccines: A Biography* as an appropriate way in which to honor each of them and pay tribute to their efforts to improve the lot of humankind.

As with any such project of this scope and magnitude, success depends on the help of a dedicated staff and colleagues who are committed to excellence. The individual chapter authors have produced truly outstanding biographical histories – many of these individuals devoted much of their professional lives to their subjects and were major contributors to the vaccines of which they write, circumstances that are transparent upon reading their work. I am indebted to them for endeavoring to produce an accurate, thoroughly readable, historical record of these stories. Margo Katz coordinated the project, and Kathy Bolleson provided reliable and constant administrative assistance; both once again showed their mettle through their devotion to its successful completion. I am fortunate to work with such excellent people. Dr. David Greer, Dean Emeritus of Brown Medical School and a close personal friend, colleague, and mentor, carefully reviewed the manuscript and provided valuable insights that improved the work. I am grateful for his guiding presence. My wife Debbie, the love of my life, and my sons, Nick and Sam, provided a constant source of support and listened as these stories came to life. I hope that those who read this work learn as much and enjoy it as much as I did in writing and editing it.

Providence RI

Andrew W. Artenstein

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# Chapter 1

## Vaccinology in Context: The Historical Burden of Infectious Diseases

Andrew W. Artenstein

*As a rule, the scientist takes off from the manifold observations of his predecessors....The one who places the last stone and steps across to the terra firma of accomplished discovery gets all the credit...*

John Enders

In a 1977 article summarizing 40 years of his involvement in vaccine research, Jonas Salk, the renowned and controversial force behind the first effective polio vaccine, coined the term “vaccinology” to comprehensively describe his chosen field (Salk and Salk 1977). The term is meant to encompass the broad areas of discipline requisite for the science of vaccines: microbiology, epidemiology, pathogenesis, and immunology. In defining this novel branch of science, Salk recognized that vaccinology formed a nexus between medicine, public health, sociology, and biochemistry. He understood the rich history of scientific accomplishments that defined the field and formed an inextricable link with the past.

Arguably, the concept and practice of vaccination against infectious diseases has resulted in greater benefits to human health than any other cultural, social, or scientific advance in the history of humanity. As a testament to their historical importance, vaccines were ranked first among the ten greatest public health achievements of the twentieth century (Centers for Disease Control and Prevention 1999). Through their use, scourges of nature have been eradicated, controlled, or rendered irrelevant, and generations of children have survived into adulthood, unscathed by diseases that would have been lethal earlier in history. Vaccines harnessed the human immune system to its fullest extent long before the fundamental tenets of immunology were described; the concepts that form the basis of vaccine science have since been extended to a plethora of infectious and noninfectious diseases.

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The formal history of vaccination, from a scientific standpoint, traditionally dates from Edward Jenner's landmark experiments with cowpox in 1796, although it would be nearly a century before the practice received its name, an honor bestowed posthumously upon Jenner by its most celebrated scientific proponent, Louis Pasteur (Moulin 1996). Jenner inoculated a neighbor's boy with purulent material from a milkmaid's hand lesion in Berkeley, England (Moloo and Artenstein 2008). The boy, 8-year-old James Phipps, was subsequently shown to be protected against a smallpox challenge. Jenner followed this initial experiment with a systematic study of the protective effect of cowpox on variola. The related concept that humans could be protected against disease by intentionally exposing them to the supposed cause of the malady probably arose many centuries before Jenner, although this remains poorly documented.

According to legend, Mithridates VI, King of Pontus on the Black Sea in Asia Minor from 120 to 63 BC, ingested daily, sublethal doses of poison in order to build his tolerance to such agents (Parish 1965). Although this behavior was presumably motivated by suspicion bordering on paranoia of his impending assassination, it may have been warranted; his mother killed his brothers and may have assassinated their father, King Mithridates V, in her attempt to usurp the throne (Marsh and Scullard 1953). It did not help matters that he was also a formidable enemy and thus a target of the Roman Empire. But the strategy may have worked; when he was eventually defeated by Pompey and "wished to die by poison, he was unable," most likely due to acquired resistance (Justinus 1853). Mithridates' concoction of plant oils and resins became the basis for the universal antidotes Mithridatium and Theriac (Griffin 2004; Norton 2006).

Various other forms of vaccination were practiced throughout pre-Jennerian history. Buddhists in India in the seventh century supposedly ingested snake venom to protect themselves from its fatal effects (Plotkin and Plotkin 2008). At least four methods of variolation were probably in use in China in the sixteenth century: placing cotton instilled with either pus or scabs from lesions in the nostrils of healthy children; blowing powdered scabs into the nostrils using a thin silver tube; and dressing healthy children in clothing worn by smallpox-infected individuals (Leung 1996). In the midst of a measles epidemic in Edinburgh in 1758, Scottish physician Francis Home attempted to inoculate healthy individuals with skin lesion material from infected individuals. Using a mixture of blood and affected skin, he inoculated a small group of children, resulting in clinically attenuated illness and protection against wild type measles (Enders 1964).

These early forays into vaccination were based on empiricism and practical realities. Jenner and his immediate predecessors also appear to have based their theories on empiric observations from nature. The common denominator was that their observations were supported by a substantial experiential tradition. Such "rational empiricism" (Hilleman 2000) was likely born from generations of struggles against epidemic and endemic infectious diseases.

Epidemic infectious diseases in ancient cultures were believed to be of divine etiology (Conrad et al. 1995; Brier 2004). Many had a profound effect upon civilizations. Ancient Greek hegemony never recovered from the devastation wrought by the plague of Athens that began in 430 BC, early in the Peloponnesian War, and was

caused by measles or perhaps another highly transmissible infectious disease (Cunha 2004). The Antonine plague of 165–169 AD, probably due to smallpox, originated in the eastern reaches of the Roman Empire (modern-day Iraq) before it became a pandemic; it played a significant role in the inexorable decline of that superpower (Fears 2004).

Recurring pandemics and sporadic, catastrophic, focal outbreaks of endemic infectious diseases have played important roles in shaping the course of human history (Zinsser 1934; Diamond 1997; Cantor 2002; Trevisanato 2004). The Justinian plague of 541–544 AD was just the opening salvo in 11 bubonic and pneumonic plague epidemics that disseminated and resurged in cycles throughout the known world of that time over a period of 200 years (Conrad et al. 1995; Asad and Artenstein 2009). It has been estimated that up to 50% of the population perished, contributing to major sociopolitical changes in the Byzantine Empire and leading Europe into the Middle Ages (Drancourt and Raoult 2002).

Plague, in the form of “The Black Death,” arrived again in Sicily in 1347 via the trade routes from Asia, devastating the population of Europe and likely changing the course of history through its impact on geopolitics, the balance of military power, medieval economics, and almost all aspects of daily and cultural life (Diamond 1997; Cantor 2002). The impact of the epidemic in Europe may have extended to the human genome, altering the genetic predisposition to future infectious diseases in that population via selective mutational pressures (Galvani and Slatkin 2003).

The intimate, complex relationship between human beings and communicable diseases was a consequence of human social evolution. Early humans operated as small bands of hunter-gatherers; their relatively short life spans were the result of food shortages rather than epidemic infectious diseases (Diamond 1997). Diseases that relied on person-to-person transmission for persistence or amplification would have either been extremely limited in their infectious range by small group size or would have been extinguished along with their hosts. With the advent of food-producing, large, dense, immobile, agricultural societies, conditions were such that epidemics of infectious diseases could be maintained (Diamond 1997). Social urbanization magnified their epidemic potential by facilitating transmission. Thus it is not surprising that infectious diseases such as smallpox, plague, tuberculosis, dysentery, and pneumonia were primarily responsible for the limited life expectancy and death of a significant proportion of the population in early modern Europe (Conrad et al. 1995).

Because communicable diseases were so prevalent, European societies became immunologically experienced in terms of their exposures to many pathogens. Hence, over time many infectious diseases persisted as endemic, sporadic threats that became part of the morbid landscape but with less explosive mortality (Conrad et al. 1995). In contrast, the effect of communicable diseases on immunologically naïve populations was potentially cataclysmic (McNeill 1976). Examples of this phenomenon abound in medical and historical literature. Columbus’ first voyage across the Atlantic in 1492 unleashed Europe’s repertoire of epidemic infectious diseases on the virgin population of the New World, a dynamic that continued with successive Old World incursions into the Americas over the next 150 years.

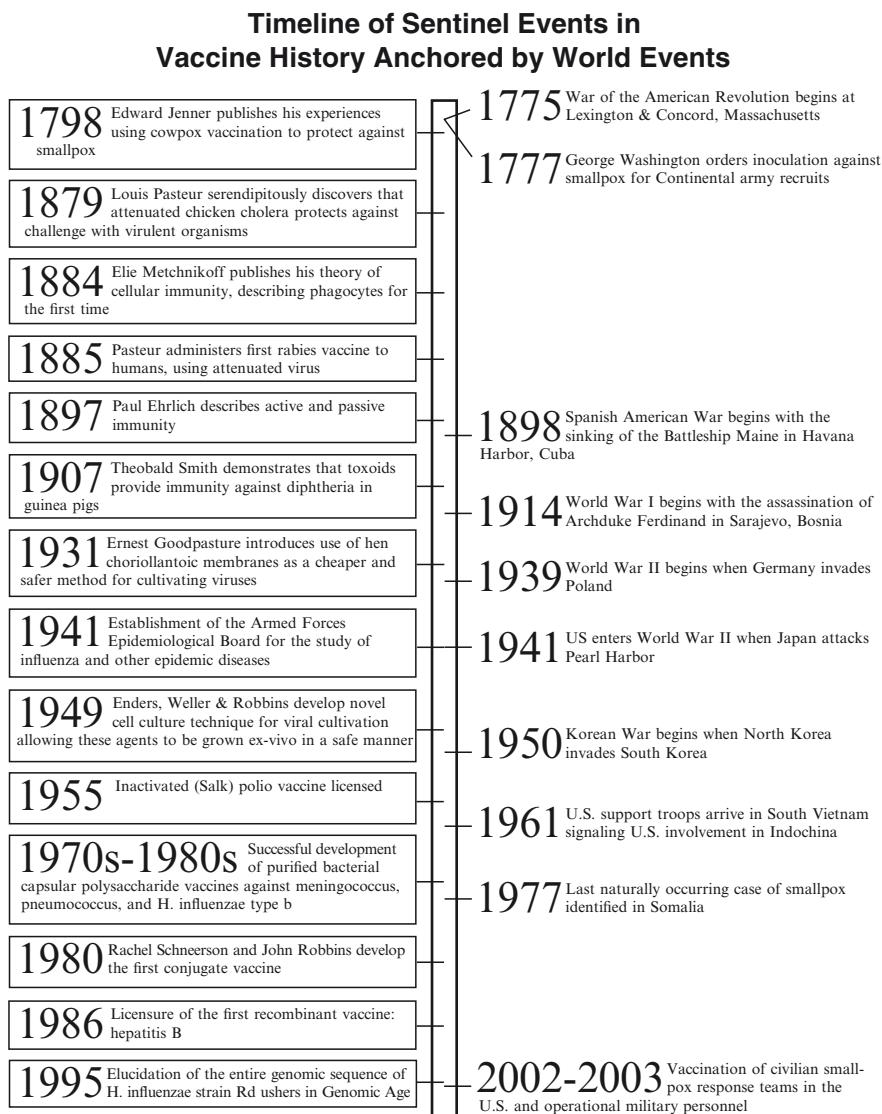
Indigenous populations were decimated; smallpox epidemics ravaged the island of Hispaniola in the first quarter of the sixteenth century, reducing the population by more than 95% (Cook 1998).

Other Native American societies of the Caribbean basin and later Mexico, Guatemala, and Brazil fell victim to additional infections: dysentery, influenza, vivax malaria, and measles among them. With epidemic smallpox, introduced by Spanish forces, rampaging through the Indian population of central Mexico, Hernán Cortés was able to easily subjugate the Aztec Empire with fewer than 500 men in 1521(Hopkins 1983; Cook 1998). His compatriot, Francisco Pizarro, was the beneficiary of a similar result against the Incas in Peru a decade later (Hopkins 1983).

An analogous fate was met by other virginal populations when novel diseases were introduced via friendly or hostile visitors from endemic areas. Yellow fever virus entered the New World through the transatlantic slave trade from Africa (Artenstein et al. 2005); it caused recurrent, highly lethal epidemics in coastal areas of the Americas from the seventeenth century to the early part of the twentieth century. In Philadelphia in 1793, the disease killed approximately 10% of the city's population (Murphy 2003); its decimation of Napoleon's expeditionary forces in Haiti in 1802 convinced the General to abandon his imperial plans for the Americas and to sell the Louisiana Territory to the United States (Artenstein et al. 2005). Yellow fever again made its mark on history in the early twentieth century as it forced the French out of the Panama Canal development process and nearly derailed the American effort there (McCullough 1977). Measles was imported to the isolated, North Atlantic Faroe Islands in 1846 by an infected carpenter and within 6 months, nearly 80% of the population of less than 8,000 had become infected (Panum 1847).

The observations of Jenner and his predecessors were informed by the historical burden of infectious diseases as viewed through the lens of “rational empiricism.” Their vaccinology descendants, beginning with Louis Pasteur in the nineteenth century, contributed to and benefited from major developments in medicine and science (Bynum 1994). Their innovations advanced vaccine research into a distinct science that produced, in numerous instances throughout its history, spectacular results (Chase 1982; Allen 2007).

The history of vaccinology (Fig. 1.1) parallels the history of human scientific endeavor and illustrates an important precept common to all scientific inquiry: major advances generally stem from incremental progress that itself derives from the accumulation of ordered, experimental observations synthesized from a variety of fields. Scientific advances are often non-linear, resulting from shifts in existing paradigms; landmark discoveries generally do not occur in a vacuum but are instead based on expansions of pre-existing scientific thought, sometimes with tumultuous consequences (Kuhn 1996). Additionally, advances in technology inherently drive advances in science, and both are frequently products of specific unmet needs. The history of vaccinology represents the individual and collective stories of inquisitive minds, thought leaders, risk-takers and those that stood “on the shoulders of giants” to improve the health of humanity.



**Fig. 1.1** Vaccine development within the historical context of world events since Jenner (Military Medicine: International Journal of AMSUS, Vol.170, April Supplemental, pp 3-11, reproduced with permission)

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# **Chapter 2**

## **Smallpox**

**Andrew W. Artenstein**

Smallpox represents an appropriate embarkation point for a historical examination of vaccines because this disease was the first against which a scientifically studied vaccine was successfully implemented in humans. During the nearly 90 years between Jenner's systematic experiments with cowpox and Pasteur's clinical use of rabies vaccines, the next to be used in humans, advances in vaccine science derived largely from the expansion of knowledge regarding smallpox vaccination and its beneficial and harmful effects. As experience with smallpox vaccines evolved through the nineteenth and first half of the twentieth centuries, it became evident that the regional successes of vaccination could be generalized to all areas of the world; thus, the global campaign to eradicate smallpox was born. Although its ultimate success established triumphant historical precedent, smallpox vaccines remain an active issue three decades after smallpox was eradicated as a natural cause of human disease.

### **2.1 A Brief History of Smallpox**

In many ways, it stands to reason that vaccination against smallpox, or variola, would be the benchmark by which future vaccines would be assessed. Historically, smallpox occupies a position as the greatest disease scourge of humankind; its impact on civilizations has been amply documented in a variety of media including written texts, works of literature, and objects of art. Although it is problematic from historical records to accurately classify diagnoses of smallpox before approximately 1,000 AD, descriptions of a clinically compatible illness appeared in ancient writings from Asia during the first millennium AD (Fenner et al. 1988). Characteristic

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findings of the disease were noted in the mummified human remains of three Egyptians dating from more than 1,000 years before the birth of Christ (Ruffer and Ferguson 1911; Hopkins 2002), but the conspicuous absence of descriptions consistent with smallpox from Egyptian medical writings of the period raises questions regarding its occurrence.

Ancient medical scholars such as Ko Hung in China, Vaghata in India, and al-Razi in Baghdad described the epidemiology and clinical appearance of smallpox in the fourth, seventh, and tenth centuries AD, respectively (Fenner et al. 1988). Epidemic disease, likely originating in Asia, entered naïve populations through invading armies and foreign traders via routes through North Africa and the Mediterranean basin (Fenner et al. 1988). Highly lethal, documented outbreaks recurred throughout the world during the latter half of the second millennium, accounting for tens of millions of deaths from this disease. Smallpox became endemic on the Indian sub-continent, throughout Asia, Africa, and Europe in this period and was imported into the New World by Old World explorers, conquerors, colonizers, and slave traders.

The scope of devastation wrought by the disease altered the course of human history. It may have been a factor in the decline of the Roman Empire (Fears 2004); smallpox was perhaps the most important of an array of transmissible diseases that resulted in the decimation of indigenous tribes of the Americas during the sixteenth century (Cook 1998; Hopkins 2002); and it altered the geopolitical landscape of the preindustrial world by deposing monarchs and halting armies. During the latter part of the eighteenth century, smallpox accounted for 10% of the mortality in some of Europe's major cities; most of this occurred in children (Hopkins 2002). Epidemics, with their attendant morbidity and mortality, continued to occur throughout the developed world until they were checked by the introduction of widespread vaccination.

## 2.2 Pre-Jennerian Smallpox Vaccine History

The concept of using inoculations of pus or scabs from smallpox-infected individuals to mitigate the severity of natural variola, the process of “variolation,” probably derived from empiric observations that smallpox survivors were protected against re-infection. Variolation generally produced a localized, less severe form of disease than naturally acquired smallpox. Although it was associated with dissemination and mortality in a small percentage of individuals, approximately tenfold lower than that following naturally acquired smallpox, variolation could lead to transmission of smallpox by contact. The procedure may have been practiced in Egypt in the thirteenth century and was known to be used in India in the sixteenth and parts of Africa and China in the late seventeenth centuries (Fenner et al. 1988).

Although various forms of variolation may have been sporadically employed in China as early as the eighth century, it is difficult to identify substantiating documentation of its practice there until the seventeenth century (Buck 2003). Inoculation, another name for the procedure, is described in a medical text from 1695. A more detailed account, *The Golden Mirror of the Medical Tradition*, published in 1742 and

apparently endorsed by the Chinese Imperial court, may have enabled variolation to become part of the mainstream medicine of the time and to be disseminated throughout eighteenth century Chinese society (Leung 1996). This text describes four distinct methods of variolation: placing pus from smallpox lesions or scabs into the nares of healthy children; dressing healthy children in clothing worn by infected individuals; and nasal insufflation of dried, powdered, smallpox crusts derived from patients in the late phases of disease via a silver tube (Leung 1996). The latter method may have been preferred, in part because of the belief that the respiratory tree provided the pathway through which the effects of variolation could circulate to the other, traditional, visceral zones of Chinese medicine (Leung 1996).

Documentation of variolation in India began in the sixteenth century; the practice gradually spread from there through parts of southwest Asia, central Europe via the Balkans, and Africa with Arab slave traders (Fenner et al. 1988). Official Russian emissaries may have learned the method from Chinese authorities and exported variolation to Eastern Europe (Buck 2003). The technique may have been introduced into the Ottoman Empire in the seventeenth century by Seljuk, ancestral Turks with connections to southwest Asia, or by Circassian traders from the Caucasus and the regions around the Black Sea, whose women were highly prized by the Turkish Sultan. These women, who were sold to the Sultan's harem, were apparently protected as children against the disfigurement of smallpox by inoculation in inconspicuous areas of their bodies (Dinc and Ulman 2007).

Turkey, ruled by the Ottomans for more than 600 years beginning in the fourteenth century, represented a geographic bridge between East and West. Its culture was mosaic, a melting pot blending parts of both societies and hosting a variety of European visitors who witnessed innovative practices introduced from exotic places throughout Asia and Africa. Variolation was one such practice. Sporadic reports of its use may have circulated via such travelers to the Far East or Africa, as references to the procedure appeared in correspondences prior to the turn of the eighteenth century (Stearns and Pasti 1950). But it became a subject of active discourse among scientific circles in the early part of the eighteenth century in Great Britain; the Royal Society of London first heard presentations describing the Chinese technique of intranasal variolation in 1700. In 1714 and 1716, independent reports by the physicians Timoni and Pylarini respectively, describing the Turkish method using the cutaneous route were read there (Woodward 1714; Huth 2006). These reports engendered mild scientific interest but did not result in acceptance of variolation or trials of the method by the conservative British medical establishment, who thought the procedure too risky and of dubious benefit (Miller 1957). This would begin to change a mere 5 years later due in part to the passion of an enlightened and politically connected layperson.

Lady Mary Wortley Montagu (1689–1762), the wife of the British ambassador to the Ottoman court, lived in Adrianople and Constantinople during the years 1717 and 1718. She was a keen observer of Turkish mores and society, attentively documenting these observations in her correspondence, poetry, and travel writings (Wortley Montagu 2000). She possessed an open mind and was favorably impressed with the practice of variolation, having experienced the death of her brother due to

smallpox and suffered herself with this disease as an adult in 1715 – leaving her somewhat disfigured and without eyelashes (Wortley Montagu 2000). While in Adrianople she wrote to a friend in England of the technique of “engrafting” in which old women scratch a small amount of material from “a nutshell full of the matter of the best sort of smallpox” using a needle into multiple veins, usually on the extremities, of children or young adults (Wortley Montagu 2000). Shortly thereafter inoculated individuals developed fevers, a brief systemic illness, and subsequently recovered. Lady Montagu (Fig. 2.1) was sufficiently impressed that she had her 5-year old son inoculated by her personal, Scottish physician while in Turkey in 1718 and after her return to England, had her daughter undergo public variolation during a smallpox epidemic, an action that piqued royal interest in the procedure in part due to the social prominence of Lady Montagu (Stone and Stone 2002).

A series of further “experiments” followed the little girl’s inoculation. With royal patronage and royal physicians directing the endeavor, experimentation with variolation was conducted on six condemned prisoners from London’s infamous Newgate prison (Miller 1957). They were offered a sparing of the death penalty if they survived the ordeal; all demonstrated protection against a smallpox challenge (Parish 1965). After a small group of orphaned children was successfully variolated



**Fig. 2.1** Lady Mary Wortley Montagu (Wellcome Library)

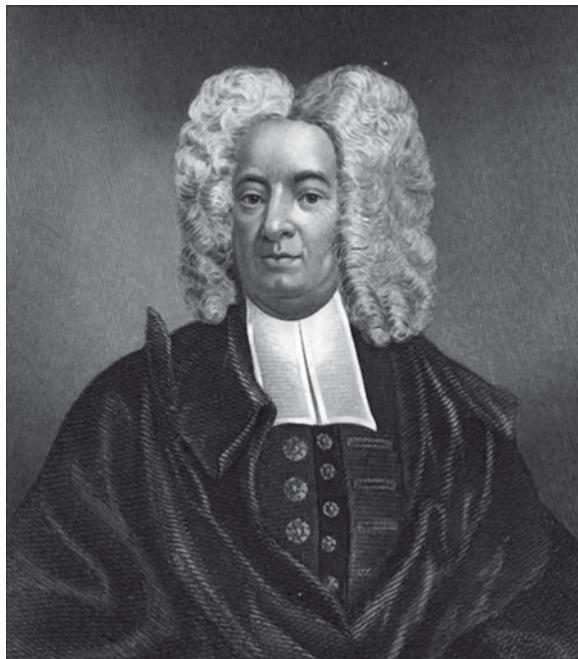
(Sloane 1756), the two daughters of the Princess of Wales were inoculated in 1722 and recovered uneventfully (Stone and Stone 2002).

The practice gradually grew in scope over the decade but was largely restricted to the upper classes in England and was still viewed by the medical profession as a somewhat risky procedure, associated with a 2% mortality rate, occasional severe morbidity, and the persistent threat of contact transmission to others, and one of unproven benefit (Stearns and Pasti 1950; Stone and Stone 2002). There was also considerable opposition from religious leaders. Nonetheless, an early, imperfect attempt to use medical statistics to justify smallpox inoculation during the latter part of the 1720s demonstrated a difference in mortality of nearly 90% comparing that of natural smallpox (approximately 17%) to that of variolation (approximately 2%) (Huth 2006).

At nearly the same time that Lady Montagu publicly introduced variolation into Great Britain, the procedure was employed in the New World in an attempt to quell the spread of epidemic smallpox. These events also reverberated in England and helped to facilitate the widespread acceptance of variolation there. In 1721, Zabdiel Boylston, a Massachusetts physician known as the first American-born physician to perform a surgical operation, the removal of a urinary stone, inoculated approximately 240 individuals in Boston with material from smallpox lesions (Harvard University Library Open Collection Program 2008). He had been introduced to the method by the Reverend Cotton Mather (1673–1726), a Harvard-educated cleric with a penchant and aptitude for medical science, who in turn had probably learned of the procedure in 1706 through one of his slaves, Onesimus, who claimed to have been inoculated in Africa (Brown 1988).

Mather (Fig. 2.2) was a controversial figure prior to his advocacy of variolation. His religious writings had served to further inflame the hysteria surrounding the Salem witchcraft trials of 1692; his views on medicine were tempered by a belief in the supernatural, apparent in his medico-religious treatise, *The Angel of Bethesda*, written but not published during his lifetime (Mather 1972). Mather's contributions in the arena of natural science had previously earned him election as a Fellow of the Royal Society of London and as such, he had read the reports of Timoni and Pylarini on inoculation and had become an advocate of the procedure (Silverman 1985). Advocacy turned to action during a smallpox epidemic in Boston in 1721 that affected half of the city's population, killing nearly 15% of its victims (Fenn 2001).

Mather appealed to Boston's medical establishment to use variolation to halt the epidemic; only Boylston heeded the call. Their actions with inoculation touched off a heated controversy in the city with both men experiencing significant public reprisals, including physical threats, from numerous quarters (Breen 1991). Nonetheless, mortality among the relatively small group of inoculated individuals, less than 3%, was significantly lower than that associated with natural smallpox infection (Stearns and Pasti 1950; Huth 2006). The New England experience, after its communication via the Royal Society to Britain, facilitated acceptance of the process with technical variations, spurred by ongoing smallpox epidemics in Great Britain during the first half of the eighteenth century and in other parts of Europe as the century progressed (Miller 1957; Fenner et al. 1988).



**Fig. 2.2** Cotton Mather (American Antiquarian Society)

Variolation continued to be employed sporadically in Colonial America during the middle of the eighteenth century, largely as a response to epidemic disease. The American thought leader Benjamin Franklin was an important advocate; he reported favorable mortality data from its use during a smallpox outbreak in the early 1750s (Franklin 1759; Huth 2006). But inoculation was not only associated with significant disadvantages, including its cost, the prolonged preparatory and recovery periods, its attendant mortality rate, and its clear transmission risk to the unexposed, it was also still objectionable on religious, ethical, or medical grounds to a substantial segment of the population; the procedure was restricted by statute in every colony except for Pennsylvania (Hopkins 2002).

Inoculation became a strategic issue during the War of the American Revolution. British troops were, in large part, immunologically experienced with smallpox, a significant advantage over the colonists. General George Washington, commander of the Continental Army, had experienced smallpox first-hand as a teenager and appears to have been appropriately concerned with its potential impact on his susceptible troops from the earliest phases of the conflict. He instituted isolation measures where possible and used immune troops in selected situations as feasible. However, dwindling troop resources due to smallpox during the campaign in Canada over the winter of 1775–1776 and attrition due to the completion of enlistment terms in late 1776 led Washington to consider extreme measures to preserve his fighting force.

With the support of Dr. Benjamin Rush, a leading voice in the Congressional Medical Department, and despite philosophical and logistical reservations, Washington adopted the unprecedented step of ordering the inoculation of all susceptible troops and all new recruits, the first mass immunization of a military force (Artenstein et al. 2005a). Thus, the Continental Army remained free of epidemic smallpox, and the British enemy was robbed of a major tactical advantage.

While variolation was becoming well established as a viable means of preventing smallpox throughout England and much of Europe during the latter half of the eighteenth century in parallel with a series of devastating epidemics, various “folk” methods of protection were probably practiced among rural agricultural communities in Great Britain earlier in the century. Such practices included the procurement of scabs from recovering smallpox patients, deliberate exposures to infected individuals, or the use of material from cowpox lesions, the latter based on empiric observations leading to local beliefs that milkmaids, known for their unblemished complexions, were somehow protected against smallpox by this bovine-acquired affliction (Fenner et al. 1988). Individuals sporadically employed this method in the later decades of the eighteenth century.

In 1774 with epidemic smallpox threatening his community, Benjamin Jesty (1737–1816), an educated tenant farmer in Yetminster in southern England, inoculated his wife and two sons with material from cowpox lesions, causing significant local outcry (Hart 1988). Jesty (Fig. 2.3), himself protected due to previous cowpox infection, based this practical “experiment” on empiric knowledge and on that



**Fig. 2.3** Benjamin Jesty (Wellcome Library)

derived from discussions with two of his dairymaids, both of whom had resisted smallpox despite intimate exposures to infected family members after experiencing cowpox infection (Hammarsten et al. 1979). Notwithstanding repeated smallpox exposures over the ensuing years, Jesty's family remained healthy; in fact, his sons failed to react to variolation years later, providing further evidence of their immune status (Pead 2003). In 1805 the Original Vaccine Pock Institute recognized him as the “earliest inoculator for Cow Pock,” but he was likely neither the first nor the last to perform such local experiments for practical reasons (Fenner et al. 1988). However, it would require an inquisitive country doctor from Berkeley, Gloucestershire to transform cowpox inoculation from folk medicine to systematic method and in so doing establish the science of vaccines.

## 2.3 Jenner and Beyond

After apprenticing with rural medical practitioners as a teenager, as was common for medical training in the mid-eighteenth century, Edward Jenner (1749–1823) went to London in 1770 to study under John Hunter, the master surgeon and physiologist, at St. George’s Hospital (Turk and Allen 1990). Jenner (Fig. 2.4) is said to have known of the popular lore among agricultural communities concerning the protective effect of cowpox against smallpox and to have discussed it with Hunter (Bailey 1996). Although his mentor did not specifically encourage a systematic study of the issue, Hunter’s general support of the scientific method may have influenced Jenner to perform his experiments with cowpox (Fisher 1991). In fact Jenner pursued a number of interests in natural science other than medicine; he earned election as a Fellow of the Royal Society not for medical research but based on his research involving the nesting behaviors of the hatchling cuckoo (Bailey 1996).

With the widely known cowpox cross-protection anecdotes as background, Jenner prepared a systematic study of the issue; *An Inquiry into the Causes and Effects of the Variolae Vaccinae, a Disease Discovered in Some of the Western Counties of England, Particularly Gloucestershire, and Known by the Name of the Cow Pox* was published at his own expense in 1798 after being rejected by the Royal Society. In it he presented a series of 23 case histories, some of them involving more than one individual and some based only on second-hand knowledge, that described epidemiological as well as experimental evidence of the protective effect of cowpox against smallpox (Jenner 1978). Jenner reported, on epidemiologic grounds, that natural cowpox infection of 17 individuals prevented their subsequent successful variolation, in one case more than 40 years later; he also reported two cases in which individuals with previous cowpox infection resisted smallpox upon exposure to active, systemic cases (Jenner 1978).

The remaining case histories in the *Inquiry* comprised Jenner’s uncontrolled inoculation experiments using cowpox and involving some of the most famous names in vaccine history. Using material obtained from a “large pustulous sore” on the dorsal hand of dairymaid Sarah Nelmes, who had been infected by milking



**Fig. 2.4** Edward Jenner (Wellcome Library)

“Blossom,” an Old Gloucester breed of cow and the source of disease, he inoculated James Phipps, a healthy 8-year old boy via two superficial incisions on the arm in May 1796 (Jenner 1978). Six weeks later, a month after the boy had recovered from the acute cowpox infection, Jenner variolated him in standard fashion and noted a stereotypical local, but no constitutional response. In 1798, after a hiatus due to lack of infected animals in the community and following a fortuitous outbreak of cowpox in the local dairy farms that provided substrate for study, Jenner again carried out a series of similar vaccinations in children in which he not only demonstrated resistance to subsequent variolation in some cases, but also showed that the infectious cowpox material – the immunizing agent – could be successfully transmitted from arm-to-arm through successive generations, thus introducing the possibility, still years away from reality, of vaccination without the continuous need for an animal intermediary.

Although inaccurate in some important respects (Baxby 1999), such as his unproven assertion that cowpox was derived from “the grease,” a disease of the heels of horses, and his conclusion that protection using cowpox was lifelong, Jenner’s *Inquiry* provided experimental data to support the popular belief that cowpox infection represented a potentially viable and safer alternative to variolation for

the prevention of smallpox. His work, although important on a number of different levels, is perhaps most significant because it represented the first systematic, scientifically rigorous study of the use of an altered form of an infectious agent of animals to provide cross-protection against a related, human pathogen. By publishing his results, Jenner widely promulgated the concept that would eventually come to be known as vaccination; vigorous scientific debate and attempts to reproduce the results ensued.

Jenner's work had an immediate impact in England and elsewhere; it was readily accepted by many because it appeared to provide the benefits of the accepted procedure of variolation without its attendant risks. Although claims for propriety regarding cowpox inoculation were made on behalf of a number of different individuals, including Jesty (Baxby 1996; Hopkins 2002), and some in the medical establishment were skeptical of the results (Beale and Beale 2005), Jenner's observations were subsequently confirmed and extended by a variety of investigators and by the early part of the nineteenth century, the concept had disseminated throughout Europe, with millions vaccinated (Hopkins 2002).

The use of smallpox vaccination in the fledgling USA proceeded in parallel to its use in Europe. It was introduced by Benjamin Waterhouse, a Professor at the newly created Harvard Medical School, who obtained cowpox from physician contacts in England (Parish 1965); Waterhouse enlisted the support of President Thomas Jefferson in 1801, who himself arranged for its first use in Native Americans. The absence of natural cowpox among animals in the USA complicated its initial experience there and injected economics – supply versus demand – into the smallpox equation, along with its associated profiteers and subsequent vaccine failures due to poorly controlled, inactive products; the situation was eventually resolved by the intervention of Jefferson. Cowpox “vaccine” continued to require importation, some of this through Jenner himself, but became widely used in the USA. By 1812, the U.S. government began mandating troop vaccination with cowpox in lieu of variolation (Grabenstein et al. 2006).

Smallpox vaccination using the Jennerian method flourished in Britain during the first half of the nineteenth century, but it was not without its detractors. The procedure initially endured significant opposition from certain physicians, who objected to their potential loss of income from inoculation; from those in Britain who subscribed to Malthusian concepts that smallpox provided natural population control by culling the poor from society; from some religious leaders who viewed vaccination as an affront to God's plan; and from those in the scientific community who remained skeptical due to an incomplete understanding of the pathogen, concern over the use of animal-derived material in humans, and weaknesses in Jenner's discourse (Hopkins 2002). Nonetheless, vaccination was widely adopted throughout Europe and America through the first few decades of the nineteenth century, and it rapidly became clear that the procedure was safer than variolation for individuals and the public and significantly reduced the mortality rate from smallpox (Fenner et al. 1988).

However, recurring smallpox outbreaks during the first half of the nineteenth century in Europe, attributable in large part to inadequate vaccine usage and waning

natural or vaccine-induced immunity, led to renewed efforts at control (Hopkins 2002). Variolation was officially outlawed in Russia in 1805 and in Britain in 1840 and replaced by vaccination, which became compulsory in the latter in 1853. The mandate of compulsory vaccination in Britain was most strictly enforced by the passage of the 1871 Vaccination Act, itself prompted by a catastrophic smallpox pandemic that began in 1870 sparked by the Franco-Prussian War, that authorized the use of punitive actions, specifically against the poor, for failure to comply with smallpox vaccination (Allen 2007). In response, a fervent, and at times violent, antivaccination movement, based in political, social, and religious roots, erupted in Great Britain, dominating nineteenth century debates about vaccination (Durbach 2005) and presaging those that would emerge, involving as-yet undiscovered vaccines, over the ensuing century and continuing into the present.

The early history of smallpox vaccination using the Jennerian method was associated with a number of problems, such as vaccine contamination with variola virus, most notably among those vaccinated in smallpox hospitals; the transmission of human adventitious agents, such as syphilis, via arm-to-arm transfer; vaccine shortages; and the need for revaccination, the latter recognized in continental Europe significantly earlier than in Great Britain (Hopkins 2002). As the nineteenth century progressed, alterations to the vaccine and vaccination procedure mitigated many of these issues. Viable vaccine material was initially maintained via arm-to-arm transfer in humans with its attendant risks; improvements in vaccine production led to the use of calves as a source of vaccine material and the dissemination of this method from Italy in the first half of the nineteenth century to the rest of Europe and the USA by century's end. This alleviated problems of vaccine shortages, variola contamination, and transmission of other infectious agents; the addition of glycerol to the fluid and tissue obtained from the animal's lesions during production provided both a suitable diluent and preservative and sterilized the preparation's bacterial content while leaving the vaccine virus intact (Parish 1965; Hopkins 2002).

At the beginning of the twentieth century, smallpox remained endemic in most areas of the world, but the widespread use of vaccination in industrialized countries kept disease incidence at low levels in those areas with broad vaccine coverage. Rapid advances in vaccine science and the dramatic expansion of microbiologic knowledge that followed the work of Pasteur and Koch in the latter part of the nineteenth century (refer to Chap. 3) had a profound effect on the most senior vaccine – smallpox. The disciplines of immunology and virology were born, and smallpox vaccine, owing to its pervasiveness, received renewed scientific attention.

Vaccine production moved from the domain of farms and individual physicians to that of industry. More refined and larger scale production methods, using scarification of mostly calf, but also sheep, or water buffalo skin and subsequent viral isolation from skin scrapings containing pus, serum, and extruded lymph were employed (Fenner et al. 1988). By the 1950s the process had become progressively more systematized and regulated with the replacement of liquid vaccine lymph preparations by lyophilized preparations that enhanced the preservation of the vaccine virus.

Although Jenner's original formulation comprised cowpox virus, at some point, most likely in the late nineteenth or early twentieth centuries and for unknown reasons, the nature of the virus changed. The resultant vaccine virus, known as vaccinia and later proven to be a distinct species of orthopoxvirus, was neither a natural cause of human nor of animal disease. The origins of vaccinia have never been completely elucidated; the virus may have either emerged as the result of genetic hybridization between cowpox and variola viruses, from selection via multiply passaged cowpox under laboratory culture conditions, or as the virologic vestige of a previously extant orthopoxvirus species, such as horsepox (Baxby 1977, 1999). Nonetheless, vaccinia, like cowpox, provided robust cross-protective immunity against smallpox, as evidenced by its illustrious track record of effectiveness (Downie 1951).

By the late 1950s smallpox had been eradicated from Europe, North America, and many countries within endemic continents due to a combination of factors but in large part due to the broad, mandated use of childhood vaccination (Fenner et al. 1988). Although indigenous smallpox transmission had been absent in the USA after 1949, surveillance data from the 1960s showed that serious complications of the routine use of smallpox vaccination occurred in approximately four per 100,000 individuals with an overall risk of death of one per million primary vaccinations (Lane et al. 1969, 1970; Fulginiti et al. 2003). As it became clear that the occurrence of vaccine-associated, serious adverse effects represented significant public health concerns in developed countries and outweighed the risk of smallpox there, the continued use of routine vaccination gradually waned. This, in concert with persistent, significant disease burdens in defined, smallpox-endemic areas in the developing world provided a fertile milieu in which to attempt Jenner's ultimate stated goal of the "annihilation of the Small Pox" (Fenner 1996).

## 2.4 Global Eradication

The concept of smallpox eradication, although initially suggested in broad terms by Jenner at the beginning of the nineteenth century, was not within the realm of scientific or practical consideration until the first World Health Organization (WHO) global campaign, proposed by the Soviet delegation to the body. The campaign was launched in 1959 (Fenner 1996). Through the use of mass vaccination targeting 80% of the population, the campaign eliminated smallpox in a number of smaller countries where the disease was endemic, but after 7 years, it was clear that eradication in the larger endemic areas in Asia and Africa would not be achievable with this level of vaccine penetration (Fenner 1996).

In 1966 the World Health Assembly adopted a multi-nationally sponsored resolution that called for renewed, focused efforts to eradicate smallpox within a 10-year timeframe; the Intensified Smallpox Eradication Programme of the WHO was born, its budget approved by a margin of two votes (Fenner et al. 1988). Extensive planning and preparations were undertaken, under the leadership of

Donald A. Henderson (Fig. 2.5), a medical epidemiologist who had been the Chief of the Smallpox Program at the U.S. CDC, and Isao Arita, who had worked in the WHO smallpox eradication program in western Africa, to develop a strategy for disease eradication targeting the 31 countries, mostly in Africa and Asia, endemic for smallpox in 1967 (Fenner et al. 1988).

The idea of disease eradication had been previously applied, with some success, to veterinary pathogens, although on a circumscribed scale. Regional efforts to eradicate human diseases such as hookworm and yellow fever had been carried out in the early parts of the twentieth century but failed to achieve global success. A WHO-sponsored program for malaria eradication achieved some successes in the 1950s and 1960s but was ultimately unable to fulfill its initial promise for a number of reasons, including biological and epidemiological ones (Fenner et al. 1988). Nonetheless, preparations for the Intensified Smallpox Eradication Programme were informed by lessons learned from the aborted malaria initiative.

That it was even considered feasible to eradicate smallpox, a human disease of massive historical scope and impact and the cause of approximately 15 million annual cases worldwide at the start of the campaign, was due to a number of factors



**Fig. 2.5** D.A. Henderson at Jenner's "vaccination cottage" (Courtesy of Dr. D.A. Henderson)

that in many ways made this infection a prime candidate for which to attempt eradication. The existence of a highly effective vaccine to control transmission and its availability in freeze-dried form for transport and storage in tropical environments that were prevalent in many endemic areas were of fundamental importance. Additional factors that contributed to the feasibility of eradicating smallpox were the absence of natural animal reservoirs of infection and the likely absence of sub-clinical infections. These properties ensured that the combination strategy proposed by the Intensified Programme comprising surveillance, isolation of cases, and vaccination of contacts in circumferentially expanding “rings” would interrupt the cycle of smallpox transmission in endemic areas.

Because of its immense scope and magnitude, the Programme was logically quite complex as were the challenges it faced; its organization, structure, function, and administration were exquisitely detailed subsequently by some of the Programme’s leaders in *Smallpox and its Eradication*, the definitive historical account of the effort. As vaccine stocks were donated from member countries or produced locally, vaccine quality control and potency in field settings were major, early issues that hindered implementation. These were addressed through the establishment of international reference centers and the development of training and testing algorithms (Fenner et al. 1988; Arita 1999). Aside from vaccine acquisition, vaccine deployment to affected areas within endemic countries required the creation of a well organized, multilevel infrastructure that had, by Programme’s end, delivered hundreds of millions of smallpox vaccine doses worldwide (Fenner 1996). Other, formidable challenges involved setting up an organized system of disease surveillance and reporting at the village level, both severely lacking in many of the endemic countries prior to the advent of the Intensified Programme, yet both essential elements of the Programme’s eventual success.

As might be expected to occur with an effort of the magnitude of the Intensified Programme, innovative approaches were devised to address a number of challenges related to the campaign. In addition to resolving issues of vaccine acquisition, quality control and delivery, and disease surveillance and reporting, the initiative required the introduction of new methods of vaccination, born of necessity to accomplish the Herculean feat within the Programme’s timeframes. The jet injector, a semi-automatic inoculating device used in early phases of the campaign in Brazil and parts of Africa, had the advantages of sparing vaccine and the ability to rapidly vaccinate large numbers of individuals, especially in areas in which multiple, different disease vaccination programs were in effect (Fenner et al. 1988). However, its use was impractical in less densely populated rural environments where potential vaccinees were separated by large distances and in areas where routine maintenance or repair of the relatively “high-tech” device was problematic (Fenner 1996).

By 1968, another mode of vaccination, the bifurcated needle, had largely replaced all other methods used in the Programme. Invented by Ben Rubin, a scientist at Wyeth Laboratories, the major U.S. producer of smallpox vaccine, in collaboration with workers at the Reading Textile Machine Company in Pennsylvania in the early 1960s, the bifurcated needle would become the standard mode of smallpox vaccination throughout the world due its use in the Intensified Programme

(Fenner et al. 1988). Designed to retain a consistent, small volume of liquid vaccine, it conserved vaccine relative to other puncture methods, was associated with take rates comparable to accepted scratch or pressure methods, and was simple enough to use that a villager could quickly master the vaccination skill and teach other non-professionals the technique (Fenner et al. 1988). Additionally, bifurcated needles were inexpensive and could be re-used after sterilization.

At the time of the Intensified Programme's inception, an effective smallpox vaccine had been in use for more than 160 years; thus, research on smallpox had been pre-empted by other, seemingly unresolved medical problems. Nonetheless, Henderson and other Programme leaders recognized the need for additional basic and applied research on smallpox in order to successfully accomplish their public health mission. Eradication, as had been learned in the abandoned malaria campaign a decade earlier, was not simply an administrative or epidemiological task but required the development of new models, the application of innovative paradigms, and the discovery of novel methods, all areas that were necessarily informed by scientific investigation. In addition to the enormous administrative challenges that were managed during the smallpox eradication campaign, findings from the Programme's concurrent laboratory and epidemiologic research initiatives contributed significantly to its ultimate success. These included the determination that mammalian reservoirs of infection were non-existent in nature, and that variola virus did not persist on fomites or in the environment for prolonged periods of time; detailed epidemiologic and virologic studies of the related monkeypox virus; an improved understanding of the primacy of containment in the overall eradication strategy; and advances in smallpox vaccine production and use (Fenner et al. 1988).

Through a defined strategy, surveillance and containment of cases and ring vaccination of contacts, systematically applied to endemic countries via a highly organized, multi-layered administrative structure that reached the most remote places on earth and “some good luck, smallpox was progressively eliminated” (Fenner 1999) from the world. The last endemic country in the Western Hemisphere, Brazil, became smallpox-free in 1971. Within 2 years, despite setbacks due to civil wars in the Sudan and East Pakistan (subsequently Bangladesh) with their resulting refugee crises, the spread of infection in parts of Asia, and a major outbreak imported into Yugoslavia from Iraq in 1972, smallpox remained endemic in only six countries in Asia and Africa (Fenner et al. 1988). The Programme accelerated in its intensity and by the end of 1975, Asia was rendered free of disease, and endemic smallpox, in the form of variola minor, was relegated to the country of Ethiopia from where it was finally eliminated in August 1976 (Fenner et al. 1988).

Smallpox appeared in nature once more, in late 1977 in Somalia, introduced from neighboring Ethiopia. With the interruption of further transmission, the process to certify eradication could be completed. Such a process, through an organized system of certification by groups of independent, international experts, had commenced in 1973 as endemic countries became persistently smallpox-free (Fenner et al. 1988). In 1977 the Global Commission for the Certification of Smallpox Eradication was formed, chaired by Australian medical virologist Frank Fenner, who had contributed to the Intensified Programme in both the research and administrative areas.

The Commission certified the global eradication of smallpox in December 1979; their report was presented to and accepted by the World Health Assembly on May 8, 1980 (Fenner 1996).

## 2.5 Post-Eradication Vaccination

The last naturally acquired case of smallpox occurred in Somalia in October 1977 (Fenner et al. 1988); the last known human cases occurred in August 1978 as a result of inadvertent laboratory exposures in Birmingham, England that caused two cases and one death (Fenner et al. 1988; Wade 1978). The routine use of vaccination in the USA and UK had officially ceased in 1971, although its selective use in certain groups, such as the military, continued for somewhat longer. With the scourge of smallpox eradicated as a natural cause of human disease, however, it was only the specter of its intentional deployment as a malicious, disease-causing agent in humans that could resurrect the broad use of smallpox vaccination.

In December 2002, after more than a 12-year hiatus, the U.S. Department of Defense (DoD) reconstituted large-scale vaccination using live vaccinia in response to the perceived threat of bioterrorism involving smallpox; a concurrent, voluntary program in civilian healthcare workers was initiated by the U.S. Department of Health and Human Services (DHHS). In excess of 1.4 million individuals have since been vaccinated in the ongoing military program and approximately 39,000 individuals in the civilian program before the latter was terminated in late 2003 due to lack of participation.

A number of serious, albeit rare, complications of smallpox vaccination had been well documented during the era of its routine use. These included postvaccinal encephalitis, a rare disorder of the central nervous system associated with a high mortality rate or severe neurological impairment in young children (Henderson et al. 1999; Miravalle and Roos 2003); progressive vaccinia, a frequently fatal complication involving regional and metastatic spread of uncontaminated vaccinia virus in immunocompromised hosts; and eczema vaccinatum, characterized by extension of the local vaccinia infection to other cutaneous areas actively or remotely affected by atopic dermatitis (Henderson et al. 1999; Fulginiti et al. 2003). Other entities, such as generalized vaccinia, congenital vaccinia, inadvertent inoculation, and bacterial superinfection represented potential causes of severe morbidity (or mortality in the case of congenital vaccinia) in vaccinees or their close contacts (Fenner et al. 1988; Henderson et al. 1999; Fulginiti et al. 2003; CDC 2006; Strikas et al. 2008). Although the incidence of serious adverse events in post-eradication mass vaccinations was potentially expected to be significantly higher than historical levels due to a larger population of individuals with vaccine contraindications and a larger proportion of vaccinia-naïve individuals in the population (Kemper et al. 2002; Neff et al. 2002), this did not materialize due to rigorous, risk-based contraindication screening and extensive education.

However, the post-eradication experience illuminated novel, vaccine-associated concerns (Lane et al. 1969, 1970; Lane and Millar 1971) that were under-appreciated

during the era of routine use of smallpox vaccine (Table 2.1). Chief among these were myopericarditis and other cardiac complications (Artenstein and Grabenstein 2008). Although vaccine-associated myopericarditis had been described during the 1960s and 1970s (MacAdam and Whitaker 1962; Ahlborg et al. 1966; Helle et al. 1978; Karjalainen et al. 1983), its occurrence at a rate of approximately 1.2 confirmed cases per 10,000 vaccinees in the posteradication experience (Halsell et al. 2003; Poland et al. 2005) resulted in an augmented set of contraindications to the use of live vaccinia virus and provided further impetus to the search for safer smallpox vaccines.

## 2.6 Future Prospects

Smallpox vaccines derived from Jenner's original model but comprising a variety of live vaccinia viruses, first-generation vaccines, were used for protection against smallpox throughout the twentieth century and the posteradication vaccinations of the early part of the twenty-first century. Yet these vaccines remain encumbered by uncommon, potentially life-threatening adverse events that limit their use in the absence of substantial disease risk (Henderson et al. 1999; Artenstein and Grabenstein 2008). Additionally, their original production method by animal scarification was abandoned more than 25 years ago and because smallpox had been eradicated as a cause of natural disease, essentially no new first-generation vaccines had since been manufactured. This, in turn, led to insufficient vaccine stockpiles, even if diluted up to tenfold (Frey et al. 2003; Artenstein 2008), to cope with a potential large-scale bioterrorist threat, prompting renewed efforts toward the development of new vaccines with a focus on enhancing safety while maintaining efficacy.

Second-generation smallpox vaccines, in which full-strength vaccinia virus is grown in tissue culture rather than in the skin of large mammals, possess theoretical advantages conferred by this modern manufacturing technique: improved sterility, lowered risk of contamination by adventitious agents (Murphy and Osburn 2005), viral genetic homogeneity, and relative ease of large scale, consistent production. In 2007, ACAM2000 became the inaugural second-generation smallpox vaccine to be licensed for human use by the U.S. Food and Drug Administration (FDA), leading to the delivery of 192.5 million doses to the U.S. government for stockpiling purposes (FDA 2007), indicated for the "active immunization against smallpox disease for persons deemed to be at high risk for smallpox infection" (Acambis, Inc. 2007). However, despite their theoretical advantages, second-generation vaccines comprise replication competent, virulent vaccinia viruses and therefore possess the potential for the uncommon yet serious adverse events associated with first-generation smallpox vaccines, including myopericarditis (Artenstein et al. 2005b; Artenstein and Grabenstein 2008). For this reason, alternative candidates based on attenuated vaccinia strains – third-generation vaccines – are under investigation.

At the current time, at least two, promising third-generation candidate vaccines are being explored. LC16m8, a replication competent, highly attenuated vaccinia strain (Hashizume and Chiba Serum Institute 1975) appears to be less neurovirulent

**Table 2.1** Noteworthy adverse events after smallpox vaccination

Event type	Events and rates among 628,414 DoD vaccinees <sup>a</sup>		Events and rates among 39,566 DHHS vaccinees		Historical rate per million vaccinees
	Events (n)	Rate per million DOD vaccinees	Events (n)	Rate per million DHHS vaccinees	
<i>Moderate or serious</i>					
Postvaccinial encephalitis	1	1.6	1	26	2.6–8. <sup>b</sup> 7
Acute myopericarditis	83 <sup>c</sup>	132	21 <sup>c</sup>	531	100
Eczema vaccinatum	0	0	0	0	2–35 <sup>b</sup>
Progressive vaccinia	0	0	0	0	1–7 <sup>b</sup>
<i>Mild or temporary</i>					
Generalized vaccinia, mild	40	64	3	77	45–212 <sup>b</sup>
Erythema multiforme major	1	1.6	0	0	NA
Inadvertent inoculation, self	73 <sup>d</sup>	116	24 <sup>d</sup>	607	606 <sup>b</sup>
Vaccinia transfer to contact	47	75	0	0	8–27 <sup>b</sup>

DOD Department of Defense; DHHS Department of Health and Human Services; NA not available

<sup>a</sup>Primarily composed of uniformed military personnel plus some DoD civilian employees; a minority of this total was healthcare workers

<sup>b</sup>Based on adolescent and adult smallpox vaccination from 1968 studies (both primary and revaccination)

<sup>c</sup>DoD events include four biopsy-confirmed, 73 probable and six suspected cases; DHHS events include none confirmed, five probable and 16 suspected cases

<sup>d</sup>DoD events include 59 inadvertent inoculations of the skin and 14 of the eye; DHHS events include 21 inadvertent inoculations of the skin and three of the eye

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in animals than unattenuated vaccinia (Hashizume et al. 1985; Kenner et al. 2006a) and shows similar surrogate clinical efficacy to that of first- and second-generation smallpox vaccines (Fujii et al. 2006; Kenner et al. 2006a, b; Wiser et al. 2007). Animal data suggest that LC16m8 may be a safer alternative to unattenuated vaccine strains in immunocompromised hosts (Kidokoro et al. 2005; Yokote et al. 2006). Modified Vaccinia Ankara (MVA), a replication defective, highly attenuated vaccinia virus (McCurdy et al. 2004) has also been advanced as a third-generation, alternative vaccine of potential utility in immunocompromised hosts in whom live vaccinia vaccines are generally contraindicated (Mayr et al. 1978; Parrino and Graham 2006). MVA appears to be less immunogenic than either first-generation vaccinia or LC16m8 (Kidokoro et al. 2005), but may be used in a priming fashion to enhance immune responses to live vaccinia products.

Although third-generation vaccines may have improved safety profiles, this has yet to be proven in adequately powered clinical studies. Similarly, advanced-generation smallpox vaccines based on DNA or viral proteins are in their early clinical development. It remains supremely ironic and unfortunate that smallpox vaccine science continues to evolve more than 200 years after Jenner's landmark work and more than three decades after derivatives of his work successfully eradicated smallpox from nature.

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# **Chapter 3**

## **A Brief History of Microbiology and Immunology**

**Steven M. Opal**

### **3.1 Introduction**

Vaccine history is inextricably linked with the histories of microbiology and immunology; evolution of the latter disciplines parallels the ongoing quest of humankind to understand the fundamental basis of life. How our species survives in the hostile world that surrounds us has been a source of fascination since the beginning of recorded time. Injury and infection likely exacted a heavy toll as our early hominid ancestors descended from the trees and adapted a predatory life style on the African plains; death from bleeding and wound infections undoubtedly plagued early humans (Opal 2003). Epidemic disease, however, probably played a minor role in shaping the primitive human immune system. Instead, the primary determinants of lethality for small, scattered bands of hunter-gatherer populations of *Homo sapiens* were starvation, predation, and hypothermia.

Our collective fate was radically altered approximately 8,000–10,000 years ago when a highly developed immune system became a major selective advantage. Inhabitants of the “Fertile Crescent”, in what is now the modern day Middle East, first successfully domesticated plants and animals, irreparably altering human history. Domestication of plant and animal species had four major impacts – reduction in the risk of starvation, establishment of fixed dwellings close to fields for farming, improved nutrition with extended fecundity rates in women associated with more successful child bearing, and proximity to animals with the attendant risk of transmission of zoonoses to humans.

Adaptation from a nomadic, hunter-gather existence to a stable agrarian society with ample food supplies spawned a massive population explosion of humans. Division of labor followed, resulting in the blossoming of civilization, science, innovation, government, and the arts (Diamond 1999). The rapid expansion of densely populated, human habitations with poor sanitation, absent sewage disposal,

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proximity to domesticated animals, and lack of understanding about the spread of infectious diseases created favorable conditions for epidemics. Since that time, waves of epidemics have been recorded and continue unabated today. Strong selection pressures created by repeated infections have promoted highly evolved innate and acquired immune systems in humans.

Although the successful domestication of animals greatly benefited humankind as a ready source of food, transportation, and work, it also exposed humans to a large set of infectious agents that were epizootic to these animal species. Crossing species barriers is a difficult process for pathogens; however, once accomplished, the pathogen enjoys unfettered access to a new host species, unencumbered by any preexisting immunologic experience, resulting in epidemic diseases. Ancient examples abound – endemic camelpox in domesticated camels became human smallpox, bovine rinderpest became epidemic human measles, bovine tuberculosis became human tuberculosis, and swine influenza became human influenza. More recent historical examples include the cross-species adaptation of human immunodeficiency virus (HIV) from simian immunodeficiency virus (SIV) of non-human primates (Kalish et al. 2004); spongiform encephalopathy from sheep to cattle and on to humans as variant Creutzfeldt-Jakob Disease (Stevens et al. 2006); avian influenza from water fowl (Herzog et al. 2004); and severe acute respiratory disease (SARS) from civet cats (Margaret et al. 2004).

Other disease transmission factors also became important in evolving human societies. Peri-domestic rodent populations, emboldened by feeding upon the enormous amounts of refuse generated by large population centers, developed into efficient reservoirs for infections such as epidemic typhus and plague. Large population densities of humans in fixed, farming communities provided the essential substrate for efficient airborne transmission of respiratory pathogens and for a sufficient number of partner exchanges to maintain sexually transmitted diseases as well (Sherman 2007). With the acceptance of the germ theory of disease, novel modalities succeeded in protecting human populations primarily through enhanced sanitation measures, public health efforts, and, as vaccine science evolved, through the use of vaccination. The fundamental historical events that gave rise to the fields of microbiology, immunology, and infectious diseases will be described in this chapter (Fig. 3.1).

## 3.2 Early Concepts of Contagion and Protection

Epidemic, transmissible diseases were documented in the recorded histories of early, yet advanced civilizations. Ancient Hebrew texts refer to “plagues” that beset the Pharaohs in Egypt more than 1,000 years before the birth of Christ; the Greeks and Romans each experienced cataclysmic outbreaks that had profound impacts on their respective Empires. With each of these “plagues” enlightened observers noted the phenomenon of resistance upon re-exposure to the same disease process. The Greek historian Thucydides recorded such observations regarding smallpox, and

## Major Milestones in Microbiology

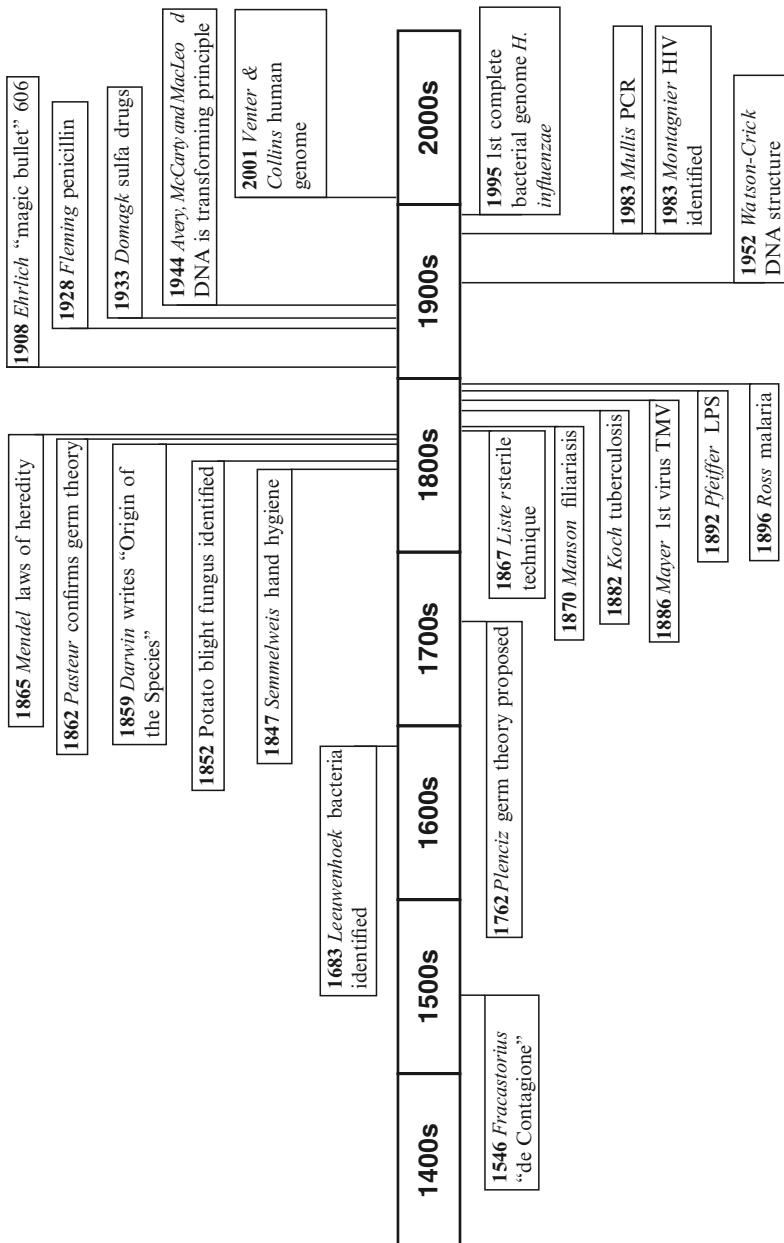


Fig. 3.1 Major Milestones in Microbiology

there is evidence that the Chinese exploited this knowledge in the sixteenth century in their practice of variolation (Leung 1996).

Much later, the intercontinental exchange of people and pathogens during the age of exploration to Africa and the New World in the sixteenth and seventeenth centuries dramatized the concept that some form of “natural resistance” to disease was often intrinsic to native populations yet lacking in the newly exposed (Diamond 1999). Africans, forcibly exported to America as slaves, were noted to be more resistant to tropical diseases such as yellow fever and malaria when compared to share croppers of European descent. This was most evident upon first arriving to the colonies, a process known as “seasoning” by landowners. European farmers died by the droves from sickness and disease in the Southern colonies, as did captured Native Americans transported from New England and elsewhere to work in the fields there and in the Caribbean (Morgan 1975), thus furthering the African slave trade as an economic expediency for the rapid expansion of a healthy labor force.

Indigenous Amerindian peoples were highly susceptible to smallpox, first introduced into the New World by the Spanish Conquistadors in the early 1500s. Cortez and Pizarro unwittingly took advantage of this phenomenon to subjugate the Aztec and Inca Empires, respectively. In 1763 Lord Jeffrey Amherst, commander of British troops in North America during the French and Indian War took this knowledge a step further, using smallpox as a biological weapon against the hostile Native American forces in Pennsylvania. Blankets were deliberately contaminated with the scabs from smallpox victims and left for the Indians in wintertime. Whether acquired from the fomites or via human-to-human transmission, smallpox devastated the Indians who had sided with the French forces, contributing to the British victory (Diamond 1999).

Back in the Old World a dramatic epidemic of another kind was underway. Shortly after Columbus’ first return voyage in 1493, an epidemic of “great pox” occurred throughout much of Europe. “Great pox” aptly described the clinical appearance of the cutaneous lesions of secondary syphilis, in contradistinction to the familiar appearance of smallpox. While it is possible, even likely, that some of Columbus’ crew contributed to the spread of syphilis throughout Europe, they were likely the vector, rather than the original source of infection. Skeletal remains found in both Britain and Greece and dated well before Columbus made his famous voyage carry the unmistakable stigmata of the osseous forms of tertiary syphilis. It is likely that syphilis existed in Europe prior to Columbus in relatively rare and localized forms, arriving from the Mediterranean via trade routes established centuries earlier. After the defeat of the Islamic Moors in the Battle of Granada in 1492, a Papal order closed all leprosaria, institutions that probably housed numerous, misdiagnosed, syphilitic patients within their confines. Release of these highly infectious individuals, coupled with the rampant prostitution practices of the time, likely contributed to the spread of the disease across Europe. This newly recognized and highly virulent form of syphilis continued to be epidemic into the first half of the sixteenth century (Sherman 2007).

The Renaissance brought forth the Age of Enlightenment with its remarkable advances in science and the arts, adding to the major advances that had already occurred in the first and early second millennia AD in China, India, Persia, and the

Islamic world. Although the fundamental principles of the scientific method were originally described by the Franciscan monk Roger Bacon in 1269, multiple factors limited the work of scientists and intellectuals during the ensuing 400 years. For the areas of microbiology and immunology the lack of tools and techniques to adequately study microscopic events was the major impediment.

Using his powers of observation and knowledge of epidemics, the Italian physician Girolamo Fracastoro, or Hieronymous Fracastorius, had written a treatise on the germ theory of disease entitled “*de Contagione*” in 1546. Fracastorius correctly surmised that tiny, free-living organisms, which he referred to as “seeds of disease,” existed in nature. Despite being invisible to human eyes, he postulated that these disease-causing organisms could be transmitted from person-to-person directly or via fomite intermediaries, thereby spreading contagion (Gensini and Conti 2004). He correctly surmised that syphilis was caused by such a microscopic organism. In his poem entitled “*Syphilis sive Morbus Gallicus*” (translated “Syphilis or the French Disease”) he described in remarkably accurate, yet mythical, poetic detail the clinical consequences of syphilis (Conrad et al. 1995). The Italians blamed syphilis on the French, hence the name “the French Disease”; the French on the other hand referred to it as “the Italian Disease.” This pattern of naming the syphilis epidemics based on local, political, or religious adversaries continued as the scourge spread throughout the western world and the Middle East (Sherman 2007).

The Dutch textile merchant and self-taught scientist, Antonie van Leeuwenhoek (1632–1723) is credited with first identifying microorganisms, or “little animals,” using his newly developed microscope in 1677, thereby confirming Fracastoro’s hypothesis (Corliss 2002). The critical significance of these tiny forms to human health was not fully appreciated until almost 200 years later when Pasteur and Koch first successfully cultured bacterial organisms from diseased tissues. Despite the technical shortcomings in the period between van Leeuwenhoek (Fig. 3.2) and Pasteur, a number of scientists and physicians correctly hypothesized the existence of microscopic organisms and their contribution to human disease.

Regrettably, theories of contagion still lacked the tools enabling scientific proof, and therefore the warnings of disease pathogenesis were largely ignored, often with tragic consequences. The Viennese physician Marcus Plenciz presented a lucid explanation for clinical observations made up to that time, proposing a germ theory of disease as early as 1762. Subsequently, Jakob Henle, a noted German physician and anatomist, further advanced the germ theory concept in 1840 (Gensini and Conti 2004). Such theories were still ahead of scientific technologies for their validation; however, empiric evidence supporting these ideas mounted dramatically with the seminal observations of two European physicians in the mid-nineteenth century.

### 3.3 Mounting Evidence for the Germ Theory of Disease

In the early 1840s a young Hungarian obstetrician embarked on an area of scientific investigation, informed by a series of observations that would eventually revolutionize the concept of disease causation (Wyklicky and Skopec 1983). Ignaz



**Fig. 3.2** Antony van Leeuwenhoek (Rijksmuseum, The Netherlands)

Semmelweis (1818–1865) was a faculty member of the Lying-In Hospital in Vienna, Austria, which consisted of two obstetrical services that alternated admissions on a daily basis. The first service was operated by physicians and medical students; the second by midwives. The mortality rate for puerperal or “child bed” fever was such that one out of ten pregnant women could be expected to die shortly after birth from this dreaded complication. Semmelweis (Fig. 3.3) observed that the mortality rate was almost tenfold higher in the physician service as compared to the second service (Nuland 1979). He recognized that the putrid odor associated with women dying of puerperal fever was similar to that emanating from corpses during autopsies by the medical faculty and students.

Autopsies were a critically important component of medical education of the time; they were employed as a primary tool to teach anatomy and pathology to medical students. Semmelweis noted that the same malodorous smell was found on the hands of doctors and students moving from the autopsy room to the labor and delivery rooms. He also observed that the death rate from puerperal fever in the physicians’ clinic decreased significantly when the medical students were on vacation and no autopsies were being performed. Lastly, he witnessed the death of one



Ignaz Philipp Semmelweis (1818–65).

**Fig. 3.3** Ignaz Semmelweiss (Wellcome Library)

of his close friends, Jakob Kolletschka, a pathologist who died shortly after cutting his finger during an autopsy of a woman who had recently died from puerperal fever. He correctly hypothesized that some form of “putrid matter” must be carried on the hands of physicians during their rounds between the autopsy and birthing tables and might be transmitted to pregnant women causing this highly lethal peripartum illness (Jones 1970).

Semmelweis made these observations with no formal training in microbiology, as the latter did not exist as a distinct area of science at the time. In fact the germ theory of disease was not taught in medical schools in Europe or elsewhere. Semmelweis found that washing hands using a dilute, chlorinated lime solution after performing autopsies would remove the putrid odor. Based on his empiric observations but lacking definitive proof of his hypotheses, he boldly introduced a policy whereby all medical students and faculty were required to wash their hands in this solution before having contact with patients. In 1847 Semmelweis showed that the introduction of hand washing between patient contacts reduced the mortality rate from puerperal fever by fourfold in 1 year (Wyklicky and Skopec 1983).

As seen throughout history, innovative ideas that contradict prevailing wisdom are vulnerable to immediate rejection; additionally, Semmelweis was guilty of poor timing. Although he had demonstrated the benefit of a simple intervention, it occurred at a moment of great geopolitical turmoil and was met with considerable acrimony, much of it politically motivated. He experienced profound, negative professional and personal consequences of his work. By 1848 the concept of revolution was spreading throughout Europe; within the Hapsburg Empire, of which Austria and Hungary were a part, the tenuous Dual Monarchy was at risk of crumbling under the separatist demands of Hungarian nationalists. A wave of political and social conservatism took hold in Austria. When this young, talented Hungarian faculty physician with his radical new ideas about health care came up for reappointment, he was passed over and forced to resign. He returned to Hungary where his novel prevention strategy against puerperal fever was implemented with success.

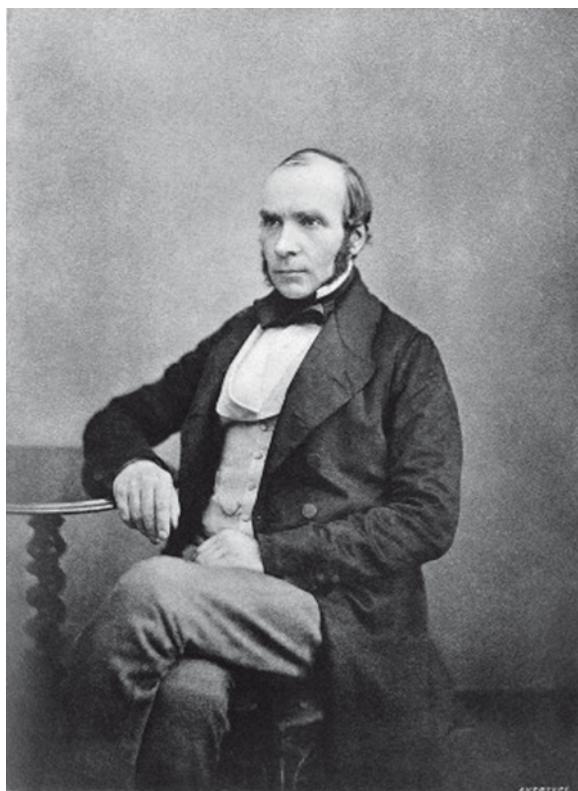
Semmelweis failed to optimize his position in Vienna. Because his oratory and literary skills in German were inadequate, it was difficult for him to effectively communicate his ideas to colleagues (Nuland 1979). Additionally, he was by reputation dogmatic and inflexible, traits that further alienated him from his peers. It did not help matters that it took him over a decade to write the definitive review of his investigations into the etiology and prevention of puerperal fever and when the manuscript was finally produced in 1861, it was a rambling, confused report that convinced few of his skeptics and was roundly criticized as being poorly formulated and unscientific. Semmelweis countered with a series of harsh diatribes against his critics, essentially accusing his fellow physicians of killing their patients through negligence and intransigence to his new ideas about hand washing. His behavior in public and private became increasingly erratic; he fell into a deep melancholy eventually resulting in his involuntary commitment to an insane asylum. The final details of his demise remain shrouded in mystery; he apparently died of bacterial sepsis from injuries sustained when he attempted to escape from this mental institution.

Semmelweis died at the age of forty-seven, never seeing his radical notions regarding transmissible microscopic organisms as the cause of disease and hand hygiene as its solution being widely acknowledged or appreciated by the medical or scientific communities. He stood firm until his final days: “In a word, the carrier is anything contaminated with decomposed animal organic material that comes in contact with the vaginal tract of the parturient. If I shall be denied the privilege of seeing with my own eyes the conquest of puerperal fever, the conviction that sooner or later this thesis will find acceptance, will cheer my hour of death.” (Wangesteen and Wangesteen 1978).

Epidemiologic evidence of microorganisms as a cause of human disease was being observed in community outbreaks as well as in hospital wards. In the late 1840s and early 1850s large, community-wide outbreaks of cholera gained much public attention. Massive population expansion into overcrowded, poorly hygienic, urban areas had occurred throughout the nineteenth century as a result of the industrial revolution. Although the flush toilet had been patented in 1819, it was not in widespread use, and the effluent from toilets and public privies was deposited into local rivers, converting municipal sources of drinking water into open sewers.

In 1849, a prominent London physician, John Snow (1813–1858), published a pamphlet in which he speculated that cholera was a waterborne or foodborne, intestinal illness (Snow 1855). In so doing he directly challenged the prevailing “miasma theory” that cholera and other diseases resulted from bad air. Such thought was widely accepted at the time through traditional teachings and the influential experimental work of the German chemist Max von Pettenkoffer. In 1854 a cholera outbreak occurred in London that provided compelling evidence in favor of Snow’s alternative hypothesis.

Snow (Fig. 3.4) carefully mapped the incident cases of cholera in the residents of downtown London and noted their proximity to public water-drawing sites. He observed that the highest incidence of disease was centered at the corner of Broad and



*John Snow*

(Autotype from a Preparation Portrait, 1856, and Autograph  
facsimile.—J. W. R.)

**Fig. 3.4** John Snow (Wellcome Library)

Cambridge Streets, the site of a pumping station for drinking water. The water intake for this pump was drawn from a location just downstream of a large sewer effluent from London in the Thames River. Using interviews of cases and contacts and statistical assessments, methods that would become standard fare for future outbreak investigators but were novel at the time, Snow deduced that the infection was transmitted by contaminated water. As a result of his evidence, the handle was removed from the Broad Street pump forcing local residents to seek water from other pumping stations. The epidemic, probably already waning, was halted. Snow is appropriately credited as the founding father of the field of epidemiology based on this work. Although he microscopically examined the contaminated water supplies and observed “small, white flocculent particles” that he speculated were the causative agent of cholera (Johnson 2002), he never obtained definitive microbiologic proof.

Snow’s recommendations included a number of other sanitation maneuvers, such as washing the clothes and bed linens of cases, isolation of sick people from healthy ones, and boiling water supplies; all of these helped to curtail further cases of cholera. It would take another 30 years before Koch and his colleagues finally isolated *Vibrio cholerae*, the etiologic agent of this dread epidemic disease (Snow 1855; Sherman 2007). Nonetheless, through careful epidemiologic study, Snow had been able to infer an understanding of the possible etiology of cholera and implement effective public health measures to prevent future outbreaks.

Around the time Semmelweis was making his seminal observations on an obstetrical infection in Vienna, the English botanist and clergyman, Reverend Miles J. Berkeley was unraveling the mysterious etiology of another devastating infection with major socioeconomic implications, the potato blight, and lending further support to the growing body of evidence in favor of the germ theory of disease. Berkeley, a mycology expert, noted the unmistakable presence of microscopic mold elements in diseased plants in 1846. The potato blight would, over the next few years, lead to the death of one million Irish and result in the mass emigration of approximately two million of their countrymen from their homeland, never to return (Sherman 2007). Berkeley’s observations were predictably mocked by the scientific community, as it was generally accepted at the time that the potato blight was due to cold and damp “miasma.” In 1861, the same year that Semmelweis wrote his now famous if flawed paper on puerperal fever, Anton de Bary, a German plant pathologist and mycologist, conclusively proved that the etiology of potato blight was in fact a fungus –*Phytophthora infestans* (literally meaning “the plant destroyer”) – by essentially following the same lines of scientific reasoning that would set the standard for microbial causation two decades later in a Berlin tuberculosis laboratory.

### 3.4 Microbiology Comes of Age: Louis Pasteur

The actual inception of microbiology as a distinct science traditionally dates to 1857, when Louis Pasteur (1822–1895) convincingly demonstrated that microorganisms were responsible for the fermentation of fluids, although incremental,

significant advances in the field had occurred in the intervening period since van Leeuwenhoek's observations using microscopy (Wainright 2001). Pasteur's work debunked the extant theory of "spontaneous generation" and showed instead that fermentation, spoiling, or contamination of organic substances was due to the presence of environmental microorganisms (Johnson 2002). With these investigations Pasteur (Fig. 3.5) essentially proved the germ theory of disease and launched the field of modern microbiology.

Although the germ theory of disease had its renowned proponents, including Jakob Henle and Edwin Klebs, both German physicians and contemporaries of Pasteur, it also attracted many influential detractors. Using early prototype microscopes, van Leeuwenhoek and Robert Hooke had clearly demonstrated the presence of unicellular protozoan and tiny bacterial organisms – the "little animalcules" – as early as 1677 (Gest 2007). Plant pathologists and mycologists had already demonstrated the essential role of microorganisms as the cause of selected diseases in plants. Yet it was still unproven whether microorganisms could actually cause human diseases. Moreover, debate smoldered as to whether these organisms arose spontaneously from substances already present in devitalized tissue or whether they derived from exogenous sources and had to be implanted to cause disease.

One of Pasteur's foremost, contemporary critics was Archimé de Pouchet, Director of the Natural History Museum in France and one of the main advocates of spontaneous generation. Owing to the scientific and even political importance of the debate, the French Academy of Sciences offered a monetary prize in 1862 to



**Fig. 3.5** Louis Pasteur (Institut Pasteur)

the scientist who could provide definitive evidence to either prove or disprove the concept of spontaneous generation. Pasteur accepted the challenge and won the award through a series of elegant and carefully executed experiments that eliminated the possibility of spontaneous generation. He showed that heat sterilization, chemical sterilization, or filtration of air and water could maintain organic materials in sterile conditions indefinitely without any microbial growth (Debré 1998).

Techniques of sterilization and “Pasteurization” of dairy products were soon introduced and undoubtedly saved millions of lives in the period that followed. Pasteur established the Pasteur Institute through a combination of major private financing and public monies. The Institute soon became an international center for microbiology, immunology, and medicine, largely due to the efforts of Louis Pasteur himself.

Pasteur’s work inspired the British surgeon Joseph Lister (1827–1912) to attempt to use sterile methods to protect the wounds of trauma patients at the orthopedic infirmary in Glasgow, Scotland in 1867. Realizing that universal air filtration or heating the patient to maintain sterility were impractical clinical options, Lister (Fig. 3.6) investigated the use of chemical disinfectants as a method of preventing wound infections. Based on the discovery by local farmers that carbolic acid decreased the fetid odor of the common fertilizer “night soil” (i.e. human excreta), Lister demonstrated the value of dilute solutions of this chemical in maintaining the sterility of dressings, surgical instruments, and the hands of surgeons caring for injured patients (Harding-Rains 1977).

Lister’s findings were favorably received by the scientific community, and the use of sterile technique in the care of surgical patients was adopted as an international standard (Bynum 1994). Lister’s work was accepted, and he succeeded in establishing principles of antisepsis where his predecessors, most notably Semmelweis, had failed because the germ theory of disease had by this point garnered widespread acceptance through the efforts of Pasteur (Wangesteen and Wangesteen 1978).

Pasteur’s celebrity and stature within the scientific community attracted talent from many parts of the world. He surrounded himself with a large number of dedicated and capable investigators, thereby greatly enhancing the prestige of the Institute that bore his name. A number of his students, assistants, and colleagues made major contributions to the fields of infectious diseases, microbiology, and immunity, including Charles Chamberland who invented the autoclave, a water purification device that was later used in the discovery of viruses, and developed a *Pasteurella* vaccine; Alexandre Yersin, co-discoverer of the plague bacillus; Emile Roux who discovered diphtheria toxin and antitoxin; Jules Bordet, who discovered the whooping cough bacillus and complement; Ilya Metchnikoff, who discovered the process of phagocytosis and provided the initial descriptions of innate immunity; and Albert Calmette, who discovered cobra antivenin and developed Bacillus-Calmette-Guérin, the first effective tuberculosis vaccine (Debré 1998).

Pasteur used his powers of experimental observation to move the burgeoning field of microbiology to its logical next steps – protection against pathogens. In an ironic nod to his own axiom concerning chance favoring the prepared mind, established earlier in his industrial chemistry career, Pasteur serendipitously discovered the phenomenon of laboratory attenuation of microorganisms and was able to



**Fig. 3.6** Joseph Lister (Wellcome Library)

extrapolate his findings as a means of developing targeted vaccines. In 1879, Pasteur observed that after serial passage the chicken cholera bacillus, now known as *Pasteurella spp.*, lost the capacity to cause lethality when injected into chickens. Because chickens were in short supply in the laboratory, Pasteur was forced to recycle the same animals in subsequent experiments using freshly passed and highly virulent strains of bacteria. Remarkably, the chickens previously exposed to attenuated bacilli survived infection with virulent strains, whereas naïve chickens died rapidly upon challenge. He surmised that serial passage of the bacteria at certain elevated temperature ranges and in the presence of oxygen resulted in organisms that could induce resistance to challenge using virulent forms of the same bacteria.

Pasteur recognized that this technique of “artificial attenuation” could replace the need to identify naturally attenuated microorganisms, as Jenner had done with cowpox in milkmaids, and that this phenomenon could revolutionize the concept of vaccines. This finding, perhaps more than any other since Jenner’s, opened up a

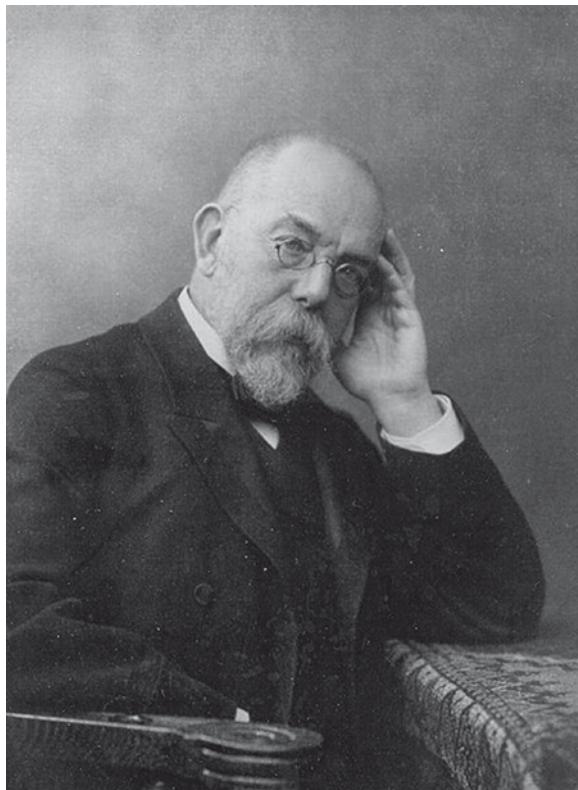
new epoch in the battle against communicable diseases, one in which the microbiology laboratory performed a pivotal function. With this technology, Pasteur rapidly developed successful vaccines against anthrax in 1881 and rabies in 1885.

### 3.5 Robert Koch and the Berlin School of Microbiology

Even as Paris was fast becoming the center of research in the nascent field of microbiology, a country doctor from Prussia was beginning his career in microbiology essentially as a weekend hobby. Robert Koch (1843–1910) studied medicine at the University at Göttingen where he came under the influence of the notable Professor of Anatomy Jakob Henle, an early proponent of the germ theory of disease, and learned the importance of careful animal experimentation in understanding disease causation. In the 1870s, as a district medical officer in the Prussian town of Wollstein, Koch (Fig. 3.7) began his investigations into the etiology of anthrax in sheep; this marked the beginning of a distinguished career in scientific research (Brock 1988). He identified anthrax bacilli in the blood of infected sheep and successfully transmitted the infection into healthy experimental animals. Using careful photomicroscopy and detailed drawings, he accurately described the life cycle of anthrax and the process of endospore formation. With the publication of this work in 1876, Koch became a major force in the fledgling field of microbiology.

Koch pioneered a number of laboratory techniques. He employed the use of the oil immersion microscope to study bacteria; developed new staining methods for bacterial identification; and he invented procedures for the isolation of pure bacterial cultures on solid media, the latter facilitated by the use of agar as the solidifying agent in flat “Petri” dishes, named after their inventor, Richard Petri (a colleague of Koch) and still in common use today. To obtain pure growth he insisted upon the use of single colony isolation, “the Koch plate technique” (Kaufmann and Winau 2005), acclaimed by even his rival and eventual antagonist, Pasteur, who was noted to remark, “C'est un grand progress, monsieur”(Brock 1988).

While serving as a senior medical officer in the Imperial Health Office in Berlin in 1882, Koch discovered the microbial etiology of tuberculosis, perhaps the most important disease cause of death at the time, making his a household name (Ryan 1992). Using differential staining techniques, careful microscopy, and solid agar methods, Koch isolated the causative agent, *Mycobacterium tuberculosis*, in pure culture (Dubos and Dubos 1956). It was in this context that he initially proposed a set of criteria that had to be satisfied to infer an etiologic role for a specific bacterial agent in a particular disease. These conditions came to be known as “Koch's Postulates” and were eventually refined by Koch: the pathogen accounts for the clinical and pathological features of the disease and must be found in every case in which the disease occurs; the pathogen is not found in other diseases as a non-pathogen; after being isolated from the body and repeatedly passed in pure culture, the pathogen can induce the disease in animal models; and the same pathogen must be re-isolated from the experimental animal (Brock 1988). These criteria remained



**Fig. 3.7** Robert Koch (Robert Koch Institute)

the gold standard upon which to judge evidence of microbial disease causation and are still valid to some extent today.

Koch, like Pasteur, surrounded himself with brilliant colleagues and collaborators and simultaneously attracted strong supporters and equally vocal detractors. Contemporary physicians who rejected the germ theory in favor of other theories of disease causation included Max von Pettenkoffer, the influential Munich hygienist and the celebrated cellular pathologist Rudolf Virchow. Pettenkoffer espoused the “sanitation theory” of disease, widely supported by social liberals, that poor sanitation, unfavorable water, soil conditions, and damp weather generated miasma poisons that subsequently caused illness, primarily in socio-economically disadvantaged populations. The cure for epidemics was therefore social progress and the elimination of poverty. Virchow, considered to be the founding father of cellular pathology and the most respected academic physician in Germany during Koch’s era, remained an ardent opponent of the germ theory of infectious diseases; he never completely embraced Koch’s discovery of the tubercle bacillus, despite the overwhelming scientific evidence (Brock 1988; Kaufman and Winau 2005).

However, the germ theory of disease was embraced by the conservative Prussian government in Berlin largely because the fundamental premise was that communicable diseases were the consequences of exogenous microorganisms invading the body, circumstances that were largely independent of socioeconomics. Pathogen control could then be viewed as possible with central governmental controls, without having to address all the ills of society. The Prussian Parliament supported Koch's work with lavish funding for the Koch Institute for Infectious Diseases, which opened in Berlin in 1891 (Brock 1988). The government's interests were more than altruistic; these were fervently nationalistic times. When Koch's team succeeded in isolating the *Vibrio* etiology of cholera in Egypt after Pasteur's group had failed, the German government hailed it as proof of the superiority of German science over French science, and Koch was welcomed back to Berlin with a hero's procession (Brock 1988; Kaufmann and Winau 2005).

Whereas Virchow eventually capitulated to at least public acceptance of Koch's theories (he returned to Berlin after The Pathological Institute was built for him on the grounds of Koch's Institute), Pettenkoffer remained a vocal skeptic of the germ theory, even in the face of overwhelming evidence. He famously ingested a culture dose of *Vibrio cholerae* from Koch's laboratory, claiming not to become ill from the disease and offering this as proof that the bacillus was not the etiologic agent of cholera. Subsequent reports suggested that Pettenkoffer did experience mild diarrhea after this oral challenge, probably the result of partial immunity from a previous bout of cholera a few years earlier (Brock 1988). History clearly sided with Koch, Pasteur, and their supporters (Sherman 2007).

A serious rift developed between the two great contemporary microbiologists, Pasteur and Koch, during this time. They were each staunch patriots in a period of strident nationalism throughout Europe; enmity between their respective countries was firmly entrenched after the French defeat in the Franco-Prussian War in 1870. But a variety of other philosophical, cultural, and scientific differences existed between these two men. Although competition in the realm of science can be healthy and provide the impetus for discovery, adversarial competition can lead to secrecy and suspicion, thus impeding scientific progress. The tempestuous relationship between Pasteur and Koch vacillated between healthy and unhealthy competition throughout their careers. Fortunately, many of their coworkers were able to maintain more reasoned and collegial professional relationships (Dubos and Dubos 1956; Kaufmann and Winau 2005).

Some of the antagonism between Pasteur and Koch was based on miscommunication. Neither spoke the other's language, setting the stage for errors in translation. At the Fourth International Congress of Hygiene and Demography in Geneva in 1882, each of these supremely accomplished scientists felt personally insulted by the other's public remarks; in both instances there appeared to be no malicious intent (Brock 1988). The result was a series of vitriolic verbal and written exchanges that played out during the 1880s at scientific conferences and in the literature. The controversy had largely subsided by 1890, although Koch was conspicuously absent from the world's celebration of Pasteur's seventieth birthday in 1892 (Brock 1988).

**Table 3.1** Fundamental differences between Koch and Pasteur

Robert Koch/Berlin School	Louis Pasteur/Institute Pasteur
Medical Training (in the MD tradition)	Scientific Training as a Chemist (in the PH.D. tradition)
Microorganisms are pathogens, one pathogen – one disease	Microorganisms are living things, some are favorable, some are pathogenic
Microorganisms need to be eliminated (from his experience caring for war wounds in the Franco-Prussian War)	Microorganisms need to be instructed and can perform useful functions for humankind (fermentation)
The “Linnaean” Approach: Microorganisms are immutable species that could not and should not be changed (The Monomorphic hypothesis)	The “Unitarian” Approach: Microorganisms are mutable and can be modified. (The Pleomorphic hypothesis of adaptation and mutability)
Use solid medium for single colony isolation is required to avoid contamination	Use of broth cultures with mixed communities of microorganisms are important to study
Darwinian concept of evolution by Random variation and natural selection	The Lamarckian view of “directed evolution of favorable characteristics”
“Humoralists”-promoted serum therapy for infectious diseases	“Cellularists” promoted cellular and phagocytic defenses against infection

Aside from their respective issues of national pride, Pasteur and Koch harbored major differences in their styles and scientific approaches (Table 3.1). Pasteur favored a vaccination approach to infectious diseases; Koch believed in a more population-based, public health approach to the problem. Nonetheless, their actions, in many ways, belied their mutual, if muted scientific respect; the Institute in Berlin, for example, was predicated on the Pasteur Institute.

Koch’s Institute flourished, attracting a superb group of investigators and collaborators to the fields of microbiology and immunotherapy. Notables included Paul Ehrlich, co-discoverer of antibodies, antigens, and chemotherapy for infectious diseases; Richard Pfeiffer, who discovered bacterial endotoxin, the phenomenon of bacteriolysis, and played a major role in the development of killed typhoid vaccines; Emil von Behring, discoverer of serum therapy for diphtheria and tetanus; and Shibasaburo Kitasato and Sakahiro Hata, Japanese scientists who made important contributions to serum therapy and the discovery of salvarsan for the treatment of syphilis, respectively (Brock 1988). Koch’s standard methodologies for bacteriology still continue to be used in clinical microbiology laboratories today, and though his classic “postulates” have been revised and revisited on numerous occasions (Relman et al. 1992; Fredricks and Relman 1996), Koch and his scientific rival Pasteur remain the two most influential figures in the history of microbiology (Kaufmann and Winau 2005).

Following on the heels of the landmark discoveries in the area of bacteriology by Pasteur and Koch, advances in other disciplines of microbiology such as mycology, parasitology, and virology developed at a rapid pace beginning in the late nineteenth century and continuing throughout much of the twentieth. In 1870, Patrick Manson, a Scottish physician working on tropical diseases in the Far East, confirmed the presence of microscopic parasites in mosquito vectors of filariasis. This discovery eventually led the British physician Ronald Ross, working in India, to definitively

prove the parasitic nature of malaria and its transmission by mosquitoes in 1896 (Sherman 2007). Contemporaneously, agricultural scientists in the Netherlands and Russia discovered the “filterable agent” responsible for tobacco mosaic disease (Mayer 1886; Ivanowski 1892; Beijerinck 1898). These infectious particles were capable of passing through submicron filters that were known to capture bacteria; hence, a new discipline within microbiology was founded based on sub-microscopic entities that did not completely conform to Koch’s well-accepted scientific dogma. The history of virology and advances in laboratory methodologies for cultivating these microorganisms are considered in detail in Chapter 9.

### 3.6 Modern Advances in Microbiology

The history of microbiology in the twentieth century was dominated by research discoveries in genetics, nucleic acid biochemistry, and molecular biology. Since Charles Darwin’s description of natural selection and variation and Gregor Mendel’s work in defining the laws of genetics in the mid-nineteenth century, scientists had sought the biochemical basis for genes that determine the destiny of life forms on earth. Oswald Avery, Maclyn McCarty, and Colin MacLeod, working at the Rockefeller Institute identified the “holy grail” of genetics in 1944 with their finding that the “transforming principle” or genetic material of *Streptococcus pneumoniae* was deoxyribonucleic acid (DNA), not protein as previously postulated (Lederberg 1994). This observation led to the elucidation of the structure of DNA in 1953 by James Watson, Francis Crick, Rosalind Franklin, and Maurice Wilkins (Watson 1968), which in turn led to the deciphering of the genetic code by the former two scientists and ushered in the modern era of molecular biology. The first complete genomic sequencing, that of a bacteriophage was accomplished in 1977 (Sanger et al. 1982); that of a free-living organism, *Haemophilus influenzae* Rd, was accomplished nearly two decades later (Fleischmann et al. 1995), followed shortly thereafter by the first draft of the human genome in 2001 (Altshuler 1995; Venter et al. 2001). Recent advances in microbiology, including the development of recombinant DNA technology, the polymerase chain reaction, and monoclonal antibodies have revolutionized clinical microbiology and permitted the use of non-culture methods to diagnose fastidious or non-cultivable organisms such as hepatitis C, *Trophycyema whippelii*, and a variety of other organisms that likely contribute to human disease (Fredricks and Relman 1996).

### 3.7 A Brief History of Immunology

Understanding the basic elements of the human immune response evolved rapidly in parallel with the acceptance of the germ theory of disease. The innate immune system evolved in multicellular organisms to defend against invasion by microorganisms.

Adaptive or acquired immunity evolved relatively late in vertebrate evolution through the acquisition of large retro-transposons within the genome to accommodate the increasing longevity of complex organisms and to provide long term immunologic memory against potential pathogens to which the host has had previous immunologic exposure. The mechanisms that underlie the capacity of the host to orchestrate an appropriate immune defense have been the focus of research for generations of scientists. Major milestones in the history of immunology are illustrated in Fig. 3.8.

The inception of immunology as a distinct discipline has its origin in the late nineteenth century with the development of the cell-mediated and the humoral immune theories of host defense. Ilya Metchnikoff (1845–1916) is credited with first recognizing phagocytosis as an important cellular defense strategy (Ambrose 2006). Metchnikoff (Fig. 3.9), a comparative zoologist from the village of Kharkov in modern-day Ukraine, reasoned that this highly advantageous host defense he observed in starfish mesenchymal cells would be found in higher species as well (Silverstein 2003). Aware of the potential significance of his findings, he changed his career path to human pathology and microbiology. With colleagues at the Pasteur Institute, Metchnikoff confirmed that phagocytosis by neutrophils (“microcytes”) and macrophages was an essential part of the innate immune response in humans. He promulgated the idea of cell-mediated immunity as a defense against specific sets of microbial pathogens in 1884.

German physicians Emil von Behring (1854–1917) and Paul Ehrlich (1854–1915), both assistants in Koch’s Institute of Hygiene laboratory in Berlin in 1890, recognized that serum factors prevented lethality from bacterial toxins such as tetanus and diphtheria (Jaryal 2001). These factors, termed “antitoxins” were subsequently shown to be antibodies; Behring (Fig. 3.10) and Ehrlich (Fig. 3.11) demonstrated that protection could be passively transferred from one animal to another using serum alone. This formed the basis for the use of serum therapy for toxin-mediated infectious diseases, a strategy that became widely used by both the Koch and Pasteur groups. Behring was awarded the inaugural the Nobel Prize in 1901 for his work on immune therapy; Ehrlich and Metchnikoff shared the Nobel Prize in 1908 for their descriptions of humoral and cellular immunity, respectively (Silverstein 2005; Gensini et al. 2007).

Another fundamental aspect of humoral immunity was discovered by Jules Bordet, a Belgian physician working in Metchnikoff’s laboratory at the Pasteur Institute in 1896, who first identified a heat labile serum factor that contributed to the protection induced by antibodies during the process of serum therapy. Ehrlich similarly observed this property and referred to it as “complement” to describe its complementary effect on the activity of antibodies (Walport 2001). It would take nearly another century for this phenomenon to be fully elucidated (Pillemer et al. 1956; Super et al. 1989).

One of the fundamental problems facing early immunologists was providing an explanation of how a seemingly infinite repertoire of diverse antibodies could be generated to maintain adaptive immunity against myriad potential human pathogens and their antigens. None of the theories advanced in the early part of the twentieth century adequately explicated experimental observations regarding antibody diversity (Weiser et al. 1969).

## Major Milestones in Immunology

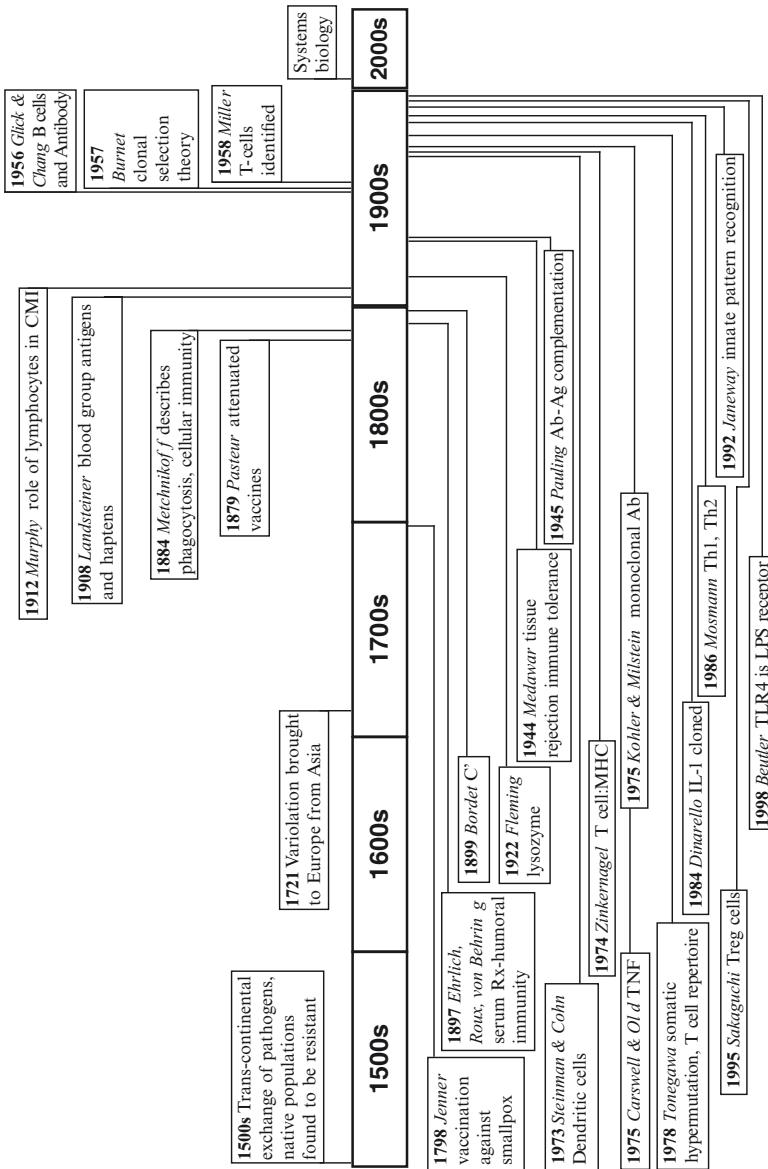
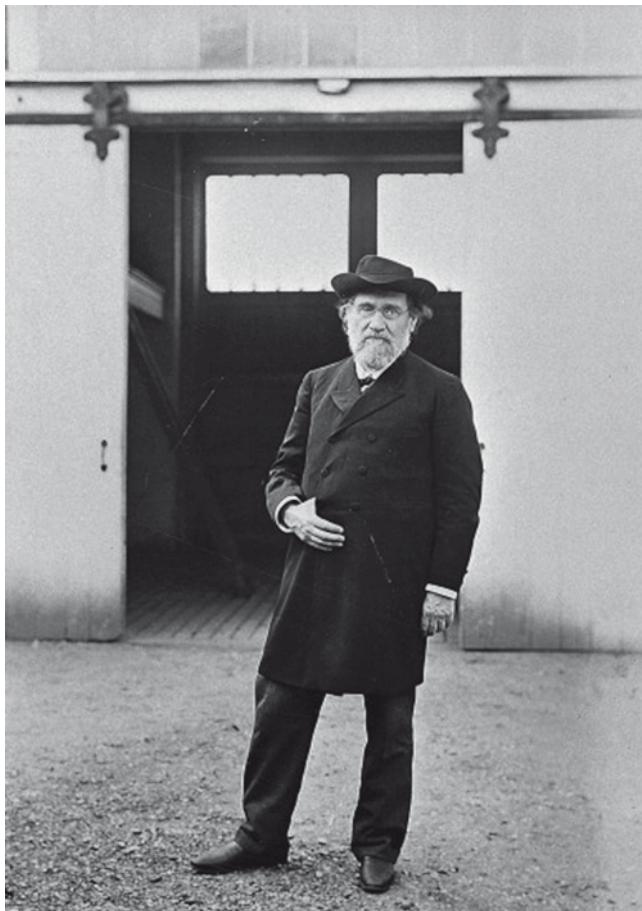


Fig. 3.8 Milestones in the History of Immunology



**Fig. 3.9** Elie Metchnikoff (Wellcome Library)

Ehrlich first proposed the selection or “side chain” theory to explain antibody diversity. He hypothesized that specialized, inducible cells of the immune system existed with antibody-like molecules on their surfaces. Upon coming in contact with a relevant antigen, cells with the highest binding affinity on the side chains of their surface antibodies would be selected, become stimulated and proliferate, releasing antibodies into the circulation. While Karl Landsteiner’s work in the early twentieth century questioned the plausibility that the human body could respond to the array of potential antigens found in the environment in this manner (Figl and Pelinka 2004), Australian virologist-turned-immunologist F. Macfarlane Burnet proposed an alternative hypothesis in 1956 based on modifications of the theories of Danish immunologist Niels Jerne. Burnet’s clonal selection theory, describing the activation, clonal proliferation, and subsequent targeted antibody secretion of



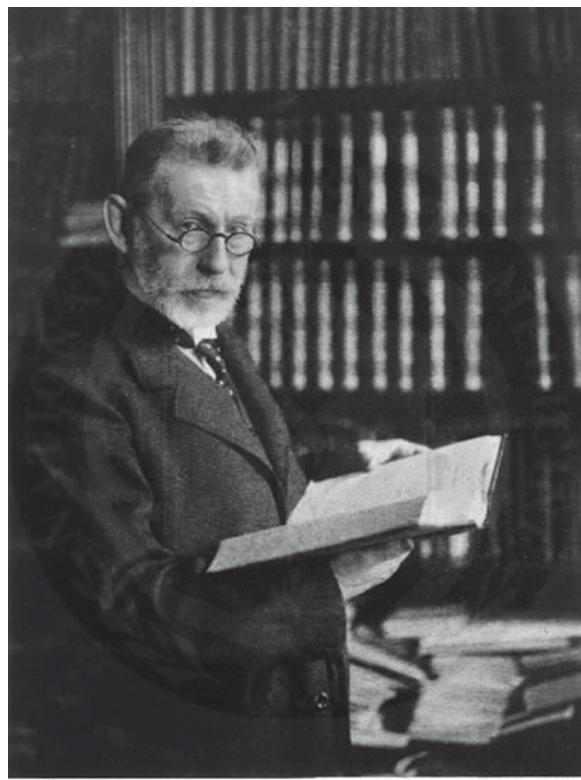
**Fig. 3.10** Emil von Behring (right) (Robert Koch Institute)

lymphocytes after binding to a matched antigen, reconciled experimental observations and was subsequently shown to be the correct explanation for the generation of antibody diversity (Burnet 1957).

Relatively rapid progress in elucidating the functional aspects of the human immune system was initiated with the discovery of the origins of B cells and T cells in the 1950s by Ohio State University graduate students Bruce Glick and Timothy Chang who serendipitously identified the bursa of Fabricius as the site of antibody formation in chickens (Chang et al. 1955; Glick 1955; Adelman 1967; Ribatti et al. 2006). Soon thereafter Francis Miller demonstrated that cell-mediated immune responses required thymic conditioning (Ribatti et al. 1965), and that thymectomy depleted the lymphoid organs of lymphocytes and abrogated these responses (Cooper et al. 1966).

The identification of human disease equivalents to B cell and T cell deficiencies of experimental animals (Stehm and Johnston 2005; Peterson 2007) introduced a new era of cellular immunology (Silverstein 2001). Novel revelations emerged in rapid succession: the essential role of lymphocytes in allograft rejection and the fundamental nature of immune tolerance (Steinman 2007); subtyping and quantitation of T cells and B cells in the mid 1970s (Köhler and Milstein 1975); the role of natural killer cells and regulatory T cells (Sakaguchi et al. 2007); the details of antigen processing (Gordon 2007), presentation, and T cell signaling by macrophages (Zinkernagel and Doherty 1974); the critical interactions between T and B cells (Claman and Chaperon 1969); and the role of dendritic cells in antigen presentation (Steinmann and Cohn 1973).

**Fig. 3.11** Paul Ehrlich  
(Wellcome Library)



*P. Ehrlich*

FOTOMAR/SCIENCE SOURCE

The last decades of the twentieth century witnessed renewed interest in the role of the innate immune system in host defense upon initial encounter with potential pathogens. The discovery of pro-inflammatory cytokines such as tumor necrosis factor (Carswell et al. 1975) and interleukin-1 (Auron et al. 1984) were major milestones in understanding immune cell signaling and response, a field advanced further by the discovery of the toll-like receptors (TLRs) in the early 1990s (Beutler et al. 2006). The definition of TLR4 as the lipopolysaccharide receptor capped a century-long search for the receptor for bacterial endotoxin and brought immunology full circle back to its bacteriologic roots (Beutler and Poltorak 2000).

### 3.8 Summary and Conclusions

Perhaps no other developments have had such a major impact on the health and welfare of humankind than those comprising the history of microbiology and immunology. Over the last 100 years, the mortality burden of infectious diseases

has decreased substantially and the average lifespan has increased by over 30 years due to advances in public health, sanitation, vaccines, and anti-infective chemotherapy – all deriving from the sciences of microbiology and immunology (Centers for Disease Control 1999). Future advances are anticipated when the genomics era in which we now live and the promise of systems biology and personalized medicine are fully realized in the next few decades. A remarkable story of directed inquiry into the fundamental nature of microbes and immune defenses preceded many of the current advances in medicine. Much work remains before the benefits of these discoveries can be applied equally worldwide.

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# **Chapter 4**

## **Anthrax**

**Peter C.B. Turnbull**

### **4.1 History of the Disease**

Microbiologists are generally familiar with the statement that anthrax has been well recognized from earliest recorded history. In the absence of any understanding of the microbial aetiology of infectious diseases prior to about the mid-1800s, the belief in the age-old recognition of anthrax has to be based on clinical descriptions in earlier writings commensurate with today's case definitions of the disease in humans and animals. A detailed review of the reports cited in papers and reviews over the past century as evidence of the ancient history of anthrax is beyond the scope of this chapter but is reviewed elsewhere (Turnbull 2009).

### **4.2 Control Measures and Animal Anthrax Vaccines**

#### ***4.2.1 Before the Formulation of True Vaccines***

Control of naturally acquired anthrax in humans has always depended on control of the disease in livestock. Consequently, the story of anthrax vaccination of animals dates from well before that of their human counterparts. Centuries before evidence-based epidemiologic principles were first articulated in the mid-nineteenth century, ancient sources addressed the issue of anthrax prevention: the Greeks in Homer's Iliad recognized the need to wash thoroughly to limit the spread of disease; Columelle, in the first century AD, recommended to "always separate sick animals from others to prevent contamination by contact" (Blancou 2000). Quarantine appears to have been practiced in some parts of Europe in the sixteenth century and by the late eighteenth century, the cleaning and disinfecting of stables, burial of

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carcasses, and destruction of contaminated meat were becoming recognized as appropriate measures for disease prevention.

#### 4.2.2 Early Livestock Vaccines

In his textbook of animal diseases written in 1847, Cole contrasts the survival of cows inoculated with “murrain pus” with the significant mortality in neighbouring, uninoculated cows during a bovine anthrax epidemic in England. William Greenfield, Superintendent of the Brown Sanatory Institution in London in the late 1870s, following up on the observations of his predecessor in that position, Dr Burdon-Sanderson, said that “bovine animals” surviving anthrax “... are less liable than others to infection by the same process...”, and demonstrated that “successive generations of artificial cultivation” resulted in “a modified virus which, when inoculated, produces much less severe symptoms and appears to be partially protective against future, more severe attacks” (Tigertt 1980). Veterinarians Jean-Baptiste Auguste Chauveau of Lyon and Jean-Joseph Henri Toussaint from Toulouse provided conceptually similar demonstrations in 1880, prior to those of Pasteur, regarding the protective effect of sublethal natural infection or laboratory-modified doses of bacteria, respectively (Wilson and Miles 1964). Toussaint believed that by subjecting infected sheep’s blood to 55°C for 10 min prior to challenging healthy sheep, he had killed the organisms (Blancou 2000).

The following year Pasteur (refer to Fig. 3.5) showed that Toussaint’s approach actually represented an attenuated, rather than killed, vaccine. Pasteur had previously observed this phenomenon in 1879 with the fowl cholera bacillus (now known as *Pasteurella multocida* and first isolated and shown to be the causative agent by Toussaint) and had speculated on the potential utility of this “artificial attenuation” for bacterial vaccines. His attempts to attenuate *B. anthracis* were complicated by the development of endospores at 35°C, the standard temperature used then for bacterial culture. Further investigation revealed that growth without spore formation occurred at 42–43°C, and that cultures held for 8 days at this temperature showed reduced virulence for guinea pigs, rabbits and sheep. Over a 6-week period at this temperature, they became progressively less virulent, finally failing to kill mice or guinea pigs and retaining this phenotype on being subsequently cultivated at 35°C (Chamberland 1883). This work, performed with his assistants, Charles Chamberland and Emile Roux, in 1880, was presented to the Academy of Sciences in Paris in February of 1881 and led directly to Pasteur’s famous public experiment in the farmyard of Pouilly-le-Fort near Melun, about 25 miles southeast of Paris.

The significance of the events at the French village of Pouilly-le-Fort is easily lost in today’s scientific climate in which the microbial nature of infectious disease is unquestioned. It was far from accepted in Pasteur’s time. The dominant theory of the day attributed the cause of all disease to physicochemical transformations of the tissue cells; associated bacteria were simply seen as opportunistic. Furthermore, Pasteur was trained as a chemist, and the incursions of this “conceited laboratory

chemist, unversed in true medical thinking" (Dubos 1951) into the field of medicine were not well received by the learned doctors and veterinarians of the day. The Pouilly-le-Fort tests resulted from a challenge issued by the President of the Agricultural Society of Melun, Baron de La Rochette, and were carried out on the farm of veterinarian M. Rossignol. Rossignol was felt to be the principal motivator behind the challenge, which he was confident would lead to the ridicule of the germ theory (Dubos 1951).

At what point Pasteur decided on the necessity of a two-dose vaccine schedule, with the second dose containing a less attenuated culture is not clear, but this became the schedule used in several demonstrations of the vaccine's efficacy beginning at Pouilly-le-Fort in 1881 and continuing into 1882. In the first demonstration, the two vaccinations were carried out on May 5th and 17th; the challenge occurred on May 31st. Twenty-four sheep, one goat, and six cows were vaccinated and twenty-four sheep, one goat and four cows served as unvaccinated controls (Fig. 4.1). Following the challenge, some of the vaccinated sheep did appear ill initially, but ultimately all vaccinated animals apart from one ewe survived with the single mortality attributed to another cause; all the unvaccinated sheep and the goat died; two of the sheep died in front of the spectators; and the unvaccinated cows showed significant local edema and febrile reactions in contrast to their unaffected vaccinated counterparts (Dubos 1951).

Although the success of the tests was widely acclaimed, not all of Pasteur's critics were silenced. Because of the liquid culture procedure used for vaccine preparation, there was inevitable batch-to-batch variation. As the vaccine became more widely used, it resulted in both vaccine failures and cases of vaccine-associated



**Fig. 4.1** Pouilly-le-Fort (Institut Pasteur)

anthrax. Antagonistic exchanges took place between Pasteur and Koch, as well as other respected scientists of the day. There were controversies over the purity of the culture used for the Pouilly-le-Fort demonstration and whether its only technical modification was maintenance at 42–43°C (Blancou 1995, 2000; Lax 2005). Certainly other methods of attenuation had been tried in attempts at improving reproducibility (Dubos 1951; Geison 1995). Furthermore, the important observations of Greenfield and Toussaint were largely ignored in the more grandiose environment established by Pasteur and his team and only became acknowledged decades later. Greenfield, for his part, more modestly noted that “M. Pasteur has recently published the results of … experiments made by a precisely similar method, with results confirming those which I published more than a year ago. And although I venture to claim for England whatever merit may be due to priority for the discovery, I none the less rejoice that the facts should have been so fully established in France” (Tigertt 1980).

#### **4.2.3 *From Pasteur to Sterne***

Following the Pouilly-le-Fort demonstrations, vaccination of livestock with Pasteur’s vaccine was rapidly and widely implemented with 90,000 animals being vaccinated in France within a year. Losses of 0.65% and 0.24% resulted from anthrax among sheep and cattle, respectively, in the first year of vaccine use compared with average annual losses of 9% in sheep and 7% in cattle over the ten previous years (Mason 1937). By 1894, the vaccine was being used on a large scale in sheep and cattle in Europe, South America and elsewhere with corresponding mortality rates in vaccinated animals of 0.94% and 0.34% (Chamberland 1894 cited by Wilson and Miles 1964). Although these were in no sense “double-blind” studies, the association between vaccination and a decrease in the anthrax-related mortality in widely disparate localities and conditions was seen as convincingly demonstrating the value of vaccination (Wilson and Miles 1964). However, by 1909 in France, the prevalence of anthrax had nearly returned to pre-vaccine levels (Wilson and Miles 1964).

The problems encountered with Pasteur’s duplex vaccine resulted primarily from the technical difficulties inherent in preparing batches with the precise degree of attenuation required for a vaccine to be used in a variety of animal species and breeds. Certain species, such as goats and horses, required weaker products. Sporulation was variable, and there was a tendency for some strains to change and become less effective on subculture (Sterne 1959). “Pasteur I type” and “Pasteur II type” were used to refer to the relative virulence of the attenuated vaccine strains being used (Sterne 1937a). Various investigators demonstrated the merits of using fully sporulated preparations suspended in 50–60% glycerol in order to inhibit the growth of contaminants, raise the immunizing efficiency, and increase the longevity of the spores (Sterne and Robinson 1939; Sterne 1945, 1959). The double vaccine thereby became replaced by a single vaccine consisting of spores suspended in 50% glycerol and attenuated such that they were non-virulent for rabbits but virulent for guinea pigs.

Vaccine failures and vaccine-induced casualties with the Pasteur vaccines, together with the perception that vaccine-induced cases could lead to animal outbreaks, led to a continuing search for improved procedures and products. By 1930 serum therapy was established as a treatment for human anthrax (Eurich and Hewlett 1930) and at least in some cases, was applied in animals also. One approach to the problem of inadequate attenuation in the livestock vaccine was the concurrent use of vaccine and hyperimmune serum. Apparently this was widely used at one time and still occasionally employed in the 1950s (Sterne 1959) but was clearly impractical for mass vaccination programmes. Evidently alternative routes of inoculation were also tried, although early authors did not typically specify the routes used (Sterne 1959).

The most important modification around this time was the incorporation of saponin into vaccines. Mazzucchi's widely used "Carbozoo" vaccine, produced both in Mazzucchi's Istituto Sieroterapico Milanese in Italy and by Lederle Laboratories in New York and which contained 2–5% saponin was the subject of numerous publications across the world during the 1930s (Sterne et al. 1939). Addition of saponin reduced the virulence of the vaccine strain while enhancing its immunizing power, thereby retarding the development of vaccine-induced infection and increasing bacillary immunogenicity (Sterne et al. 1939; Sterne 1948a, b). However, saponin is an irritant and at 2–5% caused an exuberant inflammatory reaction with necrosis, thus ultimately limiting its acceptability (Sterne 1959).

#### 4.2.4 Sterne's 34F<sub>2</sub> Vaccine

Pasteur-type anthrax vaccines included a proportion of capsulating, virulent organisms that periodically resulted in cases of anthrax. This coupled with the technical difficulty of achieving the desired level of attenuation and the fact that some animal species, particularly goats, horses and certain wild species required a vaccine so weak that it was almost non-immunogenic (Sterne 1937a) impelled research towards an effective avirulent alternative. Nungester demonstrated that capsule formation by *B. anthracis* was influenced by CO<sub>2</sub> (Nungester 1929 cited by Sterne 1937b) and Stamatin showed that non-encapsulated, avirulent dissociants arising during culture in horse blood could induce immunity in laboratory animals (Stamatin and Stamatin 1936). Max Sterne (1905–1997), working as a veterinary researcher at the Onderstepoort Institute in South Africa, utilized these observations to full effect, searching intensively for strains whose avirulent forms gave the highest degree of immunity starting with the strains already being used for vaccines (Sterne 1937b) and then testing a number of others, including fresh isolates (Sterne 1937a).

In South Africa, anthrax had been the cause of tens of thousands of annual livestock deaths prior to the advent of vaccination in the early 1920s (Sterne et al. 1942; Sterne 1959). Although highly effective initially, a resurgence of outbreaks in the mid-1930s pointed to a diminution in the immunizing power of the saponin-supplemented Pasteurian vaccine (Sterne et al. 1942). Sterne (Fig. 4.2) had qualified as a veterinarian at the Transvaal University College (subsequently University of



**Fig. 4.2** Max Sterne (Courtesy of the Sterne family)

Pretoria) in 1928 and, after successive 18-month stints in the Belgian Congo and Pietermaritzburg, South Africa, he joined the Onderstepoort Veterinary Institute, being assigned to the bacteriology department where one of his duties was to prepare the Pasteur anthrax vaccine. There he experienced problems in balancing virulence and immunogenicity during the attenuation process, an issue that together with the apparent reduction in the effectiveness of the Pasteurian vaccine convinced him of the need for a better vaccine (Alper 1996). The first challenge was to find strains that maintained their level of attenuation. In 1936, he selected a rough, non-capsulating dissociant derived from a 48 h culture of the virulent parent strain at 37°C on 50% horse serum nutrient agar under 30% CO<sub>2</sub>. The virulent parent had been isolated from a bovine hide 1 week before. This strain became the basis of the livestock vaccine used widely in most parts of the world today (Sterne 1937a).

After field comparisons in South Africa during 1937–1938 that involved 2.5 million cattle and several thousand horses and sheep, Sterne concluded that the 34F<sub>2</sub> vaccine was at least as efficacious as the Pasteur vaccine while being safer and less reactogenic; one formulation was suitable for use in most species, the principal exception being goats, in which a 1% mortality was noted (Sterne 1939). The 34F<sub>2</sub> vaccine comprised 0.6–1.2 million spores per ml in 50% glycerol-saline; from 1938, 0.5% saponin was added to improve its immunogenicity even further (Sterne 1939; Sterne et al. 1939). The incidence of anthrax again declined throughout the country in parallel with the introduction of the new vaccine (Sterne et al. 1942).

The efficacy and safety of the 34F<sub>2</sub> vaccine became accepted in South Africa in the early 1940s and by the end of that decade, it was increasingly used as the vaccine

of choice in almost all other countries (Gilfoyle 2006). In the Soviet Union, an analogous live spore vaccine for livestock had been formulated by 1940 in the Red Army Research Institute of Epidemiology and Hygiene, also known as the Sanitary Technical Institute (STI). The active ingredient was a 30% glycerol-saline suspension of spores of attenuated strain STI-1, derived from the virulent “Krasnaya Niva” strain isolated from a horse that had died of anthrax (Shlyakhov and Rubenstein 1994; Cherkasskiy 2002). The STI-1 vaccine was widely used after World War II and was associated with an almost ninefold decrease in the incidence of livestock morbidity in the period 1947–1960 (Shlyakhov and Rubenstein 1994). The strain was replaced in 1985 by strain 55-VNIIVVM, derived from a pig which died of anthrax in 1983 (Ladnyi 2008).

#### ***4.2.5 Livestock Vaccines in the United States***

In the United States, it was proposed before 1917 that the Pasteur vaccine should be replaced by a spore vaccine, and that this vaccine should be in desiccated form to enhance its longevity (Ferry 1917). However, two decades later, uncertainty regarding the optimal formulation and attenuation procedure persisted. A variety of immunizing agents, including spore vaccines of differing constitution designed for administration by alternative routes or schedules were available for animal vaccination (Stein 1942). By 1948 anthrax spore vaccine use was governed by specific regulations in 23 states (Stein 1948). The Sterne vaccine achieved only limited use in the U.S. at that time, despite its successes in other parts of the world (Stein and Van Ness 1955). However, a report of numerous vaccine-associated cases in cattle and horses in the U.S. in 1952 demonstrated the need for a safer vaccine (Bailey 1954). Following completion of efficacy studies on the Sterne vaccine in the mid 1950s, a number of companies began its production; of these, only the Colorado Serum Company continues producing it today in North America (Berrier and Huff 2008).

### **4.3 Human Anthrax Vaccines**

#### ***4.3.1 Factors and Events Leading to the Perceived Need for Human Vaccines***

In much of the world, anthrax in animals and humans declined from around 1940 due to the combined application of livestock vaccines and improved veterinary and public health measures; however, in the southern European and Asian republics within the Soviet Union the incidence of natural human cases remained high. For this reason, a prototype human version of the live spore animal vaccine, consisting

of STI-1 and “no. 3” strain spores in 50% glycerol-saline, was developed by the STI. In trials involving 3,500 volunteers between 1943 and 1950, the vaccine was deemed to be safe and non-reactogenic. Additional, controlled clinical trials were carried out in the early 1950s in endemic districts of the Moldavian Republic involving 141,663 individuals, two-thirds vaccinated by scarification and the remainder subcutaneously. Three cutaneous cases among vaccinated individuals (3.2 per 100,000) compared favourably with 47 cases in unvaccinated controls (11.3 per 100,000) ( $p < 0.001$ ) (Shlyakhov and Rubenstein 1994). Although the breakthrough cases were in the scarified group, the vaccine was licensed by the Soviet Ministry of Health for administration by scarification in 1953 and not for subcutaneous inoculation until 1959. Further trials were carried out in Moldavia in 1959 and 1960 to compare aerosol, subcutaneous and scarification routes of administration; the aerosol route, although giving satisfactory results, was designated as an emergency method to be employed in “critical situations” only (Shlyakhov and Rubenstein 1994).

Trial data and other analyses up to 1980 indicated significantly lower case rates in vaccinated groups, but protection was incomplete, particularly when STI vaccine was administered by scarification. Soviet reports claimed the complete absence of adverse effects following the use of the STI human vaccine in more than 30 years of experience (Shlyakhov and Rubenstein 1994). At some point strain “no 3” appears to have been removed from the formulation. Available information suggests that the Russian vaccine in use today contains just the STI-1 strain to be administered in two initial doses 20–30 days apart with annual boosters thereafter. Routine vaccination is administered by scarification; subcutaneous injection is used for urgent cases (Cherkasskiy 2002). The vaccine was originally manufactured at the Tbilisi People’s Institute of Vaccines and Sera in Georgia, but since the Soviet dissolution in 1991, it has been produced by the Research Institute of Microbiology in Kirov, Russia.

China developed a live spore vaccine for human use in the late 1950s for similar reasons as the Soviet Union: a high incidence of human anthrax in some regions and considerations of possible bioaggression. They used strain A16R isolated from a donkey that had died of anthrax. After trials comparing administration by scarification, subcutaneous, and inhalation routes, it was licensed in 1962 for administration by scarification (Wang 2008). It continues to be produced by the Lanzhou Institute of Biological Products in China.

While concern regarding naturally-acquired anthrax as a human disease was declining in most countries during the 1940s coincident with the deployment of effective animal vaccines, concern over the use of *B. anthracis* as a potential agent of biological warfare was growing (Turnbull 2002). Its earliest use in this context appears to have been during World War I (Wheelis 1999; Redmond et al. 1998), but it was the intelligence reports in World War II that Germany was developing biological weapons capability that led to the perceived need in the West for a human vaccine (Geissler 1999). In contrast to the Soviet Union and China, a live vaccine as used for animals was considered unsuitable for humans, leading to a search for suitable immunogens on which to base a non-living vaccine in the U.S. and U.K.

### 4.3.2 Protective Antigen and the Evolution of Human Vaccines

The idea that naturally acquired immunity to anthrax was the result of an “anti-aggressin”, with aggressin considered to be a toxin, actually dates back to late nineteenth century hypotheses of bacterial substances purported to possess immunizing power, such as “albumose” (Hankin 1889 cited by Eurich and Hewlett 1930) and “tox-albumin” (Brieger and Fränkel 1890 cited by Eurich and Hewlett 1930). The use of edema fluid extracts in immunization was suggested in the early part of the twentieth century (Bail 1904 and Salisbury 1926 both cited by Lincoln and Fish 1970) and revisited in the 1940s. This was found to be effective but, its production being something of an art, the search for more controllable methods was begun (Cromartie et al. 1947).

The aptly named protective antigen (PA) was initially noted in static cultures containing the plasma or serum of various species; yields were later increased by growing the culture in cellophane bags continuously perfused with aerated serum-containing broth (Gladstone 1946, 1948). A synthetic broth medium containing 20% serum was subsequently developed (Wright et al. 1951). Boor and Tresselt produced and tested the first practical vaccine in 1954 consisting of a dialyzed ammonium sulfate or alcohol precipitated Seitz filtrate of strain CD-2 in a serum albumin-yeast extract-bicarbonate medium (Lincoln and Fish 1970). Research on development of a protein-based human anthrax vaccine was concentrated in two institutions: the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) and the U.K. Ministry of Defence’s Microbiological Research Establishment. Vaccine development at these sites started at the common point of Gladstone’s PA preparations; however, their ultimate licensed vaccines, while analogous in many ways, developed along somewhat different routes (Lincoln and Fish 1970).

From the outset the U.S. vaccine was based on cultures in chemically-defined synthetic media without proteins or other macromolecules. The media was modified over time to improve the yield (Wright et al. 1962; Puziss et al. 1963); anaerobic incubation in a stirred broth culture was adopted to permit large-scale production (Wright and Puziss 1957); and alum precipitation of the filtrate, found to confer stability and enhance the immunizing potential in animals (Wright et al. 1954), was replaced by adsorption onto aluminium hydroxide gel (alhydrogel) as a more effective technique to precipitate PA (Puziss and Wright 1963). The vaccine was now as described in Patent 3,208,909, applied for by Puziss and Wright in 1961 (Puziss and Wright 1965), although the strain of *B. anthracis* to be used had yet to be fully established. Strain V770-NP1-R, a non-capsulating, non-proteolytic variant of a bovine isolate from Florida in 1951 1955(Auerbach and Wright ) was finally adopted. The current U.S. vaccine, “Anthrax Vaccine Adsorbed” (AVA), an alhydrogel-adsorbed cell-free culture filtrate of V770-NP1-R grown anaerobically in a fermenter, was licensed in 1972 for use in select occupational settings (Federal Register 1985). Filter-sterilization procedures changed in 1991 (Ivins et al. 1994) and thiomersal, the original preservative was changed to benzethonium chloride

with formalin added as a stabilizer. AVA remains the only currently licensed U.S. vaccine against human anthrax.

The U.K. vaccine went through fewer developmental stages and bears greater similarity to the original 1954 formulation than its U.S. counterpart (Belton and Strange 1954). A protein hydrolysate growth medium was utilized consisting of casein hydrolysate and yeast extract for nutrients, and charcoal was used to absorb an undefined inhibitory substance and increase potency (Belton and Strange 1954). The Sterne strain 34F<sub>2</sub> was adopted from the outset. Static aerobic culture in large medical flats (Thompson bottles) with alum precipitation of the filtrate was also practiced from the outset. The vaccine was introduced for those in at-risk occupations in 1965 (Anon 1965) and licensed in 1979. Changes that have taken place over the years have reflected improved membrane filter technology, the exclusion of animal-derived components to mitigate the risk of adventitious agents, and adjustments in processing in response to batch-to-batch variations in charcoal (Hambleton 2008).

#### ***4.3.3 Efficacy and Safety Issues in Relation to the U.S. and U.K. Vaccines***

With the low incidence of human anthrax in the U.S. and U.K., these countries lacked the opportunities available to the Soviet Union to assess the clinical effectiveness of AVA and the U.K. vaccine. In a study performed during the introduction of the U.S. vaccine into mills processing animal products from anthrax endemic areas of the world, it was deduced that a full course of six doses was 92.5% effective against the cutaneous form of anthrax (Brachman et al. 1962). Since the vaccine used at the time differed from the subsequently licensed vaccine in the strain used, the medium in which the strain was grown, and the adjuvant and filtration methods, evidence of the effectiveness of AVA against human anthrax is confined to extrapolated data from animal models combined with the fact that there have been no cases of anthrax in its recipients, the latter qualified by acknowledgment of the absence of exposure data.

No clinical studies were performed using the U.K. vaccine; belief in its efficacy was based on the observation in the 1960s that an overall fourfold decrease in human cases of anthrax paralleled its introduction into the wool and hide industries while infection rates among unvaccinated at-risk persons in other sectors (e.g. meat and bone meal trades, dockworkers, farm or horticulture workers) remained steady (Darlow and Pride 1969; CDSC 1981). However, the fact that anthrax cases were on the decline in other European and Scandinavian countries without human vaccine use indicated that there were other contributory factors.

Although the tripartite nature of the toxin had been elucidated by 1960 (Stanley and Smith 1961), the relative contributions of each of the components, PA, lethal factor (LF), and edema factor (EF) to vaccine-afforded protection remained unclear (Smith and Stoner 1967; Lincoln and Fish 1970). However, it was generally agreed that anti-PA antibodies were protective. This, together with a desire to minimize

reactogenicity, led to the selection of V770-NP1-R, a strain that produced detectable amounts of PA only as the strain of choice for the U.S. vaccine. When it became possible in the 1980s to measure antibody responses more precisely, it was confirmed that humans and animals vaccinated with the U.S. vaccine indeed had substantial anti-PA titers, nearly undetectable anti-LF titers, and undetectable anti-EF titers, while their U.K. counterparts had significant titers to LF and low titers to EF (Turnbull 1991). The issue of the possible contribution of LF and EF to protective immunity, and whether either antigen with or without other virulence factors should be included in an anthrax vaccine continues to be a subject of research today.

The declining incidence of anthrax in developed countries along with changing political attitudes on biological warfare and weaponry in advance of and after the 1972 Biological Weapons Convention resulted in a diminished priority for human anthrax vaccines. Revelations concerning the accidental release of anthrax spores in the Soviet city of Sverdlovsk in 1979 (Meselson et al. 1994) brought the disease sharply back into focus in the 1980s, with significant progress being made towards understanding the nature of the tripartite anthrax toxin and the importance of PA to vaccine-induced protection. A number of novel approaches for second generation vaccines ensued (Ivins and Welkos 1988; Turnbull 1991). Successive new waves of biodefense-funded research followed, driven by the first Gulf War in 1991 and renewed fears about bioterrorism sparked by the events of 9/11 in the U.S. and the anthrax letter attacks later in 2001.

The ill-defined “Gulf War Syndrome” that appeared in some veterans of the 1991 Gulf War; the suggestions by some that anthrax vaccines may have contributed to the syndrome; and the controversies surrounding the 1997 Anthrax Vaccine Immunization Program (AVIP) mandating vaccination of all active U.S. military personnel, were instrumental in drawing attention to the shortage of pre-clinical, clinical, pharmacological, and safety data on the U.S. and U.K. vaccines and illuminating aberrations in the manufacturing process of the U.S. vaccine. The full story on these controversies remains to be written but involves intense debates regarding the vaccine’s safety and efficacy, the Food and Drug Administration’s (FDA) rulings, the legality of the AVIP, judicial injunctions, and the convoluted trail of events from production by the Michigan State Department of Health to the creation of the BioPort Corporation in 1998 and the latter’s acquisition of the Michigan Biologic Products Institute, with all of the aforementioned occurring in the context of troops facing possible bioweapons abroad in a combat theatre or civilians threatened with bioterrorism at home. Ultimately, in late 2005 the FDA ruled that the scientific evidence supported the licensure of AVA for the prevention of anthrax by any route of exposure. By this time AVA was being marketed by Emergent BioSolutions under the trade name BioThrax®.

Ongoing vaccine concerns also led to a congressional mandate in 1998 for the U.S. Centers for Disease Control and Prevention to establish the Anthrax Vaccine Research Program (AVRP) in collaboration with other government health agencies. The AVRP was initiated in 2002 as a randomized, double-blinded, placebo-controlled clinical trial to determine if the route of administration of AVA could be changed from subcutaneous to intramuscular and if the number of priming doses could be reduced.

The preliminary findings from just over 1,000 participants showed that no serious adverse events have been associated with AVA, and that it will probably be possible to eliminate the dose administered at week two and to change the route of administration to reduce reactogenicity without impacting the anamnestic anti-PA immune response (Quinn 2007).

#### **4.3.4 Next Generation Vaccines**

Notwithstanding the frenetic level of scientific activity in the field of anthrax over the last two decades, the contracts issued in 2004 under Project BioShield by the U.S. Department of Health and Human Services to two biopharmaceutical companies to produce a recombinant PA vaccine mark a major milestone in the history of anthrax vaccines. The contracts represent the first attempt since the 1950s to procure a next-generation vaccine, which, in addition to meeting modern standards for formulation, safety, and efficacy, would require fewer doses, be safe to administer to pediatric and geriatric populations, and be applicable to both pre- and post-exposure scenarios.

The contract with VaxGen Inc was withdrawn in 2006 because of product stability problems and production delays. However, the VaxGen recombinant rPA-102 vaccine candidate, generated in an asporogenic, non-toxigenic derivative of the Sterne strain, was resurrected in May 2008 when the assets and rights of VaxGen were purchased by Emergent BioSolutions Inc., the parent company of BioThrax®-producer BioPort. The other contract went to Avecia in the U.K. whose vaccine candidate, a recombinant *E. coli* derived formulation, had completed phase II clinical trials and demonstrated safety and immunogenicity. The vaccine can be stored, transported and used without the need for a conventional cold chain – an important advantage for biodefense applications. Avecia's biodefence vaccines business was acquired in March 2008 by the U.S.-based PharmAthene.

### **4.4 Future History**

There is no publicly announced time line for licensure of a recombinant second generation anthrax vaccine, and in the current milieu, regulatory procedures are such that it will continue to be a prolonged process (Turnbull 2000). Nonetheless, it seems likely that the chapter on the history of anthrax vaccines will not close there. DNA and oral or skin patch vaccine alternatives and their analogs for animals, potentially targeting multiple pathogens and designed to provide lifelong protection with a single dose are probable targets of future generation human anthrax vaccines. Meanwhile, protection from anthrax remains dependent on the U.S. and U.K. human protein vaccines (and in the Russian Federation and Republic of China, on their human live spore vaccines) dating from the 1950s and on animal vaccines designed in the 1930s. Manufacture and administration of these are likely to continue for the foreseeable future.

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# Chapter 5

## Rabies

Hervé Bourhy, Annick Perrot, and Jean-Marc Cavaillon

### 5.1 Louis Pasteur: A Brief Biography

Born in Dole, France, in 1822, Pasteur was a trailblazer in the study of microorganisms and went on to become one of the most influential thinkers in the history of science. While his development of the first laboratory-attenuated human vaccine – against rabies – is well known, this major discovery was only one remarkable episode in a long and productive career that spanned numerous scientific arenas. The first 10 years of his professional life were devoted to studying the deflection of polarized light by organic substances and the relationships between this property, crystalline structure, and molecular configuration. In so doing, he would establish the foundations of a new science, stereochemistry, and describe a novel yet general law of physiochemistry: organic molecules are asymmetric. He recognized that the laws of asymmetry influenced the function of molecules and, therefore, were essential to the mechanisms of life; in their discovery, Pasteur illuminated the path for advances in biology. Thus, by the time he was in his mid-20's, Pasteur's accomplishments foretold his accession to the ranks of the great men of science.

Following his discovery of molecular asymmetry, Pasteur spent the next phase of his career devoted to studies on fermentation; studies that were directed at a specific technical problem of economic importance at the time and that had the fortuitous effect of connecting his discoveries as a chemist to his future legacy as a

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biologist. He demonstrated that microorganisms were responsible for fermentation, leading to the assertion that “fermentation has a vital origin.” Relying on his earlier work, he undertook research on diseases in wine and studied vinegar production and beer fermentation. Based on his experimental observations, Pasteur refuted the theory of spontaneous generation in favor of the germ theory of disease as detailed in Chap. 3. This, in turn, galvanized the worldwide scientific community, directly resulting in the establishment of microbiology as a distinct discipline and in the development of basic asepsis and sterilization techniques, later carried on by Lister and others. In this phase, he would also discover anaerobic life and would become interested in silkworm diseases. Each of the fundamental proposals established for fermentation was applied to the concept of infectious diseases with the same precision; a specific ferment, as yeast was known in that context, corresponds to each kind of fermentation, just as a specific “virus” (not viruses as we now know, but a term used generically to connote microorganisms) corresponds to each disease.

In 1877, Pasteur, already widely known for his landmark work in theoretical and industrial chemistry and in the fledgling field of microbiology, began focusing his research on infectious diseases. He extended and strengthened the contemporary discoveries of Koch regarding anthrax, an agriculturally important disease of large animals. Unlike Koch, however, Pasteur was immediately drawn to the implications of his work for the eventual prevention of disease, and this became his approach over the next phase of his illustrious career – one that led to the discovery of specific microbial etiologies for several human and animal diseases and of techniques that resulted in protective vaccines. While the latter began with his serendipitous but brilliant discovery of laboratory attenuation during studies of fowl cholera, the development of an effective vaccine against rabies would be the ultimate demonstration of Pasteur’s genius.

## 5.2 Attenuated Vaccines

Pasteur coined the word “vaccination” at an international medical congress in London 1881 as a tribute to Jenner’s seminal work with cowpox: “I have given to the term ‘vaccination’ an extension that science, I hope, will recognize as a tribute to the immense merit and services rendered by one of the greatest men of England, your Jenner. What a joy for me to glorify this immortal name on the ground of the noble and hospitable city of London.” During his career, Pasteur proposed vaccines for four diseases. The underlying principles upon which he based his approach derived from the observation that certain infectious diseases, such as measles and smallpox, engendered protective immunity against recurrent infection in those individuals who had experienced natural infection. Jenner’s work during the previous century had further demonstrated that a localized infection with cowpox, a naturally occurring, weaker analog of smallpox, resulted in protection against the latter. Pasteur became the first to extend this concept using laboratory manipulated, weakened, or attenuated pathogens to induce a nonlethal, immunizing infection. The first target was a bacterial respiratory disease of poultry, fowl cholera, which was rampant in nineteenth century chicken coops.

The bacillary agent of fowl cholera, eventually named *Pasteurella multocida*, had been isolated in 1879 by Pasteur's rival Toussaint, a professor at the veterinary school of Toulouse, although multiple other European veterinarians may have accomplished this task earlier. Through experimentation, Pasteur was able to define an efficient way to cultivate and maintain growth of this agent in specific, artificial media comprising a chicken muscle broth neutralized with potash and sterilized at temperatures greater than 110°C. When grown in this fashion, the bacteria were fully virulent in chickens and rabbits, killing all challenged animals. Pasteur's successful approach to a vaccine against fowl cholera is probably the best example of his famous adage, articulated during his experimental chemistry days that in the realm of observation, "chance favors only the prepared mind" (Debré 1994).

In 1879, both Pasteur and his assistant Charles Chamberland went on vacation while Chamberland was preparing virulent cultures to inoculate chickens. Upon their return, inoculation of these previously lethal, 1-month-old cultures into chickens failed to kill them. Upon reinoculation of the same chickens with a fresh culture preparation that was shown to be fully virulent for naive birds, they survived the lethal challenge. Pasteur correctly concluded that the chickens had been rendered immune to infection by the strain that had been artificially attenuated in his laboratory. When he first presented his discovery to the Academy of Sciences, he provided scant information as to the technical aspects of attenuation (Pasteur 1880a); it was revealed in a broad manner only 8 months later (Pasteur 1880b).

After chicken cholera, Pasteur addressed an infectious disease of far greater economic impact. Anthrax in his time killed thousands of farm animals, particularly sheep, all over Europe. Extending the work of Koch, who had been the first to isolate the bacillary etiology in 1876, Pasteur investigated the route of animal transmission, showing that earthworms inhabiting areas where the carcasses of animals were buried carried anthrax spores back to the surface, and that these spores could subsequently infect animals grazing in the same fields. The next step was to develop a vaccine.

Once again he was in competition with Toussaint, who reported at the Academy of Sciences in July 1880 that he had successfully protected four dogs against anthrax using a preparation of heat-killed bacilli. Pasteur, based on his chicken cholera observations, favored a live attenuated vaccine approach. It was known that chickens were resistant to the effects of anthrax, postulated by Pasteur to be a function of their relatively high body temperatures. After cooling them by plunging in cold water, they became susceptible to the bacteria's lethal effects. With the idea that higher temperatures might attenuate anthrax pathogenicity, he prepared a vaccine by maintaining cultures at 42–43°C for 8 days (Duclaux 1896).

At neither the successful public experiments with anthrax vaccine conducted at Pouilly-le-Fort in 1881 (see Fig. 4.1) nor at his presentation of the results before the Academy of Sciences later that year (Pasteur et al. 1881) did Pasteur elaborate on the technical specifications of his attenuation process. His assistants, Chamberland and Emile Roux, who had prepared the vaccine, did not provide methodological details either. Some have suggested that Pasteur used an inactivated vaccine, Toussaint's work, based on indications that he had also studied such approaches (Geison 1995), although he was clearly working on attenuated vaccines and would subsequently address his next "project" in this manner. In his 1881 report on anthrax vaccine Pasteur

wrote, “*Audentes fortuna juvat*” (translated as “chance smiles to audacious people”) (Pasteur et al. 1881), suggesting that he had indeed taken a risk on a novel approach at Pouilly-le-Fort. His public risk, though, accrued far more publicity for his new vaccine approach and was more convincing to the public than any experiment carried out in a closed laboratory would have been. By 1894, millions of sheep in Europe had been vaccinated with Pasteur’s attenuated vaccine and experienced anthrax mortality rates of less than 1% (Duclaux 1896). Pasteur addressed another disease, which was affecting the breeding, the swine erysipelas. And in 1883, with Louis Thuillier, his bright student, he developed a new efficient attenuated vaccine. Thus, the stage was set for the most “audacious” of all pursuits – a vaccine against rabies.

### 5.3 Rabies in Europe in the Late Nineteenth Century

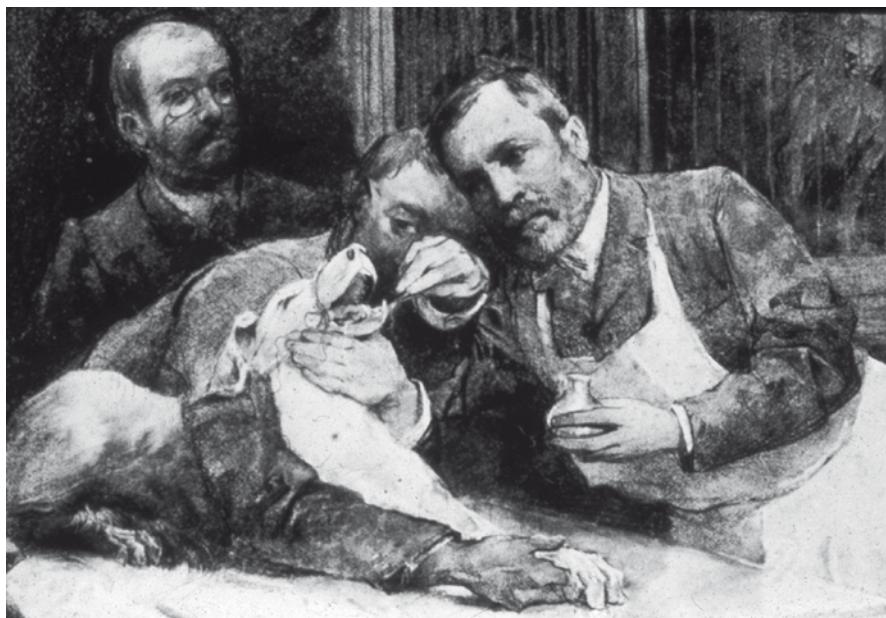
Rabies epizootics involving dogs, foxes, and wolves were a frequent occurrence in the nineteenth century. The disease was widely distributed in Europe (Steele and Fernandez 1991); numerous cases were reported among livestock, including cows, horses, donkeys, and goats. However, in the absence of available laboratory confirmation, these reports were solely based on clinical grounds, which probably led to diagnostic errors considering the overlapping clinical manifestations of other encephalitides affecting animals. Many human rabies cases were also reported, although these reports also merit caution.

Since the earliest description of rabies in antiquity, it was known that a large proportion of people bitten by rabid animals became ill and died within 5 days of the onset of neurologic signs (Theodorides 1986). Several attempts to demonstrate that rabies could be transmitted by the saliva of infected animals were undertaken during the nineteenth century (Fig. 5.1). Despite the successful transmission of disease from symptomatic dogs to uninfected animals via saliva (Steele and Fernandez 1991; Zinke 1804), the common belief that the agent appeared *de novo* under various conditions persisted.

### 5.4 Rabies Vaccine in Animal Models

Pasteur’s work on rabies vaccine occurred in the context of intense research on rabies in France. In 1879, Pierre-Victor Galtier, a veterinary professor, was able to serially transmit rabies to rabbits (Galtier 1879); the average incubation period was 18 days, and signs such as paralysis and convulsions preceded death. That same year, Maurice Raynaud successfully infected rabbits by injecting them subcutaneously in the ear with saliva from a rabid patient (Raynaud 1879), noting a shorter, 4-day incubation period. Serial transmission of this “virus” (again a general term, as such submicroscopic organisms would not be discovered for another two decades) in rabbits was successfully achieved by using fragments of submaxillary salivary glands.

Pasteur, attempting to reproduce transmission from man to animals and from animals to animals, experienced early problems with contamination due to other

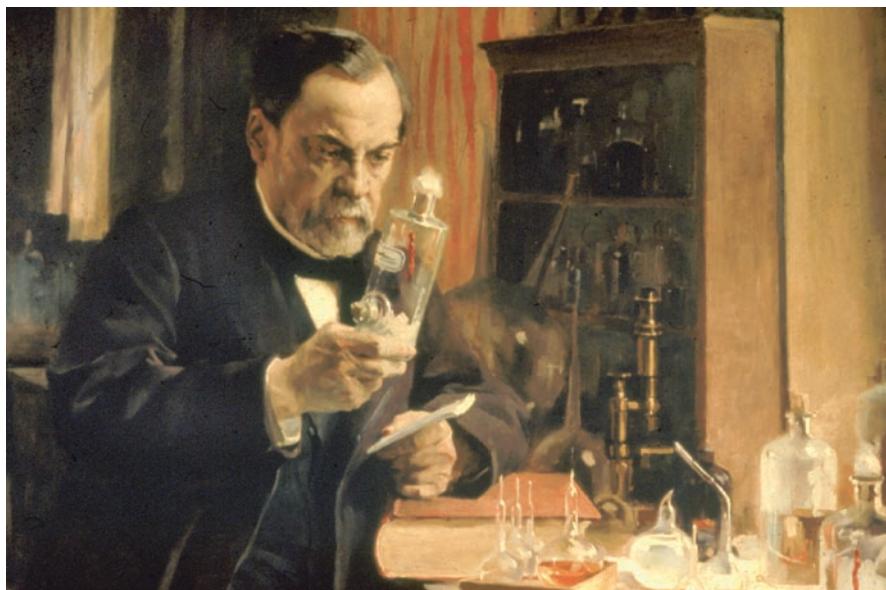


**Fig. 5.1** Pasteur sampling saliva (Institut Pasteur)

microorganisms causing septicemia of the inoculated animal instead of rabies (Fig. 5.1). Nonetheless, he reported to the Academy of Sciences in 1881 the first successful transmission of rabies in rabbits by the inoculation of central nervous system tissues and spinal fluid, demonstrating that the causal agent was not solely present in the saliva but harbored in tissues of the nervous system. He also observed in dogs that the intracerebral inoculation of infected brain material, an innovation of Pasteur's associate, Roux, shortened the usual incubation period to 1–2 weeks, making it a useful laboratory model for the disease (Pasteur 1881a, b).

The next year, Pasteur described a small number of cases of animals that recovered from rabies following experimental inoculation and which were subsequently immune to later inoculations of the virus (Pasteur et al. 1882). Galtier had presented data showing that dogs could be immunized by the intravenous injection of infected specimens (Galtier 1881); Pasteur remained skeptical in this regard until some years later, when the results were experimentally confirmed by Roux and Nocard, two of his assistants (Nocard and Roux 1888).

Following along the investigative lines of his chicken cholera and anthrax work, Pasteur accelerated his work on methods of rabies attenuation in 1884 (Pasteur et al. 1884). He did this without ever isolating the causative agent, which would eventually be recognized in the twentieth century to be nonbacterial, as the technology available to him at the time precluded such advances. Pasteur found that serial passages of dog rabies in monkeys weakened the organism, as confirmed by lengthening of the incubation period. Conversely, serial passages in rabbits produce a



**Fig. 5.2** Pasteur with dessicated spinal cord (Institut Pasteur)

more virulent form of rabies, associated with a shorter incubation period and serving as a more reliable model. By 1885, he had formulated the fundamental tenets of the vaccination strategy (Pasteur 1885a).

Through a series of passage in rabbits using intracerebral inoculations, Pasteur was able to create a form of rabies of heightened virulence and reduce and “fix” the incubation time to 7 days. The virus in the rabbit spinal cords, which populated their entire length, became progressively more attenuated when suspended in dry air (Fig. 5.2); in fact, virulence of the tissue was completely abrogated after 15 days of desiccation. Dogs that were repeatedly inoculated with a series of rabbit cord suspensions of increasing virulence, or those tissues that had been dried for shorter periods, were found to be rabies refractory, even when challenged via the intracerebral route, presumably due to the development of strong immunologic responses. Pasteur used 50 dogs for these experiments, a large number even by current standards. The animals were generally challenged with rabies only after the series of vaccinations (Vodopija and Clark 1991).

## 5.5 The First Human Vaccinations Against Rabies

Pasteur recognized that because rabies was unpredictable in its occurrence and clinical biology, a vaccine would be most beneficial if it could be given after a bite had occurred and if it induced protection more rapidly than the clinical

incubation period of the disease. His theoretical strategy, albeit not validated during the animal studies using attenuated, rabies-infected, rabbit spinal cords, actually involved the concept of therapeutic vaccination and was distinctly different from Jenner's approach or even Pasteur's own previous vaccine discoveries. In modern terms, he was describing postexposure vaccine prophylaxis to prevent symptomatic disease. Human use of the vaccine would be associated with significant risk and controversy, justified by Pasteur on the basis of the invariable fatality of rabies infections.

Although poorly documented outside of his laboratory notebooks, Pasteur vaccinated two people in the spring of 1885 with his emulsified spinal cord suspensions of attenuated rabies vaccine (Debré 1994). Both were symptomatic by the time Pasteur provided the first inoculation. One, a Parisian named Girard, received a single injection of attenuated rabies and survived, though the diagnosis of rabies was probably of dubious validity; the other, 11-year-old Julie-Antoinette Poughon, had been bitten on the face, which portended a relatively shorter disease incubation period. She presented with advanced disease and died soon after receiving a second injection of vaccine.

The first real opportunity for Pasteur to apply his novel vaccine "treatment" to human rabies occurred in July 1885 and involved 9-year-old Joseph Meister, from Alsace, who had received numerous, severe bites from a rabid dog but had not yet developed symptoms of rabies. Pasteur, who was not medically qualified, arranged for the boy to be examined by two physicians, Alfred Vulpian, a member of the French Commission on Rabies, and Jacques-Joseph Grancher, a respected pediatrician. Both men considered the child to be at high risk of death from rabies and advised the use of Pasteur's experimental vaccine (Debré 1994; Geison 1995). Grancher administered the injections, as Pasteur's laboratory associate, the physician Roux, was opposed to human use of the vaccine at the time.

The treatment started approximately 60 h after the bites, an acceptable delay for postexposure prophylaxis by today's standards. The boy was inoculated in the skin of the right upper abdomen with a suspension of 15-day-old desiccated spinal cord from an infected rabbit (Fig. 5.3), the most attenuated organisms in the regimen. He received another 12 inoculations over the next 10 days, each comprising progressively less attenuated, hence more virulent rabies virus; the last spinal cord preparation was only dried for 1 day (Pasteur 1885a). Meister returned home, feeling well, after 3 weeks in Paris. From a scientific perspective, it would have been difficult to assess vaccine efficacy against natural rabies infection in this case given the variable transmission rate of rabies from animals to humans and its dependence on a number of epidemiologic and biologic factors. It is clear, however, that the boy survived progressively more virulent rabies inoculations without evidence of disease and survived into adulthood, much later serving as a security guard at the Institut Pasteur.

While the Meister episode was a major accomplishment, it did not attract the worldwide recognition of Pasteur's subsequent human use of rabies vaccine. Later in 1885, a 15-year-old shepherd from a small rural village in Jura, Jean-Baptiste Jupille, was sent to Pasteur after being bitten several times on his left hand by a

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PARIS ET DÉPARTEMENTS : Un an, 14 fr., six mois, 7 fr.  
Trois mois, 4 fr. 50.  
STRANERS : Un an, 18 francs.UNE INOCULATION CHEZ M. PASTEUR  
LE VACCIN DE LA RAGE

29 980

Fig. 5.3 Dr. Grancher vaccinating a young child (Institut Pasteur)

rabid dog. Again the risk of rabies was high, but the treatment successful. In large part owing to Jupille's compelling story – he came from difficult family circumstances and had been attacked by the animal while attempting to rescue a group of younger children – his vaccination received worldwide attention. Jupille, like Meister, would later work at the Institut Pasteur as a caretaker (Fig. 5.4).

In short order, Pasteur was treating potentially infected people from multiple nations: four children from New Jersey who were bitten by the same rabid dog were vaccinated and remained free from rabies; a year later, 19 moujiks from the Smolensk area of Russia, all injured by a rabid wolf, arrived in Paris, some severely ill. Due



**Fig. 5.4** Jean-Baptiste Jupille standing by the statue of himself at Institut Pasteur (Institut Pasteur)

to the nature of the injuries and the treatment delay, Pasteur used twice daily vaccinations, with 16 survivors able to return to their country. By the end of 1886, more than 2,000 people had received Pasteur's rabies vaccine regimen (Geison 1995); only rare failures were reported.

But Pasteur had contemporary critics in France and elsewhere. Some, like Roux, counted among his close associates. As stated previously, because rabies was not an invariable sequelae of rabid animal bites, if rabies failed to develop after vaccination, Pasteur's critics could argue the vagaries of disease epidemiology as the explanation rather than vaccine-induced protection; if rabies developed despite vaccination, they could claim vaccine failure or worse, vaccine-induced rabies. Such logic served to magnify vaccine failures and their implications, and such was the milieu surrounding the "Rouyer affair," a legal action involving a boy vaccinated in late 1886 who died a month later of seemingly unrelated causes in which Pasteur's approach, if not the man himself, was on trial. An autopsy raised the possibility of kidney failure as the cause, but when Roux injected an extract of the child's brain into rabbits, the animals developed signs of rabies, implicating the latter as the cause of death (Adrie 1938).

At the Academy of Medicine in early 1887, the examining pathologist, with Roux's complicity, denied that rabies was involved in the boy's death. Their alleged justification for withholding the truth, recounted years later by a Pasteur assistant, was the concern that the truth would not only severely undermine public acceptance of rabies vaccination but would bring into question Pasteurian principles in general and would ultimately cost lives. The theme of vaccination as a "greater good" for society would recur multiple times over the next century during times of mass and mandated uses of vaccines against infectious diseases. With rabies, some failures, it was argued, were inevitable, such as those associated with delayed treatment or bites close to the brain. But overall, the vaccine appeared to offer benefit. In the 1898 annual report of the Institut Pasteur, 96 deaths were reported among 20,166 treated patients, a 0.5% mortality rate, as compared with at least a 16% mortality rate among the unvaccinated (Pottevin 1898).

## 5.6 Ethics and Controversies

The tumultuous nineteenth century is distinguished by its prodigious expansion in scientific and medical knowledge – advances that challenged existing paradigms of thought and therefore caused controversy. Although Pasteur received great accolades from his fellow scientists, the government, and the international public for his work, popularity and recognition were never unanimously won. His innovations and specifically his human use of an experimental rabies vaccine represented prime examples of this phenomenon and engendered the greatest enmity.

As is the case with many innovators, he had vocal detractors, many from within the scientific community. He was criticized on many fronts: authorship of some of his research was disputed; his data were questioned; he was accused of being

intransigent, dogmatic, and of being driven by monetary gain and his need for publicity. Most serious, however, were allegations that his rabies vaccine work violated ethical standards, raising an issue that Jenner and his predecessors had encountered with smallpox vaccine and that future vaccinologists would face as well.

What was demanded by the scientific ethics of that period? Clearly, it was a different time than the modern vaccine age; informed consent and human use regulations were nonexistent, to be established much later through a series of declarations. Nonetheless, ethical considerations were evolving along with the rapidly evolving arena of science, “...requiring constant monitoring to avoid errors and excesses, all the more so since scientific truth is itself subject to continuous revision” (Moulin 2008). When Pasteur proposed to the Emperor of Brazil in 1884 to use condemned prisoners in rabies vaccine experiments, ensuring their freedom if the treatment succeeded, it was more of an eighteenth than nineteenth century consideration.

Roux, one of Pasteur’s closest collaborators and staunchest allies, opposed using the rabies vaccine in humans on the grounds that it was premature, as he believed the animal studies had not been definitive (Debré 1994). In fact, he briefly left Pasteur’s laboratory for this reason. Pasteur, for his part, vacillated between impatience to try the treatment on humans – he even considered inoculating himself (Pasteur 1885b) – and apprehension concerning its risks. Additionally, a principal concern was that a public “failure would compromise future endeavors” (Debré 1994). After the successful, publicized vaccinations of Meister and Jupille, a large number of the “bitten” sought out Pasteur’s vaccine treatment. Concomitant opposition among segments of the medical, political, and lay communities, including that of a fervent antivivisectionist movement, denounced what they considered to be an insufficiently tested, potentially dangerous form of treatment; sentiments that would be echoed nearly a century later in anti-vaccination movements involving vaccinations against specific childhood diseases.

## 5.7 Pasteur’s Legacy

The success of his rabies vaccine and the intellectual support of the Academy of Sciences provided the impetus for Pasteur to create a vaccine institute (Pasteur 1886). Through a combination of public and private funding, including international sources, the Institute with its tripartite mission of scientific research in microbiology, public health, and education became a reality. It was inaugurated on November 14, 1888 in the presence of the President of the French Republic.

Pasteur’s results with the rabies vaccine were independently confirmed in experimental animals by an English commission in 1888, which resulted only in increased regulation of dogs in that country (Parish 1965). However, by the early 1900s, more than 100,000 people worldwide had been treated with Pasteur’s regimen (Parish 1965). Over the ensuing 50 years, the vaccine underwent various modifications. Early on, Pasteur’s colleagues began experimenting with variations on the number and timing of inoculations, as well as the preparation of the rabbit spinal cords,

according to their increasing experience and the individual history of exposure of each case. Later, in the first part of the twentieth century, methods were introduced to inactivate the vaccine virus using phenol so as to minimize the risk of vaccine-induced disease (Kammer and Ertl 2002). Although this approach was sporadically associated with either incomplete inactivation accompanied by cases of subsequent vaccine-induced paralysis or by serious adverse events related to hypersensitivity to nervous tissues in the vaccine, this method progressively replaced that of Pasteur and continued to be used into the 1950s.

After the discontinuation of Pasteur's method of vaccine preparation in 1953, other approaches were employed that were based on tissue culture methodologies developed earlier in the decade and improved upon by other technical modifications. Vaccines derived from nervous tissue were known to be associated with an allergic encephalomyelitis triggered by hypersensitivity to residual myelin basic protein in the preparations, causing severe demyelination in some recipients. Rabies vaccines propagated in duck embryos, human diploid cell lines, various nonhuman primate cells, and chick embryo fibroblasts mitigated this problem and generally replaced nervous tissue-based vaccines of Pasteur's type during the 1970s and 1980s, although the latter are still in use in parts of the developing world due to their low cost. Today, more than 12 million people annually receive postexposure vaccination worldwide. In industrialized countries, animal vaccination, developed in parallel with that in humans, virtually eliminated rabies as a significant problem. Unfortunately, because of a lack of access to rabies vaccines in resource-scarce, developing parts of the world, rabies remains a global public-health threat.

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# **Chapter 6**

## **Killed Vaccines: Cholera, Typhoid, and Plague**

**Charles C. J. Carpenter and Richard B. Hornick**

### **6.1 Introduction**

As reviewed in previous chapters of this work, the earliest vaccines were of the live variety, either based on a naturally occurring weaker version of pathogen, as with Jenner's use of cowpox, or the laboratory-manipulated, attenuated forms of anthrax and rabies employed by his vaccine heir, Pasteur. The next important concept in vaccine science, killed vaccines, was introduced in animals almost concurrently with Pasteur's live vaccines, but it took another decade for the realization of its clinical application. In 1886, Theobald Smith and his laboratory chief, Daniel Salmon, developed arguably the first successful, heat killed vaccine against the agent of hog cholera while working at the U.S. Department of Agriculture (Zinsser 1987). In the waning years of the nineteenth century, following closely on the heels of a half-century of stunning advances in microbiology and its sister science, immunology, killed vaccines were developed against three major bacterial causes of human morbidity and mortality of the time that flourished amidst the nineteenth century's primitive sanitation and underdeveloped public health practices: cholera, typhoid, and plague.

### **6.2 Cholera**

#### **6.2.1 Background**

Vivid descriptions of cholera-like illnesses date back to the ancient works of Hippocrates and to Sanskrit accounts in the Ayurveda of Sushrata (McPherson 1894). While periodic outbreaks on the Indian subcontinent had been documented

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for many centuries, cholera was first recognized as a worldwide threat during the first pandemic beginning in 1817 that involved broad areas of Asia, the Indonesian Archipelago, the Middle East, the Mediterranean Basin, North and East Africa and persisted as a seasonal epidemic in many regions (Barua 1992). The next five cholera pandemics, beginning with the second in 1829 and concluding with the sixth during the first two decades of the twentieth century, spanned nearly every decade during that 100 year interval and similarly involved broad swaths of Asia before spreading throughout Europe and the Americas. These outbreaks were accompanied by hundreds of thousands of deaths. The fifth and sixth pandemics, however, were associated with progressively far fewer cases in Great Britain and North America, presumably as a result of improvements in sanitation and water supplies there. Although there were no recognized indigenous cases in North America after 1911 or in Europe after 1925, cholera outbreaks continued to occur annually on the Indian Subcontinent, in Southeast Asia, and in the Middle East.

The seventh and most recent pandemic was initiated in 1961 due to *Vibrio cholerae* O1, biotype El Tor, originally identified at the El Tor quarantine station during the Mecca pilgrimage in 1906. El Tor coexisted with the classical cholera biotypes in major outbreaks in Calcutta and Dhaka in 1963 and within a year had become dominant in annual epidemics in those areas. During the next 30 years, the El Tor strain caused major outbreaks in almost every nation in which poor sanitation existed, persisting as an endemic infection in many of the sub-Saharan African countries. Especially devastating was an outbreak among Rwandan refugees in Goma in eastern Congo in the summer of 1993, in which over 40,000 persons perished within a week. Previously unrecognized in South America, El Tor cholera was introduced into Lima in 1991 and after rapidly disseminating in Peru, it extended, with varying attack rates, throughout South America by 1993 before essentially disappearing by century's end.

Cholera was a terrifying disease both for the general population and for the medical profession throughout its pandemic history, one marked by the explosive and unpredictable onset of outbreaks and by a virulent clinical disease course leading to death in a large proportion of untreated individuals. In the early nineteenth century, cholera was described as "the disease that begins where other diseases end – with death" (Magendie 1832). Common treatment approaches proved to be of no benefit during pandemics. Bloodletting and the mercury-based purgative calomel, used in a variety of disorders, contributed to the extraordinarily high mortality rates by exacerbating volume losses (Howard-Jones 1972).

The basic pathophysiologic defect in cholera, massive fluid and electrolyte depletion via the gut, was described by the Irish physician William Brooke O'Shaughnessy after the introduction of cholera to the British Isles in 1831 (O'Shaughnessy 1831), and intravenous saline therapy based on this principle was demonstrated by a Scottish physician, Thomas Latta to be at least transiently effective in the treatment of cholera shortly thereafter (Latta 1832). Similar, concurrent findings were noted in Russia (Jachnichen 1832). Despite these prescient observations, some reported in the *Lancet*, the most widely respected medical journal of the time, saline treatment was soundly rejected by the medical profession. It was not until the first two

decades of the twentieth century that field work in Calcutta would firmly establish its benefit (Rogers 1921), and it would take another 50 years for fluid replacement in the form of oral rehydration therapy (Mahalanabis et al. 1973) to be widely adopted. Meanwhile, over the nineteenth century, marked by a series of worldwide pandemics, cholera surpassed bubonic plague to acquire the dubious distinction of being the greatest killer among all epidemic bacterial diseases (McGrew 1965).

### 6.2.2 Vaccine Development

Although epidemic cholera had been prevalent for centuries in India, and pandemic disease had completed its first cycle, it was not until 1849, in the waning days of the second pandemic that John Snow proposed a water-borne route of spread (Snow 1849). Snow (refer to Fig 3.4) carried out his classic epidemiologic studies, detailed in Chap. 3, which determined the relationship between pumped, municipal water supplies and cholera and established the basis of transmission during the 1854 outbreak in London (Snow 1855). While Snow's work unquestionably demonstrated the water-borne spread of cholera, his hypothesis that the responsible agents were living microorganisms, proposed a decade before the germ theory of disease would gain widespread scientific favor through the work of Pasteur, was not widely accepted.

Concurrent with, yet unaware of, Snow's work Italian anatomist Filippo Pacini recognized a comma-like microorganism in the small intestines of persons who died with cholera but not in individuals dying of other diseases (Pacini 1854). He proposed that the “comma bacillus” was the causative agent of cholera but published his observations in an esoteric Italian medical journal; his work was therefore never recognized by scientists or physicians in Paris, Berlin or London, the epicenters of burgeoning microbiologic discovery in the latter half of the nineteenth century. Although Pacini postulated that the comma bacilli were living microorganisms, he was unable to grow them in culture.

The German physician-scientist, Robert Koch (refer to Fig 3.7), had already become widely celebrated for his definitive identification of *Bacillus anthracis* as the etiologic agent of anthrax in 1876 and *Mycobacterium tuberculosis* as the bacillary cause of tuberculosis in 1882 by the time he embarked on his studies of cholera in 1883. What began as a German government-sponsored scientific investigation of an outbreak in Egypt, essentially to counterbalance Pasteur's investigative presence there, eventually led to the identification of the bacterial etiology of the disease. However, despite demonstrating the consistent presence of comma-shaped vibrios in the intestines of cholera victims (Koch 1883), as had the geographically isolated Pacini 30 years earlier, Koch's team was unable to grow the organism while in Egypt. Koch proceeded to Calcutta, considered to be the ancestral origin of cholera, and there, using fresh clinical material he was able to grow the organism in broth culture (Brock 1988).

Koch's discovery galvanized the development of a vaccine. In mid-1884, the Catalan physician, Jaime Ferran y Clua (1852–1929), developed a live vaccine

**Fig. 6.1** Jaime Ferran (Instituto de Historia de la Medicina y de la Ciencia López Piñero, Spain)



comprising pure broth cultures of microorganisms that he had isolated from cholera patients in Marseilles. He vaccinated over 30,000 individuals in Valencia during the 1884 epidemic in Spain. While the vaccine was said to provide a high degree of protection against cholera, there were no controlled clinical trials during its widespread use (Ferran 1885). Ferran (Fig. 6.1) had initially tested the vaccine subcutaneously on himself and an assistant noting only focal pain and low-grade fever. In striking contrast to the limited attention given to Pacini's discovery in 1854, Ferran's work generated immediate worldwide interest, leading to a favorable report in the *New York Times*. Scientific commissions from several European nations came to Spain to attempt a retrospective evaluation of Ferran's work, with varying conclusions; a highly regarded group from the Pasteur Institute failed to find convincing proof of the vaccine's efficacy, yet did note the common occurrence of severe, local adverse reactions. Their findings irreparably damaged the vaccine's reputation (Bornside 1982). Nonetheless, these crude studies represented the first attempt to immunize humans against a bacterial disease. In fact the only vaccine previously shown to be effective against a bacterial pathogen was the attenuated, animal anthrax vaccine developed by Pasteur – the inoculation used for the spectacular demonstration at Pouilly-le-Fort in 1881.

By the early 1890s, despite the episode in Spain, Pasteur remained convinced that an effective cholera vaccine was possible. One of his Institute's newest members was assigned the task of its development. Waldemar Haffkine (1860–1930), a Ukrainian-born scientist fled his homeland in the wake of widespread anti-Semitism and a brief stint as a Czarist political prisoner to join his mentor and compatriot,

**Fig. 6.2** Waldemar Haffkine (Rutgers University Press)



Ilya Metchnikoff, at the Pasteur Institute in Paris in 1889. Metchnikoff would become a founding father of immunology. Haffkine (Fig. 6.2), for his part, embarked on a cholera vaccine strategy using two *V. cholerae* strains, one attenuated by growth at 39°C and given as an antecedent subcutaneous injection to mitigate the inflammatory reactions at the injection site noted in animals given the second strain, and chosen due to its enhanced virulence after multiple intraperitoneal passages in guinea pigs. The side effects, including low-grade fever, malaise, headache, and pain at the injection site, determined by a small human safety study in which he was a subject, were considered to be less severe than those observed with Ferran's vaccine.

For a number of “political” and personal reasons, Haffkine performed his initial, large-scale, uncontrolled trials in Calcutta; during the years 1893–1896, he vaccinated more than 40,000 individuals in India against cholera (Löwy 1996). Beginning in 1894 his vaccine trials in subgroups of prisoners, tea plantation workers, and other well defined populations were rigorously designed to provide the most reliable, statistically sound comparisons between vaccinated and unvaccinated subjects. The vaccine, although associated with severe reactogenicity, appeared to provide significant protection against infection (Haffkine 1906). Haffkine’s cholera studies in India are rightly regarded as the first controlled human vaccine trials (Cvjetanovic 1975).

In 1896, Wilhelm Kolle (1868–1935), one of Koch’s assistants at the Institute for Infectious Diseases in Berlin, developed an agar-grown, heat inactivated, whole-cell cholera vaccine which was considerably easier to prepare and standardize than

**Fig. 6.3** Wilhelm Kolle (Robert Koch Institute)



Haffkine's vaccine. The Kolle (Fig. 6.3) vaccine was utilized on a large scale in Japan in 1902 and purported to be over 80% effective in uncontrolled studies (Kolle 1896). Interestingly, despite his favorable results with live bacteria, Haffkine later supported the use of heat-killed vaccines for cholera and, in fact, turned to a killed vaccine approach for his next vaccine assignment – plague. Variations on Kolle's killed cholera vaccine remained the primary vaccine approach throughout the twentieth century.

Between 1920 and 1960, a period in which pandemic cholera was quiescent, a variety of killed, whole-cell vaccines was widely used, but no new approaches were developed. Such vaccines were given to most allied troops assigned to cholera endemic areas in World War II, despite the absence of definitive data concerning their effectiveness. While there were few cholera outbreaks among troops in any region, this could not be attributed solely to vaccination; improvements in sanitation, hygiene, and the absence of pandemic disease during that time were likely contributors.

In the early 1960s, in the context of the rapid emergence of the El Tor strain and recurrent, severe annual epidemics throughout the Gangetic Delta, controlled field trials of killed, whole-cell cholera vaccines were initiated in the Matlab thana area

of East Pakistan in 1963 under the auspices of the newly established Pakistan/SEATO Cholera Research Laboratory in Dhaka (Oseasohn et al. 1965), and in Manila, with scientific and economic support from the Japanese Government (Azurin et al. 1967). Both trials demonstrated significant but short-lived vaccine protection against cholera. In the rural hyperendemic area of East Pakistan (now Bangladesh), killed, whole-cell vaccine engendered more than 70% protection against clinical cholera in adults during the first year after vaccination with a steady decline in protective efficacy over the next 2 years. Protection was significantly lower in children less than 5 years of age – approximately 30% in the first year and insignificant in subsequent years (Benenson et al. 1968). The Philippines field trial comprised in excess of 500,000 subjects in a region where little or no cholera had been present for more than two decades. It showed that overall, killed whole-cell cholera vaccines provided a low level, 26%, of short duration protection during an outbreak of El Tor strain (Philippines Cholera Committee 1968).

The results in East Pakistan were similar to those described by Haffkine using a live vaccine in India and by Kolle using a heat inactivated vaccine in Japan 65 years earlier. Vaccine-induced immunity was greatest in adult populations in cholera-endemic regions and less reliable and of shorter duration in young children, confirming that parenteral killed vaccines did indeed protect to varying degrees against cholera, yet illuminating the need for a more effective vaccine. The higher degree of effectiveness in the adult population in Pakistan than in the Philippines was thought to be due to the fact that cholera had been consistently endemic in the former region for over a century, and thus the population may have harbored some degree of immunologic memory, whereas the epidemic in the Philippines was sporadic and therefore much of the population was immunologically naïve.

During the ensuing four decades, new approaches to cholera vaccine have been explored, informed by advances in the bacteriology and immunology of this pathogen (Levine et al. 2004). In 1967, the demonstration that a single enterotoxin appeared to be responsible for the pathophysiologic defect in cholera suggested the possibility of adopting an altered form of cholera toxin, a toxoid, to confer immunity against clinical disease, analogous to successful approaches with other bacterial, toxin-mediated diseases such as diphtheria and tetanus (Finkelstein 1973). Initial studies of antitoxin immunity in a canine model of cholera indicated that intramuscular immunization with two doses of crude enterotoxin administered at 28 day intervals provided highly significant but transient protection against subsequent intraluminal enterotoxin challenge (Curlin et al. 1968). Further studies based on whole-cell killed vaccines; purified Inaba and Ogawa surface antigens of *V. cholerae*; antitoxic immunity; vaccines designed to provide both antitoxin and antibacterial immunity; and more recently, studies of oral, recombinant live vaccine strains have shown evidence of significant, but not universal, immunity in adults for limited periods of time.

Recently, a major international program aimed at the development of oral vaccines comprising killed bacterial cells combined with the B-subunit of the cholera enterotoxin (BS-WC) was launched. Extensive field trials of the bivalent vaccine in rural Bangladesh in 1985 demonstrated that it engendered more than 85% protection

for at least 2 years in both children and adults, significantly improving upon the protection generally associated with killed vaccines alone (Clemens et al. 1986).

Efforts toward the development of an effective live oral vaccine have led to studies in healthy volunteers demonstrating that those who recovered from cholera induced by the oral administration of two billion live vibrios were immune to a second, tenfold greater dose of organisms (Levine et al. 2004). However, trials of orally administered, toxin-deleted mutants of *V. cholerae* have thus far failed to demonstrate significantly greater immunity than that provided by killed, whole-cell vaccines (Levine et al. 2004). More than a century after the first attempt to induce immunity against a bacterial pathogen – cholera – in humans the development of a more effective cholera vaccine that induces reliable and long-lasting immunity in both children and adults remains both a great scientific challenge and an urgent human need.

## 6.3 Typhoid

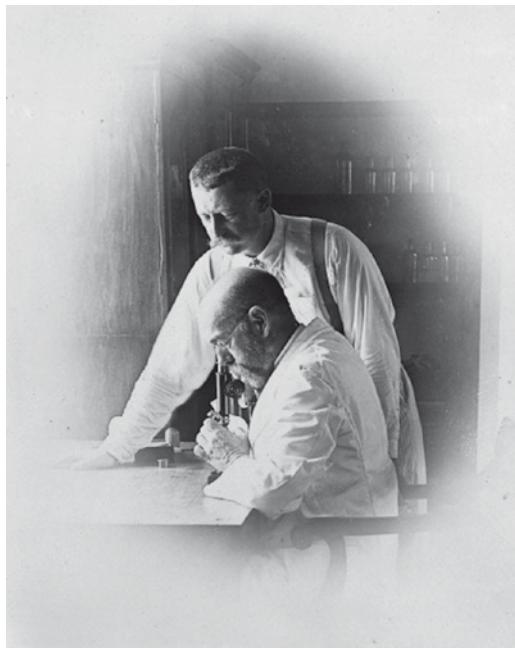
### 6.3.1 *Background*

In 1896, Almroth Wright, a British military bacteriologist (Wright 1896) and Richard Pfeiffer and Wilhelm Kolle (Pfeiffer and Kolle 1896), at Koch's Institute in Berlin, independently reported the first attempts at immunizing humans against the disease caused by *Salmonella enterica* serovar Typhi. Although clinically recognized much earlier, the disease had been recently distinguished from the myriad other febrile illnesses of no apparent identified cause. Its unique pathological features provided significant clues and, as we know now, its classic clinical symptoms and signs identified typhoid fever as a unique type of febrile disease. In 1880, Karl Eberth at the University of Zurich conclusively identified bacilli in mesenteric lymph nodes of infected patients (Eberth 1880); 4 years later, Georg Gaffky, a Koch assistant and member of the German team that would later investigate the etiology of cholera in Egypt (Gaffky 1884), successfully cultivated the organism in pure culture, advances that enabled investigators to begin studying candidate vaccines for preventing the disease.

### 6.3.2 *Vaccine Development*

The idea of producing an attenuated vaccine strain by the passage of live typhoid bacilli through rabbits and mice was introduced by Fraenkel and Simmonds in Hamburg in 1886; they demonstrated protection from re-infection in these animals (Fraenkel and Simmonds 1886). Seven years later, with heat-killed approaches to vaccines under consideration for cholera, Fraenkel used a suspension of killed organisms to treat 57 patients with typhoid during an epidemic with “considerable

**Fig. 6.4** Richard Pfeiffer with Robert Koch (seated) (Wellcome Library)



success” (Fraenkel 1893). This apparently stimulated Kolle (refer to Fig. 6.3) and Pfeiffer (Fig. 6.4) from Koch’s Institute to “immunize” two men with heat killed typhoid bacilli that had been held at 56°C for several hours (Pfeiffer and Kolle 1896). The subjects experienced significant constitutional symptoms and injection site reactions lasting approximately 1 and 3 days after vaccination, respectively. Sera collected 11 days after injection of the killed organisms demonstrated agglutinating antibodies and were successfully used to passively protect guinea pigs from a virulent typhoid challenge.

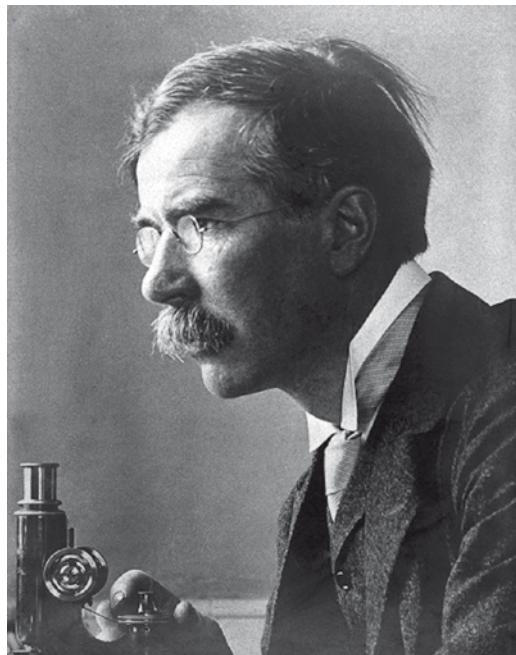
The agglutination test, as described by Georges-Fernand Widal in 1896, added serum from patients with typhoid fever in serial dilutions to broth cultures of *S. typhi* and noted flocculation at higher concentrations, interpreted to represent the presence of agglutinating antibodies (Widal 1896). Now known to identify antibodies directed against specific *S. typhi* antigens, the test remains in use in some areas as a serodiagnostic tool, but in the late nineteenth century its demonstration of such antibacterial substances in sera of individuals vaccinated with killed bacilli suggested the potential for vaccine-induced protection against typhoid.

Almroth Wright (1861–1947), a bacteriologist in the British Army Medical Services in the 1890s, had been studying calcium chloride as a treatment of “serous hemorrhages with condition of defective blood-coagulability” that included an evaluation of its effectiveness in treating injection site inflammatory responses – apparently using killed typhoid bacilli as a means to induce inflammation (Wright 1896). He reportedly inoculated killed bacilli into two British officers of the

Indian Medical Service and a horse, expeditiously publishing his data in the *Lancet* within 2 weeks of study completion and thus establishing his priority of being first in testing a killed typhoid vaccine. Pfeiffer and Kolle published their use of killed vaccine 2 months later (Pfeiffer and Kolle 1896).

Thus began a decade-long dispute between the British and German scientists regarding the primacy of the inaugural use of killed typhoid vaccine in humans (Gröschel and Hornick 1981). Wright (Fig. 6.5) claims to have based his work on a conversation with Haffkine who had suggested the use of “live attenuated organisms” to prevent typhoid fever, an approach similar to the latter’s own in ongoing cholera vaccine studies in India (Wright and Semple 1897). Due to concern over the potential risks, Wright apparently decided against the idea. But upon learning of Pfeiffer’s findings of a typhoid-specific agglutination reaction in humans given heat killed bacilli, he opted to employ a similar method of producing the preparation used in his two volunteers (Wright and Semple 1897). Although Wright appears to have primarily used killed bacilli as a means of inducing local inflammation and not for protection against typhoid, he later disputed this and pointed to an experimental live challenge in a “vaccinated” subject as evidence of his motive to induce protective immunity (Wright and Semple 1897).

A decade later, Friedberger, working in Pfeiffer’s Institute reignited the controversy, stating that Wright’s “claim to priority was inaccurate” (Friedberger 1907), leading to counter-accusations by Wright (Wright 1908). The dispute is of mainly historical interest because both groups were working with killed typhoid vaccines



**Fig. 6.5** Sir Almroth Wright (Wellcome Library)

in nearly concurrent fashion and deserve shared credit for their use (Gröschel and Hornick 1981). Actually, Fraenkel's experience with killed typhoid vaccine in humans in 1893 probably represented its first use; while this appears to have informed Pfeiffer's studies in Berlin, its impact on Wright remains unclear. Vaccines similar to those produced by Wright and Pfeiffer were used for another 60 years before presumably more effective and less reactive preparations were developed.

Despite the lack of a definitive claim to primacy in terms of vaccine use, Wright clearly deserves credit for the first large-scale vaccination using killed typhoid vaccine in nearly 3,000 British troops deployed to India; the results were inconclusive but encouraging. In 1899 due in some measure to Wright's persistence, a subset of troops embarking to South Africa in the Boer War were vaccinated. The effectiveness of vaccine was suggested by a more than 75% reduction in the incidence of typhoid among inoculated troops; however, the data were poorly controlled, and statisticians and others remained unconvinced of its value (Gröschel 1967; Parish 1965). Further, local and systemic adverse reactions were common and at times severe following the administration of the vaccine. Nonetheless, by 1915 and after vaccines had demonstrated success in reducing typhoid among soldiers in other, international military conflicts (Gröschel 1967), a re-formulated version of killed typhoid vaccine was routinely used, with good results, in enlisted British troops but not officers entering World War I.

In the United States initial efforts to immunize troops using an oral form of typhoid vaccine were undertaken in 1904 by James Carroll, who had previously worked under Walter Reed investigating yellow fever in Panama (Reed et al. 1904). He tested a vaccine consisting of broth-cultured bacilli held at 56°C for 1 h that had induced measurable antibodies in guinea pigs and rabbits. After 13 volunteers, including Carroll and two other investigators, ingested the preparation, ten developed a disease consistent with typhoid fever, one with bacteremia. The "vaccine" preparation, thought to be of killed bacilli, was found to harbor viable organisms, probably the result of nonuniform heating. This study remained a classified secret for 55 years (Tigertt 1959).

The U.S. Army initiated mandatory typhoid vaccination using killed bacilli, similar to Wright's and Pfeiffer's preparations, in 1911, as a result of the efforts of Major Frederick Russell (Artenstein et al. 2005) who had visited Wright's laboratory in 1908. Vaccinated U.S. troops deployed to the battlefields of World War I experienced a markedly decreased incidence of typhoid fever compared to that in the Spanish American War, less than two decades prior (Tigertt 1959). However, improved food and water supplies and sanitation practices among the troops were probably the major contributing reasons for the decline, but vaccination was certainly a factor in military settings. Similar typhoid dynamics emerged from World War II; rates were generally low, probably a combination of stringent attention to hygiene and sanitation and vaccination, but outbreaks occurred due to breaches in hygienic practices or suboptimal vaccine formulations (Gröschel 1967).

In fact the incidence of typhoid fever had already started to decrease by the turn of the century due to improvements in public health and sanitation and further decreased by nearly 20-fold in developed countries post-World War I (Sansonetti 1996).

Typhoid, like cholera, was an enteric infection, a disease of the gastrointestinal tract related to inadequate sewage handling with its attendant contamination of water and food supplies. Chlorination and filtration of water represented major improvements in the twentieth century that led directly to reductions in such infections.

One epidemiological study of typhoid fever in cities using untreated water from the Ohio River prior to the implementation of such public health improvements demonstrated a direct correlation between levels of *E. coli*, a marker of enteric bacterial contamination, and the incidence of typhoid (Veldee 1931). In Wheeling, West Virginia, the incidence declined by 75% and the mortality by nearly two-thirds comparing the 4 years before chlorination of water was implemented in 1918 with the 6 years thereafter. In the 3 years following the additional institution of filtration processes to water treatment in 1925, there were five cases and no associated deaths from typhoid in Wheeling despite a 50% increase in the population during this period. Typhoid vaccine was not widely used in the U.S. civilian population; disease control was therefore accomplished through these other forms of intervention.

The simmering debate regarding the actual efficacy of killed typhoid vaccines in reducing the incidence of disease was addressed in a series of large-scale, double blind, controlled studies in Yugoslavia (Yugoslav Typhoid Commission 1964), Guyana (Ashcroft et al. 1967), and Poland (Polish Typhoid Committee 1966) in the 1960s, conducted under the auspices of the World Health Organization (WHO). Two of the most effective vaccines were prepared at the Walter Reed Army Institute of Research (WRAIR). Both vaccines employed a single strain, Ty2, of typhoid. One vaccine, "L," was produced by heating bacilli to 56°C for 1 h followed by phenol treatment, the method used by Pfeiffer more than six decades earlier; in the second, "K," the organisms were inactivated by acetone and heated for 24 h at 37°C, a procedure thought to enhance preservation of the virulence (Vi) antigen on the surface of the bacteria. Both vaccines induced significant levels of typhoid immunity, but "K" was associated with better efficacy, 79–88% compared with 51–66% for "L." The Guyana study, lasting 7 years and associated with the highest efficacy, involved only children less than 15 years of age, as children are more susceptible to disease than presumably immune-experienced adults in endemic areas (Ashcroft et al. 1967). Interestingly, in one of the trials the control group receiving only tetanus vaccine experienced more than sixfold lower typhoid rates than the general population who did not participate in the study, suggesting an important role of education and nonvaccine preventive measures (Yugoslav Typhoid Commission 1964). Independent vaccine evaluations were carried out in the Soviet Union.

When tested in a nonendemic population, these killed formulations demonstrated 67% protective efficacy with a challenge dose of  $10^5$  organisms; protection was abrogated with higher challenge inocula (Hornick et al. 1970). The former inoculum may be of relevance in water-borne infection. A 1963 typhoid outbreak in the Swiss ski resort of Zermatt caused by decayed wooden sewer pipes that leaked their contents into the adjacent wooden water mains resulted in a clinical incubation period in a group of British tourists matching that associated with a  $10^5$  challenge (Bernard 1965); incubation periods for higher challenges were shorter.

However, outbreaks involving contaminated food in which transmission is initiated by a carrier may be associated with higher organism burdens and may overwhelm vaccine induced protection.

While the killed vaccines demonstrated definitive efficacy against typhoid, they were associated with high rates of systemic and local adverse events that limited their acceptability and ultimately, their availability. Two vaccines, formulated to improve upon the tolerance of parenteral, whole-cell, killed products, were developed in the 1970s: purified Vi subunit vaccine and a live, attenuated oral vaccine. They were generally well tolerated with few adverse reactions (Acharya et al. 1987; Germanier and Fuerer 1975). The Vi or virulence antigen of *S. typhi*, in purified form, was shown to engender 72% protection against typhoid fever in endemic areas when given as a single, parenteral dose (Acharya et al. 1987).

The stimulus to seek an oral attenuated vaccine is to protect against the disease derived from the curious immune status of the typhoid carrier. These individuals have a low grade *S. typhi* infection confined to the gall bladder which in some cases, may act as a nidus for gallstone formation. The organisms pass into the intestine and are excreted in the stool, in counts as high as  $10^9$  organisms per gram of stool (Merselis et al. 1964). Carrier mucosal immune mechanisms appear to prevent the bacteria from disseminating. The experimental ingestion of Ty21a, a strain of *S. typhi* mutants lacking activity of the enzyme uridine diphosphate (UDP)-galactose-4-epimerase, with reduced activities of two other enzymes, and lacking the Vi antigen (Germanier and Fuerer 1975), resulted in a significant increase in secretory IgA antibody directed at the organism's immunodominant surface O antigen (Cancellieri and Fara 1985). This leads to a process by which activated lymphocytes in the intestinal Peyer's patches migrate to local lymph nodes to mature (Kantele et al. 1997) and subsequently spread to diffuse mucosal tissues. These cells have been isolated from the blood and can secrete specific IgA antibody in the presence of *S. typhi* antigen. Thus the oral live vaccine, unlike killed vaccines, appears to stimulate both arms of the immune system.

The basis for the live oral vaccine is the attenuated strain Ty21a, employing mutant colonies derived originally from wild type strain Ty2 – the strain used to formulate “K” and “L” vaccines in the WHO trials. Live, attenuated oral typhoid vaccine was shown to be safe and immunogenic in healthy volunteers (Gilman et al. 1977); controlled field trials in 16,000 Egyptian school children demonstrated 96% protection, a higher rate than with any killed vaccine (Wahdan et al. 1982). Additional, large-scale trials in endemic areas served to define its current formulation and four-dose regimen.

The search for better oral attenuated vaccines continues, influenced by the global emergence of multiple antibiotic-resistant strains of typhoid in the last decade of the twentieth century and their association with increased morbidity and mortality. Wright and Pfeiffer provided a starting point for vaccine development and although the ideal vaccine remains elusive, highly effective ones are available. The ultimate goal is an effective, inexpensive vaccine that is practical for distribution in late developing countries. In this way the most susceptible population can be protected from this serious infectious disease.

## 6.4 Plague

Throughout history, Plague occupied a unique and terrifying position as a cause of explosive epidemics that resulted in massive death tolls and altered the course of human events. From the Justinian plague of 541 AD that sparked two centuries of resurgent disease epidemics, through the Black Death that gripped Europe in the fourteenth century and decimated its population by as much as 40%, plague had been a constant threat on that continent. In the 1800s the disease began its assault on Asia, killing millions during that century in India and the Far East.

The bacterial etiology of plague was independently discovered by Shibasaburo Kitasato and Alexandre Yersin in 1894 and named *Pasteurella pestis*, only much later to be renamed *Yersinia pestis* after one of its founders. Kitasato was a distinguished bacteriologist who had isolated the bacillary etiology of tetanus and discovered, with Emil von Behring, the phenomenon of serum antitoxin while in Koch's laboratory earlier in his career. Yersin, a disciple of Pasteur who had identified the organism during an outbreak investigation in Hong Kong, subsequently developed the first heat-killed, plague vaccine for animals. But it was another of Pasteur's disciples, Haffkine, already known in the region for his widespread cholera vaccine studies, who was called upon by the Indian government to create a plague vaccine when epidemic disease reached Bombay in 1896 (Hawgood 2007).

Haffkine moved to a temporary laboratory quarters at the Grant Medical College in Bombay, where he grew pure cultures of plague bacilli and then heat inactivated them at 70°C for 1 h. After successfully immunizing rabbits with the killed vaccine, he inoculated himself, noting only fever and injection site pain (Hawgood 2007). Based on his experiences during the previous 4 years with early, controlled cholera vaccine trials and in the setting of an outbreak of plague in the city, Haffkine performed a small, controlled trial of his new plague vaccine among "volunteers" at a local prison in 1897. The vaccine was protective. He vaccinated hundreds of thousands of individuals over the next 4 years, 200,000 in Bombay alone (Bannerman 1902; Haffkine 1897) with "strikingly good results" (Corthorn 1901). Not only did the vaccine prevent infection, but it reduced the mortality rate among plague-affected communities by nearly 50% and even appeared to reduce mortality in those incubating infection at the time of vaccination (Bannerman 1902).

As the demand for vaccine grew in India, so did Haffkine's operation. By 1901, he was the Director-in-Chief of the Plague Research Laboratory in Bombay with a staff of 53, preparing and dispensing plague vaccine as well as cholera and typhoid vaccines (Bannerman 1902); he was also appointed as the scientific advisor to the Indian government. Haffkine was a tireless advocate of vaccines for the prevention of epidemic bacterial diseases and a dedicated clinical trialist. He became somewhat of a popular hero for his antiplague efforts in India; however, his attitude, style, and foreign background probably also engendered animosity among the British authorities there.

In 1902, with the killed plague vaccine in widespread use in India, nineteen deaths due to tetanus were reported among recipients in Mulkowal within the Punjab area of the northwestern part of the country. Investigation revealed contamination

of one bottle of vaccine produced in Haffkine's laboratory. He was relieved of his duties after a government appointed Commission blamed improperly conducted manufacturing procedures for the incident (Editorial 1907) that became known by some as "the little Dreyfus affair," after the contemporary French spy case involving military secrets and antisemitism, because of its protagonist's connection to Jewish causes. Upon careful review of the investigative data and under increasing pressure from the most renowned British scientists of the day, the government relented, and Haffkine was exonerated in 1907. He returned to Calcutta to continue his research but was not permitted to test or produce vaccines, and he retired upon achieving the minimum age requirement (Waksman 1964). His reputation was eventually resurrected with the renaming of the Plague Research Laboratory in his honor.

As the central role of rats and their fleas in the transmission of plague became well established in the early part of the twentieth century, the institution of public health measures to control the vector and improve sanitation contributed significantly to the reduction in disease burden but killed whole cell vaccines in certain areas, such as India, clearly contributed to it as well (Parish 1965). Variations on Haffkine's original formulation were produced for the U.S. military beginning in the late 1940s and continued to be available through the end of the twentieth century. Live attenuated strains of *Y. pestis* were used in the former Soviet Union. Currently, subunit approaches to plague vaccines using recombinant virulence factor proteins are in clinical development to counter the threat of plague as a potential agent of biological terrorism. It is hoped that these products will be rapidly and highly immunogenic and associated with reduced local and systemic side effects as compared with killed whole cell vaccines.

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# **Chapter 7**

## **Toxoid Vaccines**

**John D. Grabenstein**

### **7.1 Introduction**

Diphtheria, tetanus, and pertussis represented important causes of morbidity and mortality well into the early part of the twentieth century. With advances in bacteriology deriving from the work of Pasteur and Koch during the latter portion of the nineteenth century, the organisms responsible for each of these serious infections were cultivated, leading to some of the earliest and most dramatic successes in vanquishing bacterial infections by immunization. Insights derived from the study of the organisms informed the fledgling field of immunology as scientists harnessed antibodies to neutralize toxins, transformed toxins into vaccines, and identified chemical substances, adjuvants, to enhance immune responsiveness. Such advances relieved humanity of great suffering and laid the groundwork for vaccines against other pathogens.

### **7.2 Historical Background of the Diseases**

Diphtheria is a potentially life-threatening disease that primarily involves tissues of the upper respiratory tract (Parish 1965; English 1985; Nezelof 2002). It was recognized in its epidemic form since ancient times, but by the late nineteenth century, diphtheria had completed the transition from epidemic to endemic disease, responsible for more than 100,000 annual cases in the United States with a case fatality ratio of nearly 15%. In 1826, the French physician and later founder of the medical school at Tours, Pierre Bretonneau, named the disease *diphthérite*, from the Greek word for “leather” or “hide,” after the appearance of the inflamed mucous membranes, and he differentiated it from scarlet fever (Relyveld 1996). The mortality associated with diphtheria resulted from the effect of this inflammation on air

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exchange, a condition especially dangerous in those with smaller caliber airways – children. Diphtheria was an especially cruel disease, as its victims died slowly by suffocation.

The German physician Edwin Klebs, who trained under the renowned pathologist Rudolf Virchow, identified the causative microbe in 1883 in stained smears of affected membranes (Klebs 1883). Friedrich Löffler, a Prussian army physician and one of Koch's assistants in Berlin extended Klebs' work by isolating the bacteria in pure culture and demonstrating it to be the etiologic agent of diphtheria in 1884 (Löffler 1884). In so doing, Löffler actually became the first to publish a set of criteria for causality that were to be memorialized as "Koch's postulates." The bacterium became known as the Klebs-Löffler bacillus, later *Microsporon diphtheriticum*, *Bacillus diphtheriae*, *Mycobacterium diphtheriae*, and finally *Corynebacterium diphtheriae*. In 1888, Émile Roux and Alexandre Yersin, Pasteur protégés based at the newly opened Institute in Paris, demonstrated that the association between the local lesions of diphtheria and its systemic manifestations resulted from protein exotoxins produced by the bacteria during infection (Roux and Yersin 1888; Dolman 1973; Winau and Winau 2002). Years later, it was proven that toxin production occurs only when the bacteria themselves are infected by a specific virus, a bacteriophage that carries the toxin's genetic instructions. Roux would figure prominently in the diphtheria vaccine story; Yersin is best known for his codiscovery of the bacterial etiology of bubonic plague, an organism named in his honor, and for his work on this pathogen in French Indochina.

Clinical descriptions of tetanus, based on a Greek word for the dramatic disease manifestations of muscular tension and contraction, appear in the writings of ancient civilizations (Wassilak et al. 2008). This noncommunicable disease acquired via wounds from environmental sources continued to be a significant cause of mortality for millennia. The spore form of the organism can survive in the environment for many years under adverse conditions yet remain capable of causing disease. Although its course usually begins with contractions and spasms of the jaw and neck muscles, earning it the moniker "lockjaw," the effects rapidly generalize and result in death by either respiratory muscle compromise or dysfunction of the autonomic nervous system.

The German scientist Arthur Nicolaier correlated the presence of anaerobic soil bacteria in wounds with tetanus in 1884 and hypothesized that the disease resulted from a diffused toxin (Nicolaier 1884). Shibasaburo Kitasato, a Japanese investigator working in Koch's Berlin laboratory, was the first to isolate *Bacillus tetani* (later *Clostridium tetani*) in pure culture in 1889 (Kitasato 1889). The Danish physician Knud Faber demonstrated the existence of tetanus toxin and its role in the pathophysiology of the disease in 1890 (Faber 1890).

Pertussis, a highly transmissible epidemic and endemic respiratory infection associated with significant morbidity and mortality was named for its striking clinical presentation of paroxysmal and violent cough by Thomas Sydenham, the father of English medicine, in 1679. Commonly known as "whooping cough" because of the sound generated as the patient attempts to inspire against a closed glottis, the risk of death associated with the disease is highest among infants younger than 6 months old (Cherry 1996).

Jules Bordet and Octave Gengou, working at the Pasteur Institute in Paris observed bacteria in tissues of infected patients in 1901 and reported the isolation of the etiologic agent in 1906 (Bordet and Gengou 1901, 1906). Bordet was the founding director of the Institute's satellite facility at Brussels in his native Belgium, and while he earned significant recognition for his work with the bacillus (initially *Haemophilus pertussis* or *Bacillus pertussis*) that was later named in his honor, *Bordetella pertussis*, his greatest scientific achievements were in the field of immunology; he was awarded the Nobel Prize in 1919 for his discovery of complement.

## 7.3 Advances in Immunology

### 7.3.1 Antitoxin as a Breakthrough

Until the late nineteenth century, there were no effective treatments for these diseases (Parish 1965). Tracheotomy, first successfully performed by Bretonneau in 1825, offered some value as a supportive treatment in diphtheria; intubation was introduced in 1886 (English 1985). In 1890, advances in immunology brightened the prognosis of untold millions of future patients.

After gaining an interest in bacteriology as a Prussian army surgeon serving in Posen (now Poznan, Poland) in the early 1880s, Emil von Behring (1854–1917) joined Koch's Berlin laboratory as an assistant in 1889 and followed him to the new Institute of Infectious Diseases 2 years later. In collaboration with Shibasaburo Kitasato (1852–1931), who had isolated the bacillary etiology of tetanus shortly after his 1886 arrival in Koch's laboratory from Japan for bacteriologic training, Behring (refer to Fig. 3.10) embarked on a line of investigation that would yield immediate and far-reaching dividends. Behring and Kitasato (Fig. 7.1) discovered that the serum of animals challenged with sublethal doses of either tetanus or diphtheria bacilli demonstrated an “antitoxin” effect when injected into other animals, preventing the lethal effects of these pathogens and their toxins (Behring 1890, 1895; Behring and Kitasato 1890; MacNalty 1954; Pope 1963; Linton 2005). Even more remarkably, the serum of the immune animal could be transferred to another animal and confer protection to the adoptive host, the concept of “passive transfer.” The experiments progressed from guinea pigs to rabbits, sheep, goats, and horses and earned Behring the inaugural Nobel Prize in 1901 for the discovery of serum therapy, specifically for its application against diphtheria. Kitasato returned to Japan in 1891 where he founded his own institute in Tokyo and shortly thereafter co-discovered the plague bacillus.

Behring's sheep-derived antitoxin made its way into clinical practice, although the story of its first use in a young girl with diphtheria in Berlin on Christmas night 1891 is apocryphal (Linton 2005). The first humans treated with Behring's diphtheria antitoxin in a rational matter were a series of children under the care of Otto Heubner, a professor of pediatric medicine at the University of Leipzig,



**Fig. 7.1** Shibasaburo Kitasato (Robert Koch Institute)

between October 1892 and May 1893. Technical issues specifically related to antitoxin concentration and potency dominated the early years of serum therapy. Production of diphtheria antitoxin on a commercial scale was undertaken by several enterprises: the German dye manufacturer Meister, Lucius & Brüning, the Pasteur Institute in Paris, the British Institute of Preventive Medicine in London, and the New York City Board of Health laboratories (Oliver 1941; Dolman 1973; Schaeffer 1985; Winau and Winau 2002). Large-scale manufacture of antitoxin tended to use serum harvested from horses, because of their large blood volume.

Soon, it became clear that diphtheria antiserum treatment had a dramatic impact on disease mortality (Relyveld 1996); reports from Germany suggested a greater than 50% reduction in this parameter. Roux confirmed its value in controlled studies in French children's hospitals in 1894 (Roux et al. 1894). Two key developments were soon introduced to boost antitoxin potency, one bacteriologic and the other immunologic.

In 1894 Anna Wessels Williams, a physician and bacteriologist working under the directorship of William Park at the New York City Board of Health, the first municipal infectious diseases diagnostic laboratory, addressed the potency issue when she isolated a strain with unusually high and consistent toxigenicity, subsequently named the Park-Williams No. 8 strain and widely used for the

production of antitoxin (O’Hern 1986). Park had been recruited to the Department by Herman Biggs, a career public health official who had been impressed with the value of diphtheria antitoxin during a visit to Koch’s Institute earlier in 1894.

Contemporaneously, Paul Ehrlich (refer to Fig. 3.11), originally working under a contractual collaboration with Behring, developed techniques to reliably standardize diphtheria antitoxin potency (Ehrlich 1897; Dale 1954; Bäumler 1984; Liebenau 1990; Linton 2005). In our modern era, in which pharmaceutical potency is so often assumed, Ehrlich’s accomplishment should not be underestimated. Ehrlich’s original standardized serum became the first international standard reference preparation. In developing the quantitative methodology for antitoxin, Ehrlich expanded his ongoing studies that laid the foundation of modern immunology, using his receptor theory initially to explain toxin-antitoxin interactions and only later, antigens and antibodies. This work subsequently earned him the 1908 Nobel Prize. Notably, Behring exploited Ehrlich’s contributions by entering alone into a lucrative commercial contract with Meister, Lucius & Brüning.

It also became clear that early diagnosis and treatment improved prognosis. Presaging modern public health maneuvers, Park created depots for diphtheria antitoxin in more than 40 drug stores throughout New York City where physicians could obtain diagnostic throat-swab culture supplies as well as case-report forms (Parish 1965; Chase 1982). Analogous arrangements were established by local health departments in other cities (Anonymous 1904; Bowman 1915; Grabenstein 1999; Liebenau 1987). Contemporaneously, in 1913 Vienna, the Hungarian-born pediatrician, Béla Schick developed a skin test to diagnose immunity to diphtheria (Schick 1913; Park and Zingher 1916; Glenny and O’Brien 1921; Pappenheimer 1958; Birch 1973). Upon his appointment in pediatrics at the Mount Sinai Hospital in 1923, Schick’s skin test was applied on a large scale in New York City to determine susceptibility to diphtheria, thus allowing antitoxin to be targeted to those most in need.

Development of tetanus antitoxin followed a path largely akin to that of diphtheria antitoxin. While diphtheria antitoxin had both therapeutic value and some degree of prophylactic value, tetanus antitoxin had limited value after the onset of symptoms (Lévy 1975). Adolf Baginsky, a noted German academic pediatrician, was the first to treat a patient with tetanus antitoxin prepared from rabbit serum by Kitasato in 1891. Although the patient did not recover, perhaps because of inadequate dosing or delayed treatment (Baginsky 1891), investigation continued. By the early years of the twentieth century it had become apparent that tetanus antitoxin was valuable in wound management, to prevent the dissemination of toxin from spores introduced into the skin.

As with early diphtheria antitoxin efforts, uniform quality standards for tetanus antitoxin were lacking. Contamination of these biologic products was well described; 14 children died in St. Louis as a result of diphtheria antitoxin contaminated with tetanus spores in 1901 (Anonymous 1901; Kondrata 1982). Based on this and similar events, the U.S. Congress enacted legislation “to regulate the sale of viruses, serums, toxins, and similar products” (Baker 2000; Coote 2005). Later known as the Biologics Control Act, it was the first federal attempt to ensure medication quality control. Fittingly, diphtheria antitoxin was the first drug licensed under this Act in 1903 (Pittman 1987).

As antitoxins derived from animal serum became widely adopted, scientists increasingly recognized a variety of adverse clinical reactions that were inherent to the products themselves and unrelated to the quality of manufacturing. Deciphering the mechanisms of these reactions contributed significantly to the development of the discipline of clinical allergy and immunology.

Experiments by French physiologist Charles Richet and colleagues in 1902 demonstrated the role of foreign proteins in inducing anaphylaxis, a life-threatening hypersensitivity reaction seen with animal-derived antisera (Portier and Richet 1902). Richet was awarded the Nobel Prize in 1913 for this work. In 1911 Clemens Von Pirquet and Schick described a syndrome of fever, rash, and arthralgias following repeated doses of equine diphtheria antitoxin (von Pirquet and Schick 1905; MacKenzie and Hanger 1930). The syndrome, serum sickness, is now understood to be a reaction to immune-complexes formed from the combination of high concentrations of antigens and antibodies. As these products were refined into globulin-rich preparations that reduced the quantity of nontherapeutic foreign proteins, they elicited fewer reactions. Eventually serum sickness was largely obviated by the use of refined antitoxins derived from human, rather than animal serum (Young and Park 1928; Weil et al. 1938). However, the frequency of adverse events associated with serum therapy, coupled with its relative brief duration of action of weeks to months, limited its use in preventing the disease.

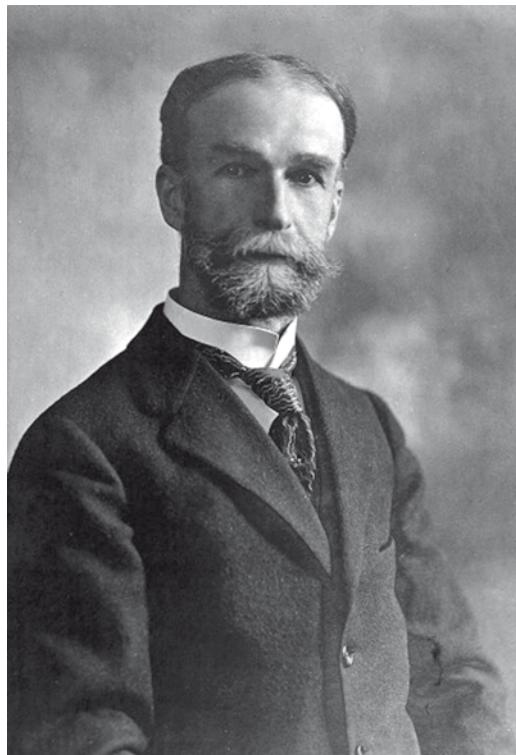
Meanwhile, developments in immunology during the early years of the twentieth century led to the discovery of forms of active immunity against these pathogens. One of the last notable episodes of the antitoxin era occurred in the winter of 1925 in the remote Arctic terrain of Alaska. A relay of 20 musher-and-sled-dog teams battled extreme conditions to deliver diphtheria antitoxin to outbreak-stricken villages near Nome, completing the nearly 700-mile trek from Nenana in a record-breaking time of less than 6 days (Wilson 1986; Salisbury and Salisbury 2003). The bravery of the teams is commemorated today in the annual Iditarod sled dog race.

### 7.3.2 *From Antitoxins to Vaccines*

The road from passive to active immunization was not direct. The first step involved combining diphtheria toxin and antitoxin in the same syringe, with a favorable effect on the diphtheria mortality rate (New York State Department of Health 1927; Harrison 1939; Emerson et al. 1940; Oliver 1941; Dolman 1973; O'Hern 1986). From about 1897, combinations of diphtheria toxin and antitoxin were used for commercial antitoxin production in horses, proving to be more efficient than toxin alone (Parish 1965). In 1909 the physician-researcher Theobald Smith (1859–1934) established a practical method to balance such mixtures for maximum effect (Smith 1909; Zinsser 1987; Chernin 1987). In the process he discovered a number of novel, yet fundamental immunologic phenomena, including hypersensitivity reactions in animals immunized with heterologous serum.

Smith (Fig. 7.2) had distinguished himself earlier in his career at the Bureau of Animal Industry of the U.S. Department of Agriculture where in 1886 he and his

**Fig. 7.2** Theobald Smith  
(Wellcome Library)



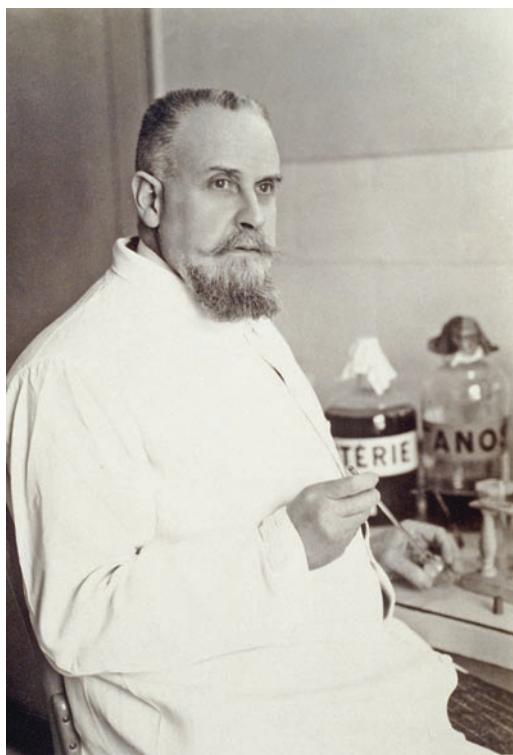
chief, Daniel Salmon, developed arguably the first successful killed vaccine against the agent of hog cholera. Smith left the federal government in 1895 to direct the Massachusetts Antitoxin & Vaccine Laboratories (now the Massachusetts Biological Laboratories). By 1914, Park, at the New York City Health Department laboratories and a prime mover in public health, began using diphtheria toxin-antitoxin mixtures for prophylaxis, guided by the Schick test (Schick 1913; Park et al. 1914; Schaeffer 1985).

Ehrlich had noted the existence of toxoids, inactivated forms of bacterial toxins, during his seminal standardization work in the late 1890s (Bäumler 1984). Ernst Löwenstein at the Vienna Serotherapeutische Institute and Alexander Glenny at the Wellcome Research Laboratories in London had used formalin-detoxified toxin to immunize horses as early as 1904 (Löwenstein 1909; Glenny et al. 1932). In 1907 Theobald Smith demonstrated that toxoids could be used to immunize guinea pigs and later, that the immunity was durable. He also suggested that these chemically inactivated products may have a potential role in human prophylaxis. However, it would take nearly two decades for medical science to realize that potential.

In 1923, Glenny and Barbara Hopkins fortuitously converted diphtheria toxin into a toxoid by the action of formalin, a reaction that resulted in cross linkages between lysine and imidazole groups within or between the toxin molecules, leading to stable bridges (Glenny and Hopkins 1923; Relyveld 1996).

However, the resultant product still required antitoxin to ensure its safety (Lévy 1975). Later that year, Gaston Ramon (1886–1963), a French veterinarian working at the Pasteur Institute and a distant relative of Roux by marriage, developed a practical method to produce diphtheria toxoid by formalin and heat inactivation and used it without antitoxin to safely induce active immunity in humans (Ramon 1923, 1960). The product, termed *anatoxine*, became the basis for modern toxoid vaccines. Ramon (Fig. 7.3) also developed a toxoid potency test based on the limit of flocculation, the dilution where the toxoid and its corresponding antitoxin first yielded visible complexes, indicating an appropriately antigenic yet not toxic concentration. The novel toxoid vaccine against diphtheria and its dosing based upon the flocculation test rapidly proved to be clinically effective.

Nonetheless, diphtheria toxoid was a relatively poor immunogen, hampered by its low level of stimulated antibodies and its lack of durable protective responses. Ramon had tried various maneuvers to render the product less “soluble” to enhance the immunogenicity and prolong the duration of immunity. After noting enhancement of immune responses against diphtheria in animals with inflammatory reactions at the site of vaccination, he experimented with the addition of sterilized tapioca (a semi-soluble starch), calcium, magnesium and aluminum salts, lanolin, kaolin, and other preparations. In 1926, Glenny and colleagues precipitated diphtheria



**Fig. 7.3** Gaston Ramon  
(Institut Pasteur)

toxoid with aluminum potassium sulfate or “alum” which rendered a preparation that persisted in the tissue and evoked durable protection (Glenny 1930; Glenny and Barr 1931; Relyveld 1996). The precipitate included a mixture of aluminum hydroxide and aluminum phosphate, because the culture medium contained phosphates. Thus began the role of aluminum salts as the most commonly employed vaccine adjuvant, an ingredient added to a vaccine to enhance the immune response to an antigen. The following years saw several chemical refinements, leading to the toxoids we know today.

The development of tetanus toxoid proceeded analogously to that of diphtheria (Jordan and Halperin 1941). Tetanus toxin–antitoxin mixtures arose first, mainly to immunize animals. Ramon and Glenny each developed early tetanus toxoids along with those of diphtheria. Ramon and Christian Zoeller immunized humans with tetanus toxoid in 1926 (Ramon and Zoeller 1926, 1927). Use of aluminum salts as an adjuvant followed within a few years. The fluid forms of diphtheria and tetanus toxoids were licensed in the U.S. in 1926 and 1933, respectively; adsorbed forms followed later. However, widespread immunization against these two diseases remained at only modest levels until spurred by military exigencies in mid-century.

The British Army began routine prophylactic use of tetanus toxoid or toxoid–antitoxin floccules at the beginning of World War II. By military doctrine, wounded troops also received a supplemental dose of antitoxin (Scheibel 1955). The effectiveness of tetanus toxoid in protecting wounded soldiers became apparent with the massive British retreat from Dunkirk in 1940. In 1941, the U.S. Army initiated routine prophylactic vaccination of soldiers with tetanus toxoid; wounded troops received a reinforcing or “stimulating” dose of toxoid, but not antitoxin (Long 1943). Among the many millions of American troops participating in World War II, there were only 16 cases of tetanus with six associated deaths. Of the total, only five were vaccinated, and none completely. Cases of tetanus among British forces were similarly rare. This represented a significant reduction in the risk of tetanus in unvaccinated casualties from World War I (Long 1955).

The German Wehrmacht used antitoxin only, rather than toxoid, and suffered high rates of disease and death from tetanus. Despite the ubiquitous presence of tetanus spores in the soil, German authorities inexplicably restricted tetanus toxoid to their Air Force – the Luftwaffe – and some parachute units, to the detriment of the land forces. German ground troops were similarly not vaccinated against diphtheria, a decision echoed in France, Britain, and other European countries in the 1930s and early 1940s (Corson 1943; Baker 2000; Dittman et al. 2000; Galazka 2000a). After the war, routine use of both toxoids became the norm in America and across Europe.

### 7.3.3 Preventing Pertussis

Early pertussis vaccines were developed shortly after the causative bacterium was isolated in 1906 (Pittman 1987, 1991). The historical record is silent on who was first, although it may have been Bordet and Gengou in Brussels. Multiple pertussis

vaccine products from different manufacturers were licensed in the U.S. in 1914, although controlled efficacy studies and even standardization of product potency was not required at that time. The American Medical Association deleted these vaccines from their list of “New and Nonofficial Remedies” in 1931, only reinstating them in 1944 as reliable potency improved (Council on Pharmacy and Chemistry 1931; Felton and Willard 1944).

Diphtheria and tetanus toxoid vaccines were relatively homogeneous, but a variety of forms of pertussis vaccine were manufactured. Most were categorized as whole-cell vaccines, comprising various preparations of bacteria killed and partially detoxified by heat and/or a chemical agent, or mixed preparations including other bacteria from among upper respiratory tract flora (Cherry et al. 1988; Grabenstein 2008). Although Louis Pillemer, an immunologist at Western Reserve University in Cleveland developed an “antigenic fraction” that represented an early form of acellular vaccine (Pillemer et al. 1954; Ecker 1958), it would be decades before such products would be viewed as improvements over traditional whole-cell pertussis vaccines.

Thorvald Madsen, director of the State Serum Institute of Denmark, conducted the first scientific investigations into the use of pertussis vaccine as prophylaxis during a disease outbreak on the Faroe Islands in the North Atlantic in 1923–1924 (Madsen 1925, 1933; Parnas 1981, Fine and Clarkson 1987; Granström 1996). Vaccination using whole bacteria inactivated by phenol or formalin mitigated disease morbidity and mortality as compared to unvaccinated controls. Another epidemic in 1929 yielded additional promising results concerning vaccination, although it also led to the observation of fatal adverse reactions to the vaccine.

Encouraged by these results, Louis Sauer at the Evanston Hospital of Northwestern University Medical School developed a more potent pertussis vaccine using freshly isolated, strongly hemolytic cultures, an approach that evolved from the discovery of four antigenically distinct phases of bacterial growth, with human disease caused by organisms in phase I (Leslie and Gardner 1931; Felton and Verwey 1955). Sauer tested this vaccine, containing billions of inactivated bacteria per dose in more than 1,300 children between 1928 and 1933 (Sauer 1933, 1935, 1946; Greenwood 1975). Four of these children were his own sons: two vaccinated some months before, two unvaccinated. Following intranasal inoculation with live pertussis bacteria, only the unvaccinated boys developed the disease.

In 1932 Pearl Kendrick (1890–1980) and her associate Grace Eldering (1900–1988), both previously working in different laboratory branches of the Michigan Department of Health, began collaborative work on developing a more effective pertussis vaccine from their base in Grand Rapids. The general belief at the time was that differences in efficacies among pertussis vaccines resulted from non-standardized bacteriologic practices that altered the antigenic components of the products (Kendrick et al. 1939; Kendrick 1943). Kendrick conducted a series of vaccine field trials, most using aluminum-adjuvanted vaccines, involving thousands of children; the trials emphasized pre-designated control groups, an approach gaining favor in the 1930s. The success of Kendrick and Eldering lay, in some measure, in their ability to assemble a community coalition in support of the trials (Shapiro-Shapin 2007).

**Fig. 7.4** Pearl Kendrick (Michigan Women's Hall of Fame)



The results were encouraging, but not decisive, in large part due to the lack of a standard measure of potency (Eldering 1971).

Kendrick (Fig. 7.4) began her career as a science teacher and school principal before studying bacteriology at Columbia University under Hans Zinsser and joining the Michigan Department of Health in Lansing in 1920. Three years later, she moved to open and subsequently direct the Western Michigan branch laboratory in Grand Rapids. Eldering (Fig. 7.5) also started as a school teacher, returning to her undergraduate alma mater, the University of Montana, to study bacteriology. In 1928, she went to Lansing for a short-term, volunteer stint in the public health laboratory there; 4 years later, Kendrick recruited Eldering to join her group in Grand Rapids. Their productive partnership would persist for more than 40 years.

Working collaboratively, Kendrick, Eldering, and Margaret Pittman (1901–1995), the latter an Arkansan bacteriologist working at the Laboratory of Biologics Control within the National Institute of Health (NIH), developed an intracerebral mouse-protection potency bioassay in 1946 to standardize whole-cell pertussis vaccines. Further work by this team led to the development of an optical standard for the bacterial content of vaccines that was adopted as the International Reference Preparation in 1958. This triumvirate of leading female scientists working on one specific pathogen and its vaccine was distinctly unusual in an era in which the fields of science and medicine were dominated by men. The parallels that existed between Pittman (Fig. 7.6) and her Grand Rapids colleagues are uncanny; she too left a career as a school teacher and principal to study bacteriology, receiving a

**Fig. 7.5** Grace Elderding  
(Michigan Women's Hall  
of Fame)



**Fig. 7.6** Margaret Pittman  
(Food & Drug Administra-  
tion)



doctoral degree from the University of Chicago in 1929. After making productive contributions to the bacteriology of *Haemophilus influenzae* at the Rockefeller Institute, she joined the NIH as a regulatory scientist in 1934 and later became the first woman to direct an NIH laboratory (Pittman 1990).

Additional field trials of more consistently potent pertussis vaccines demonstrated its efficacy (Edwards and Decker 2008). But despite its success in early clinical trials, pertussis vaccine still met with skepticism in various quarters. It would take a series of randomized, placebo-controlled studies in the 1940s and 1950s conducted by the British Medical Research Council to convince authorities in the United Kingdom of the vaccine's value. Overall, the data showed the vaccine to be more effective at preventing the disease than the infection (Kendrick and Weiss 1942; Cockburn 1955a, 1957; Medical Research Council 1959; Lévy 1975).

### 7.3.4 Contemporary Era

Refinements to toxoid formulations over the past five decades have resulted in higher purity products and reduced booster doses of diphtheria toxoid for adolescents and adults (Pillemer 1948), the latter maneuver mitigating the burden of painful injection-site reactions (Edsall 1952; Edsall et al. 1954). Hence, "Td" became the designation for the adult/adolescent combined, tetanus-diphtheria toxoid product, in contrast to "DT" or "DTP" to describe products for infants and young children. Studies of the durability of toxoid-induced tetanus antibodies from the 1960s informed the currently recommended routine booster interval for adult Td vaccination of 10 years (Gottlieb et al. 1964; Edsall et al. 1967). Active immunization of children with a combined DTP product increasingly became the norm in industrialized countries beginning in the late 1940s. In 1974, DTP was included in the original set of vaccines recommended for the Expanded Programme on Immunization for developing countries (WHO 1977; Aylward et al. 1994).

From their inception whole-cell pertussis vaccines had been associated with significant safety issues, notably their frequent injection-site reactogenicity and sporadic association with acute, reversible neurologic events, such as febrile seizures in infants (Walker et al. 1988). As is the case with most preventive vaccines, once the burden of a targeted disease declines to very low levels due to widespread childhood vaccination, concerns regarding adverse effects predominate. With whole-cell pertussis vaccine, these concerns involved events such as sudden infant death syndrome (SIDS) and chronic encephalopathy (Cockburn 1955b; Kulenkampff et al. 1974; Miller et al. 1982; Stewart et al. 1984; Cherry et al. 1988). Rigorous assessments have found no scientific basis for a causal relationship between vaccine and SIDS and insufficient conclusive evidence regarding chronic neurologic damage (Griffith 1989; Griffin et al. 1990; American Academy of Neurology 1992; Miller et al. 1993; Gale et al. 1994). However, burgeoning litigation over alleged vaccine-induced injuries spawned a modern vaccine safety movement in the 1980s that eventually resulted in the congressionally legislated National Childhood Vaccine

Injury Act to compensate families for selected adverse events potentially related to mandatory childhood vaccinations.

In assessing the body of evidence, the Institute of Medicine, an advisory body of the U.S. National Academy of Sciences, concluded that no etiologic link between pertussis vaccine and chronic encephalopathy was apparent (Institute of Medicine 1994). Nonetheless, pertussis vaccine acceptance had declined dramatically in some areas due to persistent safety concerns of the public and physicians; use in the United Kingdom had declined from 79% in 1973 to 31% in 1978, resulting in an epidemic of 102,500 pertussis cases and 36 deaths during the late 1970s and early 1980s (Gangarosa et al. 1988). After years of vaccine-associated disease control, similar pertussis outbreaks occurred in Japan and Sweden as immunization rates waned. These events provided further impetus towards subunit vaccine strategies.

Pittman had hypothesized that the disease was largely toxin mediated and that toxoid might be a protective subunit approach, as with diphtheria and tetanus (Pittman 1979). Based on a conceptually similar line of reasoning, Yuji Sato and colleagues at the Japanese National Institute of Health in Tokyo developed an acellular pertussis vaccine in the early 1980s (Sato et al. 1981; Sato, Kimura and Kukimi 1984; Noble et al. 1987; Pittman 1991; Granström 1996). Unlike whole-cell vaccines, these products comprise specific bacterial components, such as formaldehyde-treated toxin, filamentous hemagglutinin, and other pertussis proteins. They are relatively free of lipopolysaccharide, considered a major contributor to whole-cell reactogenicity. Although methodologic differences render it difficult to compare the efficacies of various acellular products, it is clear that as a group they are effective for the control of pertussis. These products are currently the primary or exclusive pertussis vaccines in use in industrialized countries, typically in combination with tetanus and diphtheria toxoids.

## 7.4 Conclusion

Diphtheria, tetanus, and pertussis represent early successes in vanquishing severe bacterial infections by active immunization, developed in parallel with advances in immunology. Once each of the component vaccines had been developed, evaluated, and accepted for routine public health practice in the mid-portion of the twentieth century, the combined DTP vaccine became among the most widely used vaccines in the population. The cumulative result, achieved over decades, was widespread immunity across all age groups. Sustaining such levels of population immunity requires vaccinating each sequential birth cohort and delivering booster vaccinations to adolescents and adults.

The recent resurgence of diphtheria in the former Soviet republics (Dittman et al. 2000; Galazka 2000a, b; Golaz et al. 2000; Tatochenko and Mitjushin 2000; Relyveld 1996) and pertussis in developed countries including the United Kingdom, Japan, and Sweden illustrate the delicate control we maintain over certain vaccine-preventable diseases and how this balance can tip in the pathogen's favor when

vaccine use declines (Cherry et al. 1988; Feikin et al. 2000). Pertussis morbidity and mortality, for example, is 10- to 100- fold higher in settings where immunization programs have been compromised by antivaccine movements (Gangarosa et al. 1988). Global recommendations call for routine immunization of children against all three diseases, with continued attention to sustaining immunity in adolescence and adulthood (NACI 2000, 2003, 2004; ACIP 2006a, b; WHO 2005, 2006a, b). The advent of acellular pertussis vaccines has aided adherence. Although these diseases remain prevalent worldwide, they are kept in check wherever active immunization is practiced widely.

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# Chapter 8

## Tuberculosis and BCG

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### 8.1 Tuberculosis History

#### 8.1.1 Introduction

Tuberculosis occurred in humans probably as early as 8,000 BC in its sporadic form. Indeed, it is mentioned in India's Vedas, the most sacred texts of Hinduism, and later by Hippocrates, Celse D'Aretée de Cappadoce (170 BC), and Avicene (Calmette 1923; Calmette et al. 1928). Recently, genetic studies of the tubercle bacillus have found its progenitor to come into existence possibly as early as 35,000 BC (Gutierrez et al. 2005). Tuberculosis became an epidemic problem once humans settled and crowded into permanent, food-producing social networks. Thus, Egyptian mummies from the Rhamses period (3,000 BC) showed spinal deformities consistent with tuberculosis – Pott's disease. Hippocrates used the term “phthisis,” the Greek term for “consumption,” to describe the wasting away experienced by individuals with tuberculosis. Swollen cervical lymph nodes were known as “scrofula” or the “King's Evil” in England (Artenstein et al. 1995). The belief that they could be healed by the King's touch, although coincidentally true in some cases, likely had more to do with host immune responses than regal intervention.

The European Middle Ages, Renaissance, and nineteenth centuries exhibited a dramatic increase in the burden of human tuberculosis. Known as “The Great White Plague” (Dubos and Dubos 1952), epidemic tuberculosis was the result of industrialization with its attendant increased population density and poverty; the spread of the disease into American and Asian populations was triggered by European migration and colonization (Daniel et al. 1994). John Bunyan, the seventeenth century author of *The Pilgrim's Progress*, dubbed tuberculosis “the captain of all of these men of death,” on the basis of the high burden of mortality attributed to the disease. The well-described cases of tuberculosis among luminaries such as

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musicians (Frédéric Chopin), poets (Alfred Musset and John Keats), writers (Henry David Thoreau and Robert Louis Stevenson), and scientists (René Laennec, Paul Erlich and Max Lurie) attest to its impact on all aspects of daily life at that time (Daniel 2004).

### 8.1.2 Etiology

René-Théophile-Hyacinthe Laennec (1781–1826), the French physician, who invented the stethoscope in 1816 to mediate chest auscultation (Laennec 1928), became the first to demonstrate, through his anatomical and pathological observations, that tuberculosis was, in all of its myriad forms, a unique disease (Calmette et al. 1928; Bynum 1994). The infection had killed his mother, and he himself ultimately died of it. The infectious nature of the disease and its respiratory mode of transmission were suspected from early times. It was another French physician, Jean Antoine Villemin, who demonstrated that injecting tuberculous material, either in the form of caseous exudate or sputum, to healthy rabbits produced the disease (Brock 1999). He presented these results in an 1868 note to the Académie de Médecine – Paris, entitled “Etudes sur la tuberculose” (Calmette et al. 1928).

The discovery of the causative agent of tuberculosis was announced by Robert Koch (see Fig. 3.7) in 1882, within 8 months of his initial experiments on the disease and after much trial and tribulation (Brock 1999); it represented a defining moment in the new field of bacteriology and a culmination of Koch’s work on culture and staining methods over the preceding 6 years. Koch had already achieved significant stature as a scientist because of his groundbreaking investigations into the bacteriology of anthrax, a zoologic disease of major economic import. Koch’s intellect, his skill and patience as a laboratory investigator, his persistence, and his scientific dedication to the germ theory of the disease acted synergistically to ensure a successful outcome of his tuberculosis research.

He described the bacillus, *Mycobacterium tuberculosis*, later known as Koch’s bacillus, before a rapt scientific audience of the Berlin Physiological Society on March 24, 1882 (Koch 1882). Koch’s findings were published within 3 weeks and rapidly accepted by many in the international medical community (Grange and Biship 1982). Another German physician, Paul von Baumgarten, had independently and contemporaneously discovered the tubercle bacillus in infected tissue specimens (Brock 1999), but Koch received the public’s acclaim. Rudolf Virchow (1821–1902), the father of cellular pathology and the most respected academic physician in Germany during Koch’s era, had been a vocal opponent of the germ theory of infectious diseases, viewing the tubercle as a type of tumor instead. Although probably absent from Koch’s evening lecture, Virchow reviewed the experimental data shortly thereafter; he begrudgingly acknowledged Koch’s rigorous methodology, results, and their interpretation, yet he continued to publicly express scepticism as to the bacterial etiology of tuberculosis (Brock 1999; Murray 2004).

Koch performed detailed investigations into the nature of the organism, greatly expanding the technical and microbiological understanding of tuberculosis. His original method of staining the bacillus was improved upon by Paul Ehrlich, who later became an assistant in Koch's laboratory and a founder of modern immunology, and further by a pair of German physicians, Franz Ziehl and Friedrich Neelsen, who modified the technique to the form that is currently applied to sputum smears for the diagnosis of pulmonary tuberculosis (Brock 1999). Using tuberculosis as a model, Koch delineated criteria for the proof of bacterial causality. Later known as Koch's postulates, they soon became cornerstones, if not dogma, of the rapidly developing distinct field of microbiology: the organism must be present in diseased tissues; it must be isolated and grown in pure culture; and the cultured organisms must induce the disease when inoculated into healthy, experimental animals (Brock 1999).

### 8.1.3 Epidemiology

Tuberculosis was the most important and lethal communicable health problem of the eighteenth and nineteenth centuries, accounting for nearly 15% of human deaths across all socioeconomic groups (Dubos and Dubos 1952). Its epidemic prevalence in Europe began to rise at the inception of the Industrial Revolution and peaked in the late nineteenth century; it then declined steadily over the next century, probably related to improvements in hygiene and public health, including the pasteurization of milk, until it resurged in close association with the burgeoning epidemic of human immunodeficiency virus in the 1980s (Stead 1996). Tuberculous mortality in turn-of-the-twentieth-century Europe remained at 20–43% (Calmette et al. 1928). Such a high death rate from a prevalent disease spurred research towards a tuberculosis vaccine.

## 8.2 Vaccine History

### 8.2.1 Background

Apart from his seminal work on the etiology of tuberculosis, Koch discovered a substance in 1890, referred to as "tuberculin," that he initially claimed had demonstrated success in guinea pigs as a preventive measure and therapeutic agent for tuberculosis (Burke 1993). Upon clinical trials of the agent among patients at Charité Hospital in Berlin, it became evident that while infected patients generally demonstrated exuberant cutaneous responses to the intradermal inoculation of tuberculin, the formulation was ineffective as a therapeutic (Brock 1999). In fact the cutaneous reaction Koch had observed, later known as the "Koch phenomenon,"

actually represented a delayed-type hypersensitivity response that became the basis for the diagnostic skin test of infection.

Despite the missteps associated with the tuberculin affair, leading bacteriology groups of the day continued to toil towards the ultimate goal of a vaccine against the tuberculosis scourge. On 28 December 1908, Albert Calmette and Camille Guérin, working at the Pasteur Institute in Lille, presented a note to the “Académie des Sciences” of Paris announcing that they had obtained a “new race of biliated tubercle bacilli” that could be used to vaccinate against tuberculosis (Calmette et al. 1921). This new *Mycobacterium bovis* strain was discovered within the context of rapidly evolving scientific understanding of tuberculosis initiated by Koch’s discovery and amid a growing body of work on vaccines against other infectious diseases, much of the latter emanating from Pasteur and his disciples.

By the late nineteenth century vaccine science was experiencing rapid growth. The hypotheses of Jenner and his historical predecessors had proved to be correct (refer to Chap. 2); the use of cowpox to protect against natural smallpox infection had become well established in society (Artenstein 2008). In fact Pasteur had used the occasion of the centennial celebration of Jenner’s work to recognize the English physician by applying the term “vaccination” to the procedure. By that time, Pasteur himself had already made landmark contributions to the fledgling vaccinology field through his experimental work with anthrax vaccines, rabies, and microbial attenuation using poultry cholera virus (Pasteur 1880; Pasteur et al. 1881). Researchers, inspired by both Pasteur’s successes with attenuated organisms and the work of Behring and Kitasato using antibody-inducing toxins against diphtheria (Chap. 7), focused on applying these principles to tuberculosis vaccines.

The failure of Koch’s tuberculin to prevent or treat tuberculosis led to the concept of using non-pathogenic, killed, or attenuated tubercle bacilli from different origins, including human, bovine, and equine sources, as opposed to components of the bacillus as vaccines. Such vaccine approaches were attempted in animals; two may have influenced Calmette. The “bovo-vaccine,” described by Behring in 1902, was prepared with human tubercle bacilli attenuated by aging them in the laboratory for more than 6 years, followed by desiccation under vacuum conditions. The vaccine was then used for the “Jennerization” of bovines. It was the first product to show protection against tuberculosis but was abandoned due to its transient immune responses, variable attenuation, and the risk of human contamination from vaccinated animals. The second anti-tuberculosis vaccine was prepared by Koch using both human and bovine attenuated tubercle bacilli and was called “tauruman” (Gheorghiu 1996). Further development of this product was also halted as its limitations were similar to those of the “bovo-vaccine.”

### **8.2.2 BCG Discovery**

Leon Charles Albert Calmette (1863–1933) served as a physician in the French Navy in the Far East, North Atlantic, and French West Africa before accepting the

directorship of the first branch of Pasteur's Institute to be located outside of Paris in Saigon, French Indochina in 1891, on the personal recommendation of the great scientist himself (Sakula 1983). During his productive 2-year stint there, Calmette presided over the smallpox vaccination of half a million Vietnamese people; developed the water buffalo as a source of smallpox vaccine "lymph"; implemented a successful rabies vaccination program; and investigated cholera and dysentery outbreaks (Gelinas 1973). However, his most significant scientific contributions in Saigon involved his studies on cobra venom and its attenuation which led to the development of the first, effective snake antivenins.

In 1895, following work in Émile Roux's laboratory in Paris on snake bite immunization and plague antiserum, Calmette was chosen to head a second European Pasteur Institute in Lille, France. There, in addition to completing his landmark studies on snake antivenins, Calmette initiated the experimental and clinical studies of tuberculosis that would define the rest of his career and assure his professional legacy.

Calmette recognized the need for animal expertise at the new Institute and on the recommendation of Edmond Nocard, a former pupil and close associate of Pasteur, he arranged for Camille Guérin, a young veterinary surgeon, to join him in Lille in 1897 (Hawgood 2007). Guérin had studied and subsequently worked under Nocard at the national veterinary school at Alfort near Paris (Sakula 1983). Upon Guérin's arrival, he and Calmette, (Fig. 8.1) commenced a collaboration on tuberculosis vaccines that lasted for 36 years.

The pair of researchers observed that ingestion of relatively low virulence, equine tubercle bacilli by guinea pigs made them resistant to infection with virulent strains. Thereafter, they worked towards developing a vaccine for oral use, fashioning their experiments on Behring's hypothesis that pulmonary tuberculosis could be acquired as efficiently by oral as respiratory routes.

In 1904, Calmette and Guérin began working with a strain of *M. bovis* that had been isolated from a cow with tuberculous mastitis by Nocard. A well dispersed bacillary mass was required in order for the bacilli to translocate across the intestinal mucosa and reach the lungs via lymphatics and blood. As Guérin reported, "the bacilli grown on glycerinated potato were tightly clumped and very difficult to homogenize in an agate mortar" (Guérin 1948). Adding a drop of sterile beef bile onto the bacillary mass made the in vitro homogenization procedure "remarkably easy." After many fruitless attempts "to grow the Koch bacillus on a strongly alkaline medium simulating bile," they proposed a beef bile potato medium: "potato slides were immersed into 5% glycerinated beef bile, heated it in a water bath at 75°C for 3 h. They were then placed into a tube with a narrow waist at the base of which a new glycerinated bile was added to make contact with the potato slides without submerging them, and autoclaved 30 min at 120°C" (Guérin 1948).

On this culture medium successive passages at 21-day intervals modified the initial bacterial strain: "the first passages were poor and then became abundant. The morphology changed from a hard, rich scaly to a smooth, glossy, pasty bacillary mass" (Guérin 1948). The virulence of the strain at the origin was known; an inoculum of 0.0001 mg killed guinea pigs in 40–60 days. Following a slight increase in virulence after the first year of passages on their novel culture medium, successive



**Fig. 8.1** Camille Guérin and Albert Calmette (Institut Pasteur)

passages during subsequent years resulted in progressively decreased *M. bovis* virulence (Gheorghiu 1996). By the 39th passage, the strain no longer killed animals.

It was these findings that Calmette and Guérin presented to the “Académie des Sciences” in 1908 as a new “race of bililiated tuberculous bacilli with a fixed attenuation of its virulence while keeping its antigenicity.” They referred to it as “bacille tuberculeux bilié”; the strain later became known as “bacille bilié Calmette-Guérin,” simplified to Bacille-Calmette-Guérin or BCG. During the maintenance of this strain over the 13 years and 230 passages between its first description and its first use as a vaccine in 1921, BCG was found to remain avirulent in different animal species tested: bovines, guinea pigs, mice, horses, rhesus monkeys, and chimpanzees. In fact it conferred resistance to challenge with virulent mycobacteria by 30 days after vaccination.

The primary reason that the early research efforts of Calmette and Guérin on BCG vaccine took so long to progress to clinical application was due to their interruption by the start of World War I and the German army’s occupation of Lille.

In the midst of long-term studies in calves in 1915 to determine whether BCG protected against natural infection, the researchers chose to sacrifice the remaining animals so that they could complete the autopsy assessments before the Germans could requisition the cows to feed their soldiers (Hawgood 2007). The war years were also personally difficult for Calmette; his wife and 24 other French women from Lille were held hostage by the Germans for more than 4 months outside of Hanover (Chung and Biggers 2001). In 1919 he returned to Paris to assume the assistant directorship of the Pasteur Institute there; Guérin stayed in Lille to run the Tuberculosis Service. They continued their productive collaboration for 14 more years; during the last five, they were reunited in Paris.

### 8.2.3 *BCG Vaccination*

Immunity to tuberculosis required that the “vaccin-bacille was viable and able to disseminate through the lymphatic system into the host” (Calmette et al. 1928). Although Calmette and Guérin observed that tuberculin hypersensitivity preceded acquired resistance and that only sensitized animals were immune, they recognized that hypersensitivity and immunity were distinct states of infected or vaccinated animals respectively. A complete explanation of the complex cellular pathogenesis and the mechanisms and nature of the immune responses to both tuberculosis infection and BCG vaccination would not begin to be fully elucidated until the latter part of the twentieth century, more than four decades after the work of Calmette and Guérin (Table 8.1).

Once the safety and efficacy of BCG had been demonstrated in animals, the vaccine began to be used in humans. In the early part of the twentieth century, tuberculosis remained a major public health problem and was still associated with excessive mortality among symptomatic individuals; hence, prospects for a potential vaccine were viewed as a health imperative. In contradistinction to the current approach to experimental vaccines, the initial human vaccinations were performed in children. The first baby, a newborn contact of a family member with tuberculosis, was vaccinated orally with BCG by Dr. Benjamin Weil-Hallé at the Hôpital de la Charité (presently the Hôtel Dieu) in Paris in 1921 (Weil-Hallé and Turpin 1925) (Fig. 8.2). Neonates were fed a small amount of BCG on a little spoon followed by the ingestion of milk. Initially, the dosing regimen of BCG involved 2 mg administered on the third, fifth, and seventh days of life, representing a total of 240,000,000 colony forming units of bacteria; the dose was increased shortly thereafter by fivefold.

After it was demonstrated that the first thirty vaccinated babies had been safely protected from family contacts with tuberculosis, the use of BCG vaccination began to rapidly diffuse throughout France and the rest of Europe. Between 1921 and 1926 more than 50,000 children were vaccinated. The mortality rate from tuberculosis among vaccinated tuberculosis contacts was 1.8% as compared with a rate of greater than 25% among unvaccinated Parisian children (Calmette, Bocquet and Nègre 1926). At the Conference of the League of Nations in Paris in 1928, the

**Table 8.1** TB immunity knowledge, major contributors and chronology

Contributors	Knowledge	Historical events
1891 – Koch R	Koch phenomenon = delayed type hypersensitivity (DTH)	1870s French-German war (Napoleon III/Bismark) – Parisian's revolution – La Commune
1909 – Helmholtz HF	TB resistance to second infection	United States post-Civil War reconstruction
1910 – Bail O	DTH by blood transfer	1914–1918 World War I
1921 – Calmette A	DTH by spleen cells transfer	1929–1933 world economical crash
1945 – Chase MW	Only live BCG protects	1933–1945 Hitler's nationalist – World War II
	Cellular-DTH cutaneous transfer	1945 – Antibiotics era
1953 – Suter E	Immune Macrophages inhibit TB-bacilli multiplication	1945– 1991 Cold War
1942–1964 – Lurie M	Macrophages inhibit TB-bacilli	
1967–1969 – Mackaness GB, Blanden RV	Lymphocytes activate macrophages for TB-bacilli killing	
1966 – Bloom BR, David JR	Lymphokines = lymphocytes soluble products activating macrophages	
1970–2000 – Orme JM, Collins FM, Kaufman SHE, Bevan MJ, Flynn J, Rook, Gaw (see references)	Role of Th1 cells (CD4 +, CD8 +, $\gamma\delta$ ) in macrophages activation	Fall of European communism 2001 terrorism
	Cytokines = IL-2, IL-6, IL-7, IL-10, IL-12, IFN- $\gamma$ , TNF- $\alpha$ , etc.	United States Iraq War



**Fig. 8.2** Calmette and Dr. Benjamin Weil-Hallé performing the first oral BCG vaccination of an infant (Institut Pasteur)

vaccine was recognized as safe, endowed with a certain degree of “pre-munition” (from the Latin *pre-munire*), or protection against severe tuberculosis, and its use was encouraged (Calmette et al. 1926; Société des Nations 1928).

However, not long after BCG vaccine had received the general endorsement at the Conference, a catastrophe occurred that cast aspersions on its safety. In the German port city of Lübeck in 1929, 252 infants received BCG from the Pasteur Institute, Paris, but prepared for administration in the tuberculosis laboratory in Lübeck and later found to be inadvertently contaminated with a human tuberculosis strain (Sakula 1983). The majority of the children became infected; 72 died within a year as a result of the disease (Gheorghiu 1996). A subsequent investigation carried out by German tuberculosis experts revealed that the vaccine was contaminated with the distinct, human Kiel strain during its preparation at a local laboratory in Germany (Lange 1931). In the absence of modern molecular diagnostic techniques, the morphological aspects of cultures and virulence in guinea pigs helped to differentiate the attenuated BCG phenotype from that of the virulent Kiel strain.

Although BCG was exonerated as the cause of the Lübeck disaster, its use declined for several years thereafter, and both Calmette and Guérin came under intense public scrutiny. Eventually, in association with the epidemic resurgence of tuberculosis during the Second World War, BCG vaccine was again used on a massive scale, and public confidence in its safety was restored. Calmette died in 1933, disheartened by a prolonged lawsuit related to the Lübeck affair but having devoted most of his career to the prevention of tuberculosis (Bernard 1931).

Guérin carried on the BCG vaccine work at the Pasteur Institute until his retirement; in the postwar years; in fact, he lived in a small, converted apartment on the premises so that he could be close to his laboratory (Hawgood 2007).

The use of oral BCG vaccination was widespread in many European countries at the time of the First International BCG Congress held at the Pasteur Institute in 1948 and presided over by Guérin. Great Britain was a notable exception to its use, until a large clinical trial among adolescents in the early 1950s confirmed its efficacy in preventing serious tuberculosis infections (Sakula 1983). Oral vaccination was discontinued in most countries worldwide during the 1960s. The main reasons advanced at the time were twofold: oral vaccination induced only inconsistent and ephemeral delayed-type hypersensitivity responses at a time when the association between protection and hypersensitivity was believed to be of potential import (Orme and Collins 1984); and the oral route was associated with cases of cervical lymphadenitis.

Parenteral routes of vaccination were thereafter applied (Rosenthal 1980). The methods most often used were Bretey's cutaneous scarification, Rosenthal's multiple puncture, or Mérieux's ring, the latter used until recently in France. The intradermal route became the preferred parenteral one, as it required injection of a minimal dose of BCG to induce durable delayed-type hypersensitivity in more than 90% of vaccinees (Wallgren 1928). However, because the complication rates among oral and intradermal routes of BCG vaccination were similar, approximately 3% (Lotte et al. 1984), and their comparative efficacies had not been assessed in humans (Gheorghiu 1996), there was renewed interest in oral vaccination. The oral route also possessed potential advantages in terms of simplicity of administration, lower cost, and the induction of mucosal immunity, the latter being recognized as an important component of mycobacterial immunity.

It has been shown that wild-type or recombinant BCG induces protective immune responses whether administered by respiratory or oral routes (Lagranderie et al. 1993; Gheorghiu 1994). The immune responses are both local and systemic, and they involve mucosal IgA antibody and cytotoxic T lymphocytes of intestinal, intraepithelial cell origin (Lagranderie et al. 1997, Gheorghiu et al. 1998). Calmette had experimented with inhalation of tubercle bacilli by guinea pigs; investigation of the aerosol route of BCG vaccination was subsequently pursued by others. A relatively low burden of aerosolized BCG is safe and appears to protect animals as effectively as larger doses given intradermally (Cohn et al. 1958; Barklay et al. 1973). Additionally, aerosolized BCG has been shown to be more effective than the intradermal route at inducing pulmonary macrophage activation in guinea pigs (Lagranderie et al. 1991).

### **8.2.4 BCG Efficacy**

The prevention of tuberculosis has been the subject of continuous controversies. An early evaluation of the protective efficacy of BCG vaccine in children demonstrated a mortality of 25% among unvaccinated subjects as compared to less than

2% in vaccinated ones (Calmette et al. 1926), although it has been argued that mortality in children represents an insensitive indicator of vaccine efficacy (Bonah 2005). In more recent studies, using tuberculosis infection in lieu of mortality as an endpoint, protection has varied from 80% among Native American and British children (Springett and Sutherland 1970; Aronson et al. 2004) to 14% in the southeastern United States (Comstock et al. 1976) to essentially no protection in Madras, India (WHO 1979). Multiple meta-analyses concur that the protective efficacy of BCG appears to vary widely in different parts of the world; hence, vaccine impact on the control of tuberculosis worldwide remains unclear (WHO 1979; Clemens et al. 1983; Bloom and Fine 1994; Colditz et al. 1994).

A number of methodologic, epidemiologic, and immunologic factors likely have contributed to the discordant results from efficacy studies. Factors such as the prevalence of environmental mycobacteria among study subjects may confound the assessment of immune responses. Epidemiologic factors including the duration of cohort follow-up; the heterogeneous genetic background of vaccinated populations; their living conditions; and the influence of prevailing climate on vaccine viability could have interfered with BCG efficacy assessments (ten Dam 1993; Bloom and Fine 1994). Nevertheless, BCG prevents approximately 80% of miliary tuberculosis and tuberculous meningitis in children and 50% of pulmonary disease in adults (Colditz et al. 1994). Numerous case-control studies demonstrate similar levels of protection against severe forms of tuberculosis (Colditz et al. 1994) and greater than 50% protective efficacy against extra pulmonary tuberculosis and leprosy in endemic populations (Brewer and Colditz 1995).

### 8.2.5 *BCG Strain Diversity*

The BCG vaccine strain was disseminated throughout the world in the late 1920s. Each host country maintained it by passages on natural culture media. These continuous passages may have been the cause of observed changes among these BCG substrains, or “daughter strains” that became designated by the laboratory, country, or person’s name with which they were associated. The first difference in “vitality” between Pasteur and Copenhagen strains was reported by Orskov in 1948 at the First BCG Congress (Gheorghiu 1996). Purportedly, it was due to successive passages on Sauton’s medium in Copenhagen without intermediate passages on bile or glycerinate potato as was the case with the Pasteur Institute strain. It was later reported that BCG strains differed with respect to morphology and multiplication capacity in mice, and that residual virulence correlated with protection (Dubos and Pierce 1956), a finding confirmed through additional research performed under the aegis of the World Health Organization (WHO) and the International Association for Biological Standardization (Sekhuis et al. 1977; Smith et al. 1979).

Divergent results of efficacy studies have focused attention on strain differences. Comparative studies of different BCG strains produced in different laboratories have shown differences in morphology as well as cell-mediated immune responses

(Gheorghiu and Lagrange 1983; Osborn 1983). Additional investigations, aided by modern molecular and genetic tools, have demonstrated strain-specific differences in structural mycolic acids (Minnikin et al. 1983), secreted proteins (Abou-Zeid et al. 1986), genomic sequence (Fomukong et al. 1992), origin and genomic content (Behr and Small 1999; Mostowy et al. 2003). Whole genome microarrays have revealed certain differences within genetic regions of various BCG strains (Pym et al. 2002). For these reasons the WHO has recommended the maintenance of BCG strains as freeze-dried seed lots for vaccine production that have been derived from a single colony as opposed to a mixture of colonies (Gheorghiu et al. 1983).

While clinical trials have shown reactogenicity differences among various BCG strains, there is no clear correlation between strain variation and protective efficacy (Brewer and Colditz 1995). In fact more evidence exists to support a genetic basis for efficacy differences (Gros et al. 1981); the same BCG strain may induce robust immune responses among one population and markedly poorer responses in a different population (Comstock 1964; WHO 1979; ten Dam 1993; Corbel et al. 2004).

### 8.2.6 New Tuberculosis Vaccines

BCG vaccine is variably effective at protecting against some forms of tuberculosis in certain populations. It is ineffective against endogenous reactivation of disease. Therefore, it is not useful in protecting infected adults or preventing tuberculosis dissemination. Since the 1960s, BCG has shown value as an immune modulator, specifically in the chemotherapy of bladder cancers (Chung and Biggers 2001).

Modern vaccinologists have endeavored to develop novel approaches to vaccines against tuberculosis (Ginsberg 2002; Orme 2005; Baumann et al. 2006; Orme 2006). The elucidation of the complete genomic sequence of *M. tuberculosis* may further the understanding of the organism's biology and suggest new approaches to vaccines (Cole et al. 1998). Such strategies could include subunit vaccines, live attenuated mycobacterial mutants, viral attenuated recombinants, and DNA constructs (Huygen et al. 1996). Those under clinical trials are summarized in Table 8.2.

**Table 8.2** Clinical trials of phase 1, novel anti-TB vaccines

Origin	Vaccine	Type
USA: (Skeiky YA, et al. 2004)	Mtb 72F in AS02A	Subunit (fusion protein + adjuvant)
Denmark: (Langermans JA, et al. 2005)	Ag85-ESAT-6 in ic31	Subunit (fusion protein + adjuvant)
England: (McShane H, et al. 2005)	r-MVA-Ag85	r-Modified Virus Ankara (MVA) prime BCG/Boost r-MVA Ag85
USA: (Hortwitz MA, et al. 2005)	r-BCG-Ag85	Viable r-BCG
Germany: (Grode L, et al. 2005)	r-BCG ure-Hly +	Viable r-BCG

A strategy employing live BCG recombinants in a prime/boost platform may be best suited if the ultimate goal is to prevent endogenous tuberculosis reactivation, as the majority of children worldwide have received BCG at birth.

Globally, tuberculosis continues to be a leading cause of mortality, accounting for over two millions deaths per year, and morbidity, as a substantial proportion of the world's population is infected and their access to treatment is poor. The disease's impact has extended due to the expanding prevalence of individuals with immune deficiencies, a group at higher risk of tuberculous morbidity and mortality, and the emergence of multi-drug resistance in mycobacteria (WHO 1997; Kaufmann and McMichael 2005). Although imperfect, BCG remains a relatively inexpensive, safe, available agent that is still the only vaccine effective for the prevention of human tuberculosis (Gheorghiu 1996).

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# **Chapter 9**

## **The Discovery of Viruses and the Evolution of Vaccinology**

**Nicholas C. Artenstein and Andrew W. Artenstein**

The early part of the twentieth century produced significantly fewer major advances in vaccinology than had the latter part of the previous century. However, beginning with the landmark work of the German physician Robert Koch, who in 1876 definitively demonstrated the transmissible nature of *Bacillus anthracis*, both periods witnessed a number of important scientific advances that would serve to build a firm foundation for the future of vaccines. As described in Chaps. 4 and 5, Louis Pasteur extended the work of Koch to formalize the concept of microbial attenuation, leading directly to his audacious experiments with live, attenuated anthrax vaccine in livestock and rabies vaccine in humans.

In parallel with the significant developments in bacteriology (rabies was also felt to be a bacterial infection until its viral etiology was discovered in the early part of the twentieth century) that were taking place during the late nineteenth century, scientific investigations were unfolding that sought to explain the well-recognized but poorly understood observations regarding immunity to toxins and other diseases. The work of Ilya Metchnikoff and Paul Ehrlich established the basis for the concepts of cellular and humoral immunity, respectively and launched the field of immunology (Chap. 3). Incremental scientific developments by others in the fledgling arena led, almost synergistically, to further refinements in vaccine science during the early part of the twentieth century. Concurrent with these developments in microbiology and immunology, another line of scientific inquiry was evolving, led by the work of a group of agricultural chemists and plant biologists in the countryside of Western Europe, that would have the fortuitous consequence of revolutionizing vaccinology.

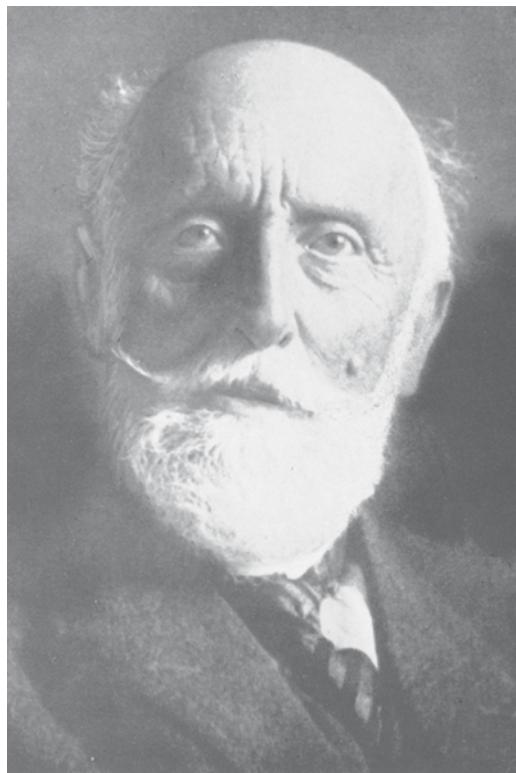
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The town of Wageningen, located in the province of Gelderland in the west-central Netherlands adjoining the eastern border of Germany, was initially settled in the twelfth century (EconomicExpert 2008). Today, the town is at the center of academic and commercial developments in food innovation, a pathway that was initiated with the establishment of an Agricultural School there in 1876 (Bos 1999). Adolf Eduard Mayer (1843–1942), a German scientist trained in agricultural chemistry, was appointed the first Director of the Agricultural Experiment Station at Wageningen. It was there, in 1879, at the request of local farmers, that Mayer (Fig. 9.1) began working on a disease of tobacco plants that would leave his imprint on history.

Although the fundamental tenets of microbiology had begun to be elucidated prior to Mayer's arrival in Wageningen, by Antonie van Leeuwenhoek in the seventeenth century and subsequently by Koch, Pasteur, and Lister earlier in the nineteenth century, the emphasis remained largely on bacteriology, as no evidence of submicroscopic entities existed at that time. Koch's postulates, formulated through the studies of the anthrax bacillus in animals but articulated by one of Koch's assistants, Friedrich Loeffler, in an 1883 paper on diphtheria and by Koch in an 1884 paper concerning the etiology of tuberculosis, stipulated the criteria for establishing a causal relationship between a microorganism and a disease: the



**Fig. 9.1** Adolf Mayer  
(Reprinted from  
Phytopathological  
Classics, No. 7, 1942  
(reprinted 1968),  
American  
Phytopathological  
Society, St. Paul, MN)

organism must be found in pathologic lesions of the disease; it must be isolated in pure culture; inoculation of the pure culture into a host must reproduce the disease entity; and the organism must be again recovered from the lesions (Brock 1999; Levine 2001). With these rules in tow, there was general agreement among early microbiologists that a readily identifiable microorganism could be associated with each infectious disease. Mayer's observations demonstrated a major exception to this newly minted dogma.

Mayer, a disciple of the von Liebig school of thought of the mid-nineteenth century that started the field of agricultural chemistry through an emphasis on the importance of minerals in plant nutrition (Bos 1981), was appointed Professor in Botany at the new Agricultural School in Wageningen. Upon beginning experimental work on tobacco there, he named the plant affliction tobacco mosaic disease, in recognition of the heterogeneously pigmented spots on the leaves (Mayer 1886) (Fig. 9.2). Despite the absence of an obvious bacterial etiology, thus failing to satisfy Koch's postulates for causality, Mayer succeeded in transmitting the disease to 90% of healthy plants by inoculation with sap expressed from diseased tobacco leaves (Mayer 1886). He noted infectivity after filtration, with the loss of transmission only after passage of the sap through two layers of filter paper (Mayer 1886). In a preliminary report published in 1882 Mayer hypothesized the causal agent of tobacco disease to be a "soluble, possibly *enzyme-like contagium* (author's emphasis), although almost any analogy for such a supposition is failing in science" (Levine 2001), thereby acknowledging the potential novelty of his findings. Although his subsequent definitive treatise on tobacco mosaic disease recanted his previous theory in favor of a bacterial etiology, he continued to call attention to the failure of his findings to fulfill Koch's postulates (Mayer 1886).

While Mayer speculated on the existence of ill-defined, infectious agents as the cause of tobacco mosaic disease, a young botanical science student at St. Petersburg University in Russia was contemporaneously investigating epidemic tobacco



**Fig. 9.2** Tobacco leaves infected with Tobacco Mosaic Virus (Reprinted from Phytopathological Classics, No. 7, 1942 (reprinted 1968), American Phytopathological Society, St. Paul, MN)

**Fig. 9.3** Dimitri Ivanovsky  
(Reprinted from Phytopathological Classics, No.  
7, 1942 (reprinted 1968),  
American Phytopathological Society, St. Paul, MN)



diseases in his native country. Dimitri Ivanovsky (1864–1924) (Fig. 9.3) began work on tobacco mosaic disease approximately 5 years after Mayer demonstrated its transmissible nature (Lustig and Levine 1992). In a presentation to the Academy of Sciences of St. Petersburg in 1892 that was largely devoted to the phenotypic properties of the affliction and in which he took issue with Mayer on some accounts, Ivanovsky reported that the sap from mosaic disease-infected tobacco leaves reproduced the disease in healthy plants after passage through an unglazed, Chamberland porcelain filter (Ivanowski 1892). The Chamberland filter candle, developed in 1884 by Charles Chamberland from Pasteur's lab to purify water, was known to trap bacteria, and it became a common instrument of microbiologic research at the time (Lustig and Levine 1992). Ivanovsky appropriately concluded that the filtrate in his experiments was infectious. However, because of the supremacy of Koch's recently accepted postulates, he interpreted his findings cautiously, preferring to consider sources of laboratory error, such as filter defects, or the presence of potentially filterable substances such as bacterial toxins (Ivanowski 1892) in lieu of novel organisms.

In fact Ivanovsky's observations of a filterable substance significantly extended those of Mayer and introduced an experimental definition for what would later

become known as a new class of infectious agents. He is credited by some authorities as the discoverer of viruses (Lustig and Levine 1992). However, Ivanovsky apparently failed to completely appreciate the significance of his findings at the time of his work (Bos 1995). The comprehensive account of his work on tobacco mosaic disease, published in 1903, notes two conflicting findings: multiplication of the agent in artificial media, a characteristic that is inconsistent with the true biological properties of viruses; and intracellular, crystal-like inclusions in the cells of infected plants, which would prove to be a significant clue to the presence of submicroscopic organisms, albeit poorly understood by Ivanovsky at the time (Bos 1999).

Prior to Ivanovsky's work in the field, Mayer had shared his early experimental results regarding the infectious nature of tobacco mosaic disease with a younger colleague at the Agricultural School at Wageningen, Martinus Beijerinck (Fig. 9.4). Beijerinck (1851–1931), originally from Amsterdam, had graduated from the Delft Polytechnical School in 1872 with chemistry as his primary academic focus but botany as his main interest (Chung and Ferris 1996). Following graduate work in biology at the University of Leiden, in the Netherlands, and a series of secondary school teaching jobs, he received a faculty appointment at the newly founded



**Fig. 9.4** Martinus Beijerinck  
(Reprinted from  
Phytopathological Classics,  
No. 7, 1942 (reprinted 1968),  
American Phytopathological  
Society, St. Paul, MN)

Agricultural School in Wageningen, arriving the same year as Mayer. Together, the two academics founded the local Natural Science Society (Bos 1999). Beijerinck, upon reviewing Mayer's experimental results and apparently attempting his own experiments, admitted his inability to "prove the presence of microbes in the diseased plants to which the disease could be ascribed" (Beijerinck 1898). He attributed his failings to an incomplete knowledge of bacteriology at the time.

Said to be a demanding and unpopular teacher, Beijerinck left the academic life at Wageningen in 1885 to pursue a career in industrial microbiology at the well-resourced Netherlands Yeast and Spirit Factory at Delft (Theunissen 1996). It is fitting that in this South Holland city, the birthplace of Antonie van Leeuwenhoek, the seventeenth century inventor of the microscope and a founding father of microbiology, Beijerinck advanced the field and achieved significant renown in the laboratory; important basic microbiologic discoveries derived from his search for solutions to industrial problems related to the production of yeast. Having made a name for himself in the field he returned to academics in 1895 as a Professor and Chairperson of Bacteriology at what is now known as the Technical University of Delft (Bos 1999). Through his 26 year tenure there, he solidified his reputation as a rigorous and innovative scientist, and he founded the Delft School of Microbiology that arguably established general microbiology as a distinct subject area within biology (la Rivière 1997) and spawned generations of international, academic microbiologists (Chung and Ferris 1996).

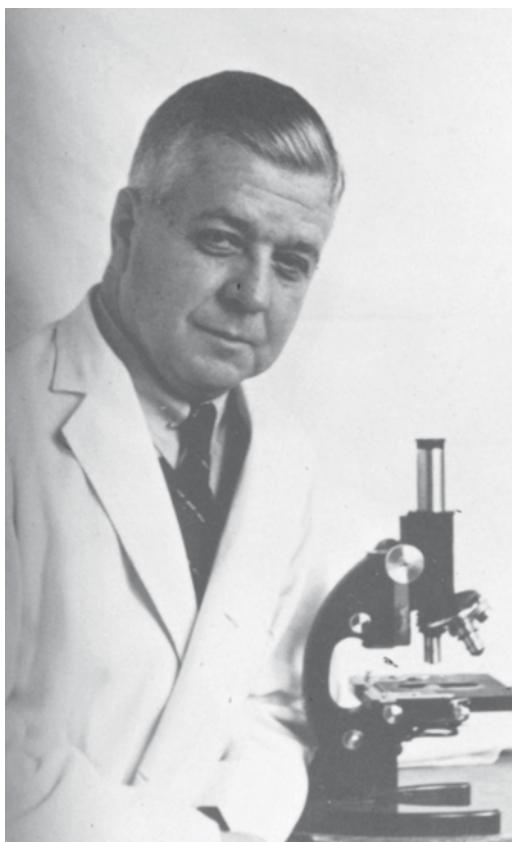
During his first few years at the Technical University, Beijerinck resumed work on tobacco mosaic disease, embarking on a series of experiments that explained the observations of both Mayer and Ivanovsky, although he was unaware of the findings of the latter scientist, and ushering in a new paradigm in microbiology. His initial work, while still at Wageningen, confirmed Mayer's results and led Beijerinck to conclude that "the spot disease is an infectious one that is not caused by microbes" yet discovered (Beijerinck 1898). Renewed investigations at Delft further characterized the causative agent. After proving that the sap from affected plants could transmit infection despite passage through porcelain filters, Beijerinck hypothesized a *contagium vivum fluidum*, or a soluble living germ, as the culprit of tobacco mosaic disease. Additional laboratory work by Beijerinck elucidated a number of properties of this agent and the disease it produced in plants, most notably that it was incapable of independent growth, requiring the presence of living and dividing host cells (Beijerinck 1898).

The incremental, important advances of Mayer, Ivanovsky, and Beijerinck not only opened a new arena of microbiology, but they also resulted in a redefining of Koch's postulates to accommodate the newly discovered class of pathogens (Bos 1981). Additionally, their pioneering work led to a flurry of scientific developments in the early part of the twentieth century that further defined the biochemical and physiochemical nature of viruses and refined the laboratory techniques for their study (Stanley 1935; Levine 2001). Such discoveries laid the foundations that eventually ushered in the era of modern molecular biology.

Foot-and-mouth disease of cattle became the first viral disease of animals to be identified by Loeffler and Frosch in 1898, the year after Beijerinck's famous

experiments (Brown 2003). By the end of the first quarter of the new century, a causal link between various filterable agents—viruses—and multiple diseases of animals and humans had been suggested. In late 1926 Thomas Rivers (1888–1962), a renowned physician-researcher in the nascent field of virology at the Rockefeller Institute, described the state of knowledge of the “filterable viruses” to the annual meeting of the Society of American Bacteriologists. He categorized at least 65 diseases of animals or humans as potentially belonging to this group of pathogens and discussed their various chemical and physical properties (Rivers 1927). He then proceeded to review selected features that distinguished viruses from “ordinary bacteria”: small size, accounting for their general filterability and invisibility by light microscopy, and the inability to cultivate these agents in the absence of living cells (Rivers 1927).

Rivers (Fig. 9.5) was recruited from Johns Hopkins to the Rockefeller Institute in 1922 and spent more than 30 years there, most of the time as the Director of the Department of Bacteriology and the final three years as the Director of the Institute (Benison 1967). He went on to coordinate the research efforts of the



**Fig. 9.5** Thomas Rivers  
(Tom Rivers:  
Reflections on a Life in  
Medicine and Science,  
edited by Saul Benson  
and published by The  
MIT Press)

National Foundation for Infantile Paralysis through the period of successful polio vaccine development. Rivers is widely considered to be one of the preeminent figures in the history of virology. His career spanned the crucial period between the early years of virology as a distinct science and the heyday of vaccines; he was a prominent force in the field throughout these periods. But making the transition from the basic research advances in virology to the development of vaccines against the major viral diseases of the time was dependent on solving a key technical problem in the laboratory – the inability to grow viruses on standard nutrient media.

Cultivating microorganisms was an essential component of the laboratory investigations of infectious diseases that permitted the characterization of pathogens, their diagnosis, and prevention steps as well as their epidemiology and correlation with their clinical features. This fundamental principle had been well established in the discipline of bacteriology by Koch and others in the latter part of the nineteenth century; most bacteria grew readily on nutrient media. Because viruses behaved as obligate parasites, they required host cells in order to replicate, a significant technical obstacle that stood in the path of progress towards viral research in diagnosis and vaccines.

As early as 1908, Landsteiner and Popper had succeeded in reproducing the lesions of poliomyelitis in the central nervous systems of two species of Old World monkeys by injecting them with spinal cord material from an infected boy (Eggers 1999). Despite the importance of this finding, neither monkeys nor smaller mammals provided a practical, renewable, or cost effective resource for large scale virologic research. Additionally, the use of human vaccines derived from animal tissues, especially nervous tissues, was potentially dangerous, as discovered by Pasteur in his work with rabies (refer to Chap. 5), and again by yellow fever researchers subsequently (refer to Chap. 10). The virologists' dilemma awaited a practical solution that was to come from Vanderbilt Medical School in Nashville, Tennessee.

Ernest W. Goodpasture (1886–1960) was born on the family farm in rural Tennessee and was raised in Nashville where he attended Vanderbilt prior to completing medical school at Johns Hopkins in 1912. Following faculty stints at Johns Hopkins and Harvard, he returned to Vanderbilt in 1922 and remained there for more than 30 years as a Professor of Pathology, and later, Dean of the School of Medicine (Dawson 1961). Following his retirement from Vanderbilt, Goodpasture, (Fig. 9.6) spent 4 years as the Scientific Director of the Armed Forces Institute of Pathology before returning to Nashville. His research interests were significant and varied; his seminal contributions to virology were many.

Early in Goodpasture's career, while serving in the Naval Medical Service during late 1918, he noted the conspicuous absence of bacteria in the post-mortem lung tissues of influenza victims, thus suggesting an alternative infectious etiology for the lower respiratory tract infection (Burnett 1973). Although more than a decade passed before the culprit influenza virus of the 1918 pandemic was isolated from pigs (Shope 1931), these early observations proved prescient. It was also during his military service in 1919 that Goodpasture described a progressive and usually fatal syndrome characterized by glomerulonephritis and hemoptysis that came to bear his name and signify an autoimmune disease of an unclear etiology.

**Fig. 9.6** Ernest Goodpasture  
(Eskind, Biomedical Library,  
Vanderbilt University)



Goodpasture's work with the "filterable viruses" progressed throughout the 1920s. He performed extensive investigations into the nature of the microscopic intracellular inclusions observed in poxviruses such as fowlpox and molluscum contagiosum (Goodpasture 1928), and in other diseases of presumed viral origin (Goodpasture 1925; van Helvoort 1994). He correctly deduced that these pathologic lesions represented microscopic evidence of viral pathogens. Goodpasture studied viral tropism and produced landmark findings on the spread of herpes viruses along neural pathways (Burnett 1973). He was, in the words of Tom Rivers, "a great investigator" (Benison 1967) whose most important work in experimental virology was yet to be accomplished.

In 1931 Goodpasture and his colleague Alice Woodruff reported the successful cultivation of fowlpox on the chorioallantoic membranes of chick embryos (Woodruff and Goodpasture 1931). The concept of using embryonic tissue for experimental biology probably originated from the field of embryology but was first exploited by Peyton Rous in the early part of his career in the laboratory of cancer research at the Rockefeller Institute in 1911. He demonstrated, in a series of elegant experiments using embryonic tissues from chickens as well as mice, that various malignant tumors could be successfully implanted and grow in the developing embryo (Rous 1911).

Rous (1879–1970), who discovered that certain sarcomas of chickens were transmissible through cell-free filtrates, was the first to make the connection between viruses and cancer. Although it took decades for his scientifically rigorous hypotheses to be fully accepted and longer for them to be completely appreciated, he was awarded the Nobel Prize in Medicine in 1966 in recognition of the momentous impact of his work on the fields of biology and human disease (Dulbecco 1976).

In the 20 years that followed the publication of Rous' seminal findings, the use of chorioallantoic membranes gained ground in the areas of experimental graft and tumor implantation; however, Goodpasture became the first to successfully employ this technique in the direct context of viral infections, and this proved to be a major breakthrough that stimulated progress towards new vaccines against infectious diseases. Using viruses from the pox group, fowlpox followed by vaccinia, he showed that inoculating chorioallantoic membranes of ten to 15-day old chick embryos provided a regenerative source of large quantities of concentrated virus that was free from bacterial contamination (Goodpasture et al. 1931; Goodpasture and Buddingh 1933).

Goodpasture's work represented a major advance in the field of virology and created a new approach that jump-started research on a variety of viral pathogens. In the decade that followed, Sir MacFarlane Burnet extended Goodpasture's findings to other embryonic tissues and other viruses, culminating in the isolation of influenza A in the amniotic sacs of chick embryos in 1940 (Burnett 1973). The chick embryo proved to be a safe, practical, cost effective and more efficient means of growing virus as compared with using whole animals; vaccinologists seized upon the technique, resulting in the development of effective vaccines against yellow fever (Chap. 10), influenza (Chap. 11), and typhus (Chap. 14). This medium is still used today for the production of influenza and yellow fever vaccines. However, within 15 years dramatic advances in tissue culture techniques would redirect the field yet again and provide the impetus that would propel vaccine research into its golden age.

John Franklin Enders (1897–1985) was raised in Connecticut, the son of a banker and a family of substantial means. As a high school student he preferred subjects in the humanities to those in quantitative science; in the latter he apparently expended great effort with only “mediocre” results (Weller and Robbins 1991). After graduating from Yale, his education interrupted by a voluntary stint in the Naval Reserve, his interests wandered for a time. He tried his hand at real estate and later enrolled in a doctoral program at Harvard with the goal of eventually teaching English. His ultimate career path shifted in the mid-1920s when he shared living space with a group of medical students and junior faculty members of Harvard Medical School. One of these young instructors introduced Enders to microbiology, laboratory research, and Hans Zinsser, the inspirational and brilliant Chairperson of the Department of Bacteriology at Harvard, who achieved worldwide recognition for his landmark work on rickettsial diseases and immunity (Weller 2004). The laboratory “became a way of life” in the eclectic, scientifically exhilarating environment of Zinsser's Department (Weller and Robbins 1991).

Enders obtained a Ph.D. in bacteriology at the age of 33 in 1930, yet despite the relatively late start to his research career in microbiology, he gradually rose up the

academic ranks at Harvard and expanded his research areas of interest (Robbins 1991). Enders spent most of the 1930s investigating the pathophysiology and immunology of the pneumococcus; by 1939, he had turned his attention to viruses, a focus that would continue for the remainder of his illustrious career. The initial virus he addressed was mumps at the request of the military; Enders developed the first tests to measure the immune response to this virus (Weller 2004). In 1946 he moved his laboratory to Boston Children's Hospital, establishing the Research Division of Infectious Diseases, where he focused on using the newly described techniques of Goodpasture and Burnet to isolate the etiologic agents of various common viral diseases and on developing new techniques to facilitate the growth of these viruses (Weller 2004).

Although not new, the technique of tissue culture enjoyed little penetration or impact on the field of virology prior to the late 1940s. Other than its use, along with Goodpasture's technique, in the attenuation of yellow fever virus by Max Theiler at the Rockefeller Institute in 1937 leading to a live vaccine (Artenstein et al. 2005), it had not yet found a formal niche in virology. Alexis Carrel, the renowned French surgeon, biologist, and later philosopher, who received the Nobel Prize in Medicine or Physiology in 1912 for his research on tissue preservation, vascular grafting, and organ transplantation, had developed techniques for the preservation of living tissue explants while working at the Rockefeller Institute in the early 1900s (Corner 1964). He was mainly interested in using cultured tissue as a substrate for studying wound healing and organ regeneration. Carrel had been further motivated by the work of Ross Harrison, Head of the Department of Zoology at Yale, who in 1908 reported the first successful cultures of animal tissue, nerve cells from the spinal cord of a frog embryo, outside of the body (Harvey 1975). Carrel, continually improving upon Harrison's techniques, undertook a series of investigations in 1910 that eventually led to the successful cultivation of embryonic chicken fibroblasts, initially derived from chick heart cells, through thousands of tissue generations and 34 years of growth (Carrel 1911; Corner 1964). In fact the explanted chick cultures outlived Carrel by 2 years.

Tissue culture techniques underwent incremental but important technical modifications through the work of Montrose Burrows, Carrel's assistant, and later Albert Ebeling, who supervised Carrel's cultures from 1912 at Rockefeller until 1946 during his tenure at Lederle Laboratories (Corner 1964). Because viral replication depended on dividing host cells, such technical improvements were necessary before tissue culture methodologies could be successfully applied to the viruses. Steinhardt, Israeli, and Lambert in 1913 reported success growing vaccinia on rabbit and guinea pig corneas in tissue culture but were only able to propagate the virus through three generations (Steinhardt et al. 1913). In 1925 Raymond Parker, a former student of Harrison's at Yale, and Robert Nye, both working in the Thorndike Laboratory at Boston City Hospital, successfully cultivated vaccinia *in vitro* in rabbit testes, demonstrating not only the presence of virus through 36 generations of growth, but also that the viruses multiplied to more than 50,000-fold their original content (Parker and Nye 1925a). Parker and Nye, attempting the same techniques using herpes virus, were able to demonstrate growth in tissue culture for

briefer periods than with vaccinia, but they were not able to prove that viral multiplication had occurred (Parker and Nye 1925b).

The next incremental, yet major advance in tissue culture of viruses came in 1928 when Hugh and Mary Maitland, working at Manchester University in England devised a simplified method of growing vaccinia in a substrate of fresh minced adult hen's kidney, serum, and organic salts in flasks (Maitland and Maitland 1928). Rivers and colleagues introduced further refinements to the Maitlands' technique (Rivers et al. 1929; Li and Rivers 1930), and over the next decade studies demonstrated, with variable, relative success, the cultivation of fowlpox, foot-and-mouth disease virus, yellow fever virus, and the rickettsial agent of typhus (Eagles 1933; de Bruyn 1942). Certain other viruses, such as polio, or presumed viruses, such as the causative agents of mumps and measles, defied definitive evidence that they could be grown in tissue culture (Eagles 1933), although Albert Sabin and Peter Olitsky, working on neurotropic viruses at the Rockefeller Institute in 1936, were able to successfully cultivate poliovirus on human embryonic brain tissue (Sabin and Olitsky 1936).

The development of the roller tube culture technique by George O. Gey, Director of the Tissue Culture Laboratory at Johns Hopkins, in 1933 represented an attempt to standardize and automate at least some of the technical aspects of tissue culture (Harvey 1975). The technique, in which glass tubes containing tissue substrates and nutrient media were placed horizontally in slots in a metal or wooden wheel that rotated slowly, was designed to optimize oxygenation and cell growth, and was adapted by virologists to enhance their tissue culture yields (Gey 1933; Weller 2004). Gey was a major force in the technical aspects of tissue culture throughout his career and is perhaps best known for his establishment of the continuous line of human carcinoma cells, HeLa, from a patient with cervical cancer (Hanks and Bang 1971).

Contemporaneous tissue culture methods near the mid-twentieth century suffered from a number of limitations including their tendency to yield inconsistent and non-reproducible results, the inability to maintain cultures for prolonged periods of time, the complexities and nuances of the various culture systems in use, and the inherent difficulties in proving the occurrence of viral replication versus simply viral persistence (Eagles 1933; Katz et al. 1996). In the latter regard, inoculation of whole animals was still required for confirmation of viral replication in some instances, even in the setting of successful tissue cultivation.

The Enders laboratory began working with tissue culture in earnest in the late 1940s. They had previously demonstrated the ability to cultivate vaccinia virus in high titer for up to 9 weeks using Gey's roller tube culture method (Feller et al. 1940). The next successful application of the technique came with mumps, a viral disease of childhood to which Enders had already made significant scientific contributions. In early 1948, Weller and Enders successfully propagated both mumps and influenza A using a modification of the Maitlands' tissue culture method (Weller and Enders 1948). Despite these forays into experimental methodology, the full impact of tissue culture in the field of virology and subsequently vaccinology was as yet indeterminate. It would take a series of well-crafted experiments,

conceived on the heels of a fortuitous laboratory finding, to essentially revolutionize the fields of virology and vaccinology. Scientific breakthroughs, while often dependent on some measure of good fortune, tend to favor disciplined, meticulous investigators working in a research environment that is conducive to discovery. Such conditions co-existed at Enders' Research Division of Infectious Diseases.

Thomas H. Weller (1915–2008) was the son, grandson, and nephew of physicians; his father was a faculty member in the Department of Pathology at the University of Michigan and a major influence on Weller's interests in biology and medicine (Weller 2004). He matriculated at Harvard Medical School in 1936, with a master's degree in zoology and strong leanings toward biologic investigation, publishing papers in the fields of ornithology, ichthyology, and parasitology prior to completing his medical education (Ligon 2002). Weller, interested in pursuing parasitology research, obtained a research elective as a fourth year medical student in the Enders lab. His intention was to learn tissue culture techniques being studied there in the context of viruses and apply them to grow parasites (Weller 2004). In 1947, following clinical training in pediatrics interrupted by 4 years of service in the Army Medical Corps during World War II, Weller joined Enders in the Research Division of Infectious Diseases at Children's Hospital, becoming the Assistant Director there in 1949.

Frederick C. Robbins (1916–2003) was a classmate and roommate of Weller's at Harvard. Robbins was born in Alabama, reared in Missouri, and was educated at the University of Missouri, from where he transferred to complete his final 2 years at Harvard Medical School (Katz 2005). His father was a well known, academic botanist and for a time, director of the New York Botanical Garden. His pediatric residency at Boston Children's Hospital, too, was interrupted by military exigencies; Robbins was awarded the Bronze Star for his service as the Chief of the Virus and Rickettsial Disease Section of the 15th Medical General laboratory in North Africa and Italy, where his clinical and microbiologic descriptions of Q fever represented major contributions to the field (Bendiner 1982; Katz 2005). After completing his clinical training in pediatrics and at Weller's urging, he joined the Enders lab in January of 1948 through a 1 year fellowship from the National Foundation for Infantile Paralysis, with the specific tasking of isolating a possible viral etiology of infant diarrhea.

Weller and Robbins were both closely mentored by Enders, who while rarely involved in the actual technical performance of experiments, was responsible for strategic planning of the experiments, laboratory supervision, and review of all experimental data (Weller 2004). On March 30, 1948, Weller was pursuing the use of tissue culture to grow varicella virus, the causative agent of the childhood disease chickenpox and herpes zoster in adults. Using spontaneously aborted human embryos, he prepared the culture flasks by suspending minced skin and muscle tissue in media, inoculated some of the cultures with throat washings from a child with chickenpox, kept some as negative cell controls, and so as not to waste the remaining four flask cultures, inoculated them with a suspension of mouse brain that had been infected with the Lansing II strain of poliovirus – specimens that happened to be in the freezer at the Enders laboratory (Weller 2004).

Although Weller was unable to isolate varicella virus in this manner, the intracerebral inoculation of mice with material from the cultures containing poliovirus caused paralysis and death in four out of five animals (Weller 2004).

Further experimentation, including neutralization by specific antiserum and spinal cord histopathology of monkeys inoculated with the cultured material, confirmed that the cause of disease and death in the mice was in fact the Lansing strain of poliovirus (Weller 2004). This represented the first time that poliovirus had been successfully cultivated ex-vivo in non-neural tissue (Enders et al. 1949) and turned out to be a monumental discovery that would yield immediate dividends. In a later reflection Enders claimed that the experiment may have emerged from discussions among virologists at the time that poliomyelitis viruses “might not be strict neurotropes...it was becoming increasingly difficult to visualize the nervous system as site of manufacture of the enormous quantities of virus that were found in the feces of many patients” (Enders et al. 1980). Nonetheless, all three researchers concurred that serendipity undoubtedly played a part in their findings (Enders et al. 1980; Bendiner 1982; Weller 2004).

The investigators were able to maintain cultured growth of poliovirus for prolonged periods, through multiple subcultures and dilution procedures. They estimated that the viral titer increased by more than 15 logs of growth during the initial 2-month period of the experiment (Enders et al. 1949). Additional, derivative investigations rapidly followed from their initial, landmark findings. The Research Division of Infectious Diseases, although not previously engaged in a significant poliovirus research effort (Enders et al. 1980), turned its short-term, scientific focus to this pathogen with swift and remarkable results.

Over the next few years, they reported a number of highly significant findings: the growth of poliovirus in non-embryonic, human foreskin tissue (Weller et al. 1949) and a variety of other fetal and differentiated human tissues, including embryonic intestine (Weller et al. 1952; Robbins et al. 1952); the growth of all three antigenic types of poliovirus in non-neural tissues (Weller et al. 1949); the use of roller tube culture methodology to efficiently produce high titers of virus (Robbins, Weller and Enders 1952); the phenomenon of degenerative changes induced in poliovirus-inoculated cells in tissue culture, termed the “cytopathic effect,” that became a *sine qua non* of viral pathogenicity and obviated the need for animal inoculation to assess viral replication in clinical virology (Robbins et al. 1950); and the direct isolation in tissue culture and serotyping of poliovirus from patient specimens (Robbins et al. 1951). The latter efforts led to the identification of novel, non-polio enteroviruses from patients as well as to a serum diagnostic assay for polio. Finally, the team demonstrated that multiple passages in tissue culture led to attenuation of even the most virulent strains of polioviruses (Enders et al. 1952) – a finding that directly led to the development of live attenuated vaccines for this and other viral pathogens (Katz et al. 1996; Lepow 2004; Weller 2004).

In recognition of the enormous impact of their achievement, a discovery that “had a revolutionary effect on the discipline of virology” (Norrby and Prusiner 2007), Enders, Weller, and Robbins (Fig. 9.7) were awarded the Nobel Prize in Physiology or Medicine in 1954, a mere 5 years after the publication of their findings.



**Fig. 9.7** Thomas Weller, Frederick Robbins, and John Enders (l-r) at the 1954 Nobel Prize ceremony (Francis A. Countway Library of Medicine, Harvard University)

It was apparent from the deliberations of the Nobel Committee and primary recollections of those involved in the experiments that all three researchers played a major role in the work (Norrby and Prusiner 2007). The implications of their studies for the field of virology were profound and immediate, igniting an explosion of “restless activity in the virus laboratories the world over” (Gard 1954) and launching the modern era of this science. What followed was a tremendous period of productivity in vaccinology; their work set the stage for the development of effective poliovirus vaccines as well as vaccines against other major viral causes of human and animal disease.

The impact of their work and the accolades that followed it were equally profound for the three scientists. Enders continued as the Director of the Research Division of Infectious Diseases for the remainder of his career. After a flurry of poliovirus research activity evolving from their landmark discovery, Enders used their scientific approach to pursue other important issues in virology. His achievements were myriad: the initial description of diseases caused by Coxsackie

virus and Echovirus; the isolation of measles virus and the development of an effective, live attenuated measles vaccine that was licensed in 1963 and remains in use today; and groundbreaking studies on viral pathogenesis and oncogenesis (Weller and Robbins 1991). Weller went on to isolate the viral causes of varicella-zoster, cytomegalovirus, and concurrently with the group at the Walter Reed Army Institute of Research, rubella (Weller 2004). He then devoted the final 30 years of his illustrious career in medicine to other problems of global importance – tropical diseases and public health, serving as Chairman of the Department of Tropical Public Health at the Harvard School of Public Health until his retirement. Robbins left the Enders lab in 1952 to resume a career in academic medicine at Case Western Reserve University School of Medicine in Cleveland, first as the chief of pediatrics and contagious diseases and, from 1966 to 1980 as the Dean of the medical school (Katz 2005). He left to become president of the Institute of Medicine yet returned to Case Western for the remainder of his career where he endeavored to bring vaccines, the fruits of his and his colleagues' research successes, to resource-poor environments in order to improve human health.

In the 67 year period that began with Pasteur's animal and human experiments involving rabies and Mayer's initial observations regarding a filterable agent causing transmissible disease in tobacco plants, and ending with the landmark work growing these agents – viruses – ex vivo in tissue culture in Enders' laboratory, the foundations of vaccinology had been created. This enabled the rapid development of successful vaccines against many of the major viral scourges of the era and the eradication of diseases such as measles, rubella and polio from developed countries (Roush and Murphy 2007). The scientific advances of this period continue to provide an approach to the prevention of novel, emerging infectious challenges.

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# Chapter 10

## Yellow Fever

Thomas P. Monath

In 1951, Max Theiler (Fig. 10.1), a Rockefeller Foundation scientist, became the only person to be awarded the Nobel Prize in Medicine and Physiology for the development of a virus vaccine (Norrby 2007). His live, attenuated 17D vaccine was not the first yellow fever vaccine to be tested in humans, but it was by far the most successful one. More than 500 million doses have been distributed since the late 1930s. Yellow fever vaccine history, told briefly in this chapter, provides numerous lessons to those interested in the science of vaccinology; it is enriched by the cast of colorful characters who were involved in vaccine discovery and introduction into routine use, and informed by the investigation of various mishaps in manufacturing and application.

### 10.1 The Disease and the Need for a Vaccine

Yellow fever, caused by a small, enveloped, single-stranded RNA virus of the same name is the original viral hemorrhagic fever – a highly lethal infection resulting in hepatic, renal, and myocardial injury, hemorrhage, and shock. The virus is maintained in a forest cycle involving nonhuman primates and tree-hole breeding *Aedes* mosquitoes and currently occurs only in tropical parts of South America and Africa. Since yellow fever is a zoonosis, the only reliable way to prevent the disease is through human vaccination.

The early history of the disease is uncertain. Henry Rose Carter, a U.S. Public Health Service physician, noted the first description of epidemic yellow fever in a seventeenth century Mayan manuscript from the Yucatan. His hypothesis that the virus and mosquito vector were introduced from Africa via the slave trade (Carter 1931) was later supported by molecular epidemiology. During the eighteenth and nineteenth centuries, yellow fever posed a major problem throughout the

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**Fig. 10.1** Max Theiler  
(Rockefeller Archive Center)



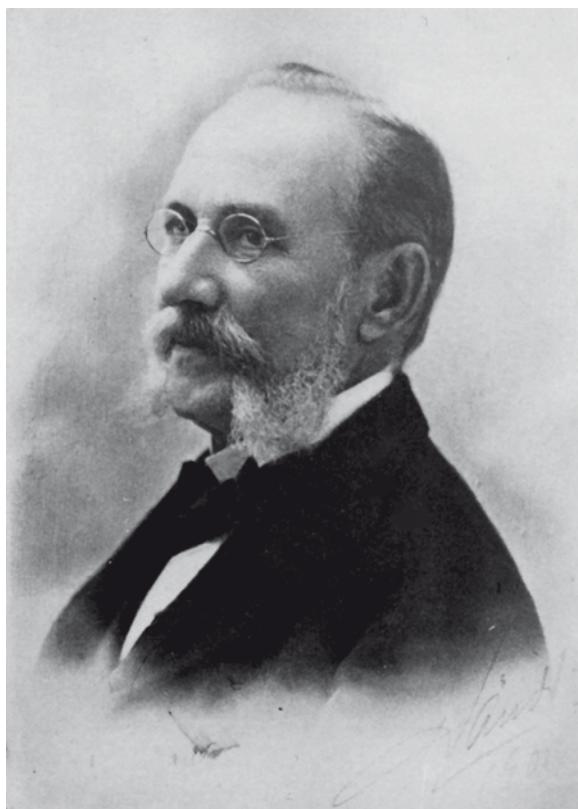
Americas and West Africa. Seaports in locations as far north as Nova Scotia and England experienced repeated epidemics because of viral importation aboard mosquito-infested sailing vessels. Infamous outbreaks occurred in 1793 in Philadelphia, the fledgling federal capital of the U.S., in which 10% of the population perished (Powell 1949), and the lower Mississippi Valley in which 20,000 died in 1878 (Bloom 1993).

Yellow fever was widely held to be an airborne “miasma” arising from filth, sewage, and rotting organic matter. Benjamin Rush, the first academic American physician, attributed the 1793 epidemic to the offensive odor of a large quantity of rotting coffee that had been thrown on the wharves at Philadelphia (Rush 1794). Many similar accounts over the ensuing century implicated the presence of damaged coffee, sugar, beans and “...deposits of filth made up of shavings and various refuse of other kinds” (Government Printing Office 1880), but in the absence of a causative agent, vaccination remained an elusive goal.

## 10.2 First Attempts at Vaccination

Several physicians during the latter half of the nineteenth century had suggested that yellow fever was transmitted by mosquitoes (Warren 1951). Carlos Finlay (1833–1915), working in Havana during the 1870s and 1880s, a time when the

germ theory of disease and Koch's postulates of microbial causality were becoming accepted by mainstream European science (refer to Chap. 3), proposed that mosquitoes carried the "germ" of yellow fever (Finlay 1881). He attempted to prove this by feeding mosquitoes that had previously fed on yellow fever patients upon nonimmune individuals. He inoculated 33 immigrant Spanish priests using the single-mosquito method, none of whom developed yellow fever, and followed 32 uninoculated controls, five of whom died of the disease, leading Finlay to conclude that his immunization method was effective. In the context of the rapidly expanding science of vaccination – originally established by Jenner at the end of the eighteenth century and gaining momentum in the 1880s through the work of Pasteur and others -Finlay (Fig. 10.2) believed that a "controlled" dose of "germs" introduced via the bite of a single mosquito would immunize against subsequent severe infection acquired by multiple bites (Finlay 1891). But his thesis was based on flawed methodology; he was unaware of the transmission requirement for an incubation period in the mosquito, as this would not be discovered for another 15 years.



**Fig. 10.2** Carlos Finlay  
(Phillip S. Hench Walter  
Reed Collection,  
Historical Collections  
and Services, Claude  
Moore Health Services  
Library, University of  
Virginia)

**DR. CARLOS J. FINLAY**  
who first promulgated the theory of the transmission of yellow fever  
by the mosquito

In this pre-virology era a number of contemporary attempts to recover the causative agent – an appropriate embarkation point for a vaccine – focused on bacteria and fungi. Professor Domingos José Freire in Rio de Janeiro identified a microorganism resembling *Cryptococcus xanthogenicus* as the cause of yellow fever in 1879 and created a vaccine by applying a Pasteurian “ageing” process of attenuation. The French Academy of Sciences embraced Freire’s vaccine; within a year, it was used in 12,000 Brazilians and in 1887 was recommended for use in all persons living in countries affected by yellow fever. Shortly thereafter, Freire’s fungal agent was discredited in favor of an alternative, bacterial etiology (Benchimol 2001).

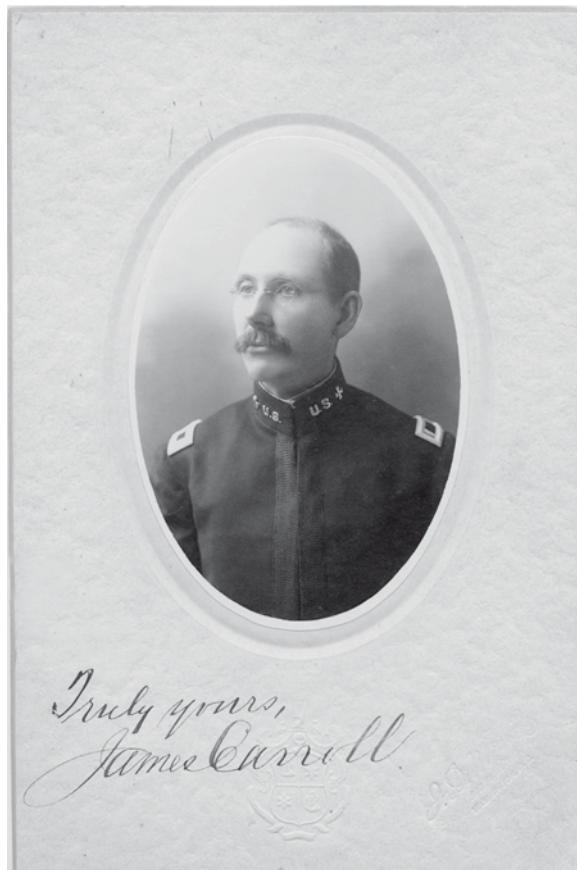
In 1897, Giuseppi Sanarelli, a prominent Italian bacteriologist working in Uruguay, announced that he had discovered a bacterial agent, *Bacillus icteroides*, in over half of the yellow fever cases he investigated, findings widely embraced by contemporary scientists during an era in which yellow fever was a burgeoning and devastating epidemic disease and in which there was the belief that microbiology could provide definitive solutions to infectious diseases. However, Sanarelli’s work lacked scientific rigor, and his agent did not satisfy Koch’s postulates. Hence, a main objective of Walter Reed’s expedition to Cuba in 1900, guided by U.S. Surgeon General George Sternberg, was to prove or disprove Sanarelli’s *Bacillus* theory.

The history of key discoveries by the Yellow Fever Commission, led by Walter Reed and comprising Jesse Lazear, Aristedes Agramonte, and James Carroll (Fig. 10.3) in 1900 is well known (Government Printing Office 1911) and will be



**Fig. 10.3 a** Walter Reed  
(Phillip S. Hench Walter  
Reed Collection,  
Historical Collections and  
Services, Claude Moore  
Health Services Library,  
University of Virginia)

**Fig. 10.3 b** James Carroll  
(Phillip S. Hench Walter  
Reed Collection, Historical  
Collections and Services,  
Claude Moore Health  
Services Library, University  
of Virginia)



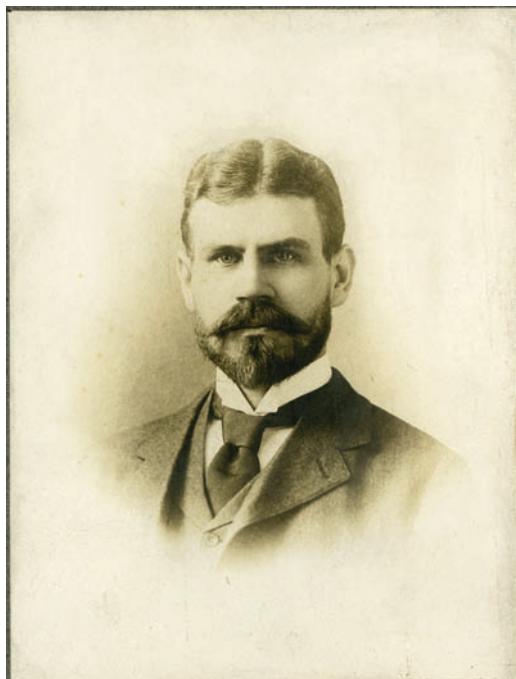
described only briefly. Yellow fever was both endemic and epidemic on the island of Cuba and represented a grave threat to the occupying U.S. Army forces during the Spanish American War. Once autopsies and bacteriological studies disclosed no evidence for Sanarelli's *Bacillus*, the Commission's attention turned to Finlay's mosquito theory of transmission. Reed's experiments were aided by using eggs of the suspected mosquito vector (now called *Aedes aegypti*) provided by Finlay and informed by the work of Henry Rose Carter from 1898 which indicated that an "extrinsic incubation period" of about 2 weeks separated the appearance of initial and subsequent yellow fever cases (Carter 1900).

Reed and colleagues allowed mosquitoes to feed on the blood of yellow fever patients, held the mosquitoes for a period of time and subsequently fed them on non-immune human volunteers. None of the experimentally infected volunteers died, and few suffered severe illness. They demonstrated the agent to be a filterable virus, a group of agents only recently discovered (refer to Chap. 9), transmissible by direct injection of blood or by the agency of *Ae. aegypti* mosquitoes (Government Printing Office 1911), the latter mode requiring an extrinsic incubation period of

**Fig. 10.3 c** Aristides Agramonte  
(Phillip S. Hench Walter Reed  
Collection, Historical Collections  
and Services, Claude Moore  
Health Services Library,  
University of Virginia)



**Fig. 10.3 d** Jesse Lazear  
(Phillip S. Hench Walter Reed  
Collection, Historical Collections and  
Services, Claude Moore Health Services Library,  
University of Virginia)



12 days in the mosquito and confirming Finlay's hypothesis. Additionally, they described the clinical incubation period of 3–6 days and the lack of fomite-based or person-to-person disease transmission in the absence of mosquitoes.

The Commission's studies appeared to corroborate Finlay's earlier suggestions that immunization by mosquito bite might provide a means of protection. Subsequently, Juan Guiteras, Professor of Pathology and Tropical Medicine at the University of Havana, conducted a series of experimental infections by mosquito bite "... with the hope of propagating the disease in a controllable form, and securing among the recently arrived immigrants, immunization, with the minimum amount of danger to themselves and to the community" (Guiteras 1901). In contrast to those of Reed, his experiments led to clinical yellow fever in eight volunteers, three of whom died, apparently due to the inadvertent use of a virus strain that produced a low infection-to-case ratio and a high case-fatality ratio. The fatalities in Guiteras' series put an end to "mosquito vaccination."

### 10.3 Isolation of Yellow Fever Virus

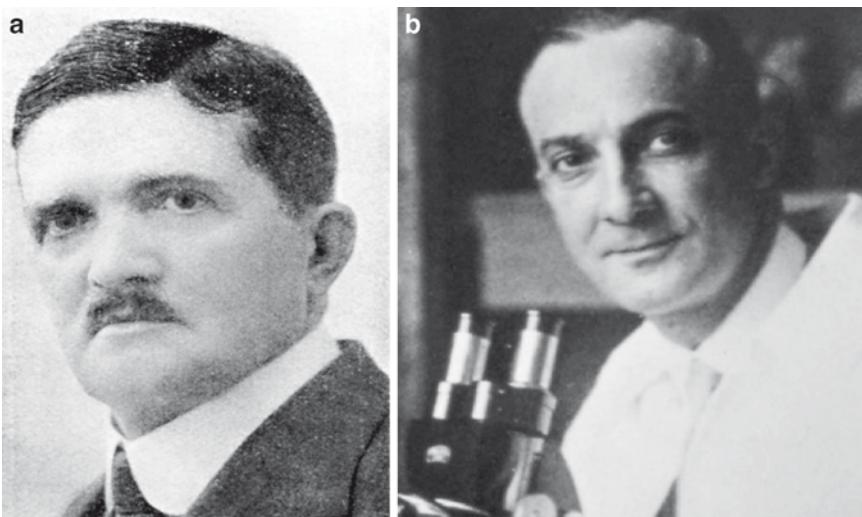
During the first decade of the twentieth century, others confirmed the Reed Commission's findings, but the etiologic "virus" eluded isolation and characterization. It was therefore surprising when a prominent Japanese scientist, Hideyo Noguchi proclaimed in 1925 that he had isolated a bacterial agent, *Leptospira icterooides*, from cases of yellow fever and reproduced the disease in guinea pigs, fulfilling Koch's postulates (Noguchi 1925). In that year the Rockefeller Foundation, chartered in 1913 and the first such entity to focus on international health problems, established the West African Yellow Fever Commission laboratory in Yaba, near Lagos, Nigeria (Fig. 10.4a), primarily to determine if the disease in Africa was due to yellow fever, if Noguchi's *Leptospira* was responsible for the disease and, if not, to isolate the specific etiologic agent. The Commission's physician staff included Johannes Bauer, Alexander Mahaffy, Adrian Stokes, and N. Paul Hudson (Fig. 10.4b–e).

An extensive epidemic of yellow fever swept through coastal Africa in 1927 providing study material for the Commission. When attempts to isolate *Leptospira* directly from patients or animals failed, blood from human cases was inoculated into a variety of experimental animals (Stokes et al. 1928). Indian crown monkeys were noted to be a susceptible host, developing illness and in some cases hepatic pathology consistent with yellow fever, yet the investigators were unable to recover a causal agent until Mahaffy inoculated the blood of a 28 year-old patient named Asibi from Ghana (Fig. 10.4f), obtained 33 h hours after the onset of mild yellow fever, into a rhesus monkey at the field laboratory in Accra (Stokes et al. 1928). Four days later the animal was moribund and demonstrated hepatic lesions consistent with yellow fever. Blood from this monkey caused clinical yellow fever in a second animal following intraperitoneal inoculation.

Commission investigators established the Asibi strain, ultimately the parent strain of the 17D vaccine, by continuous direct passage in monkeys and indirect passage through *Ae. aegypti* mosquitoes. In addition to identifying the causative agent of yellow fever and confirming that it was a filterable viral agent, they also



**Fig. 10.4** (a) The Rockefeller Commission on Yellow Fever laboratories at Yaba (near Lagos), Nigeria, 1925; Rockefeller Foundation's West Africa Yellow Fever Commission (b) Johannes Bauer (c) Alexander Mahaffy (d) Adrian Stokes (e) N. Paul Hudson (f) Ghanian patient Asibi



**Fig. 10.5** (a) Constantin Mathis (b) Jean Laigret

showed that rhesus monkeys were susceptible hosts, providing an invaluable tool for testing future vaccine candidates. Tragically, Stokes succumbed to laboratory-acquired disease in 1927, another victim in a line of yellow fever researchers that included Jesse Lazear from the Reed Commission; he would not be the last. While attempting unsuccessfully to resurrect and validate his *Leptospira* theory in Accra, Noguchi too acquired yellow fever in the laboratory and died, disillusioned, in 1928.

Contemporaneous to the work of the Commission in Nigeria and equally important to vaccine research, Constantin Mathis and Jean Laigret at the Institut Pasteur, Dakar (Fig. 10.5), recovered a virus – the “French strain” – from a Syrian patient with mild yellow fever and again showed that rhesus monkeys were susceptible to the disease (Mathis et al. 1928). Isolation of the Asibi and French strains was the key to vaccine development. Many years later, comparison of the genomes of the Asibi and French viruses confirmed that, despite differing passage histories, they were 99.8% identical at the sequence level, divergent at only 23 nucleotides and nine amino acids (Deubel et al. 1986; Jennings et al. 1993), consistent with their isolation amidst the same epidemic in 1927. Despite their common ancestry, however, the methods used to attenuate the Asibi and French strains and to manufacture their respective vaccines would be fundamentally different, resulting in quite divergent product profiles.

## 10.4 Early, Inactivated Vaccines: 1928–1930

The principle that immunity against yellow fever could be effective was established as early as 1903, when convalescent serum was shown to confer protection (Marchoux et al. 1903). The earliest attempt to use the newly isolated virus for

active immunization was by Edward Hindle at the Wellcome Research Laboratories in London in 1928, using vaccine prepared from monkey liver and spleen tissues infected with the French strain and inactivated with formalin or glycerin-phenol (Hindle 1928). Rhesus monkeys inoculated subcutaneously with the inactivated vaccine survived challenge with a large dose of virus a week later; unvaccinated animals died. A similar experiment was conducted in France (Petit 1931).

In Brazil human trials with an inactivated vaccine were initiated during a devastating epidemic of urban yellow fever in Rio de Janeiro in early 1929. Henrique Aragão at the Oswaldo Cruz Institute and Lemos Monteiro at the Butantan Institute prepared vaccine from macaque liver and spleen tissues infected with the Asibi strain, inactivated according to Hindle's method, and administered it in uncontrolled fashion to 25,000 recent immigrants to and residents of the city (Benchimol 2001). Their results were inconclusive; approximately 25 cases of yellow fever occurred in those vaccinated, and a vaccinated laboratory worker developed disease.

Preparation of inactivated vaccines at the time was hampered by the lack of efficient methods for viral propagation, potency measurement, and control of the inactivation process. Some preparations probably contained residual live virus responsible for immunization, whereas others were degraded during the inactivation process and lacked efficacy (Okell 1930). In 1929, Wilbur Sawyer (Fig. 10.6), later Director of the International Health Division Laboratories at the Rockefeller Institute, and colleagues at the Rockefeller Foundation in New York produced vaccines derived from the chemical treatment of infectious rhesus monkey serum;



**Fig. 10.6** Wilbur Sawyer  
(Rockefeller Archive Center)

the behavior of these preparations was similarly inconsistent, leading the researchers to conclude that chemical treatment of virulent virus was unlikely to produce a safe and dependable vaccine. Additional impediments to the development of a successful inactivated vaccine included a poor understanding of immunologic responses and their metrics, the unappreciated requirement for priming and boosting, and technical limitations on vaccine production. The latter forced the Oswaldo Cruz Institute to suspend work on the project (Benchimol 2001).

## 10.5 Partially Attenuated live Vaccines and “Sero-immunization”: 1931–1934

In 1928, Andrew Watson Sellards, Head of the Department of Tropical Medicine at Harvard Medical School returned from the Institut Pasteur, Dakar with samples of infected rhesus monkey liver containing the French strain of yellow fever virus. Max Theiler (1899–1972), recruited to Sellard’s department in 1922 as an Instructor was entrusted with this material for his work on developing a small animal model of the infection. Theiler, born in Pretoria, South Africa, the son of a recognized veterinary researcher and Director of the Onderstepoort Institute, trained in medicine at St. Thomas’ Hospital in London. He was familiar with the work of Pasteur and Roux, nearly 50 years prior, who had infected dogs, monkeys, guinea pigs, and rabbits with rabies by the intraspinal or intracerebral routes during their vaccine efforts (refer to Chap. 5) and also with that of Andervont who in 1929 had shown that mice were susceptible to herpes simplex encephalitis after intracerebral inoculation (Andervont 1929).<sup>1</sup> Theiler inoculated adult Swiss mice intracerebrally with the infected monkey liver suspensions from Dakar; all of the animals died of encephalitis without signs of liver damage. Subsequent inoculation of brain tissue from these mice into rhesus macaques caused typical, fatal yellow fever with hepatic disease. The successful mouse model represented a landmark in yellow fever research (Theiler 1930a).

Pasteur and Roux had further demonstrated a modification of the virulence of rabies by serial passage in rabbit brains. Based on this work and his erroneous understanding that smallpox had been attenuated naturally via passage through a bovine host (it had not; Jenner’s vaccine was cowpox, not attenuated smallpox), Theiler attempted to attenuate yellow fever virus through a series of sequential passages using intracerebral inoculation of mice. Monkeys inoculated with mouse brain material passaged 29 and 42 times survived infection without clinical signs of disease and were resistant to challenge with virulent virus (Theiler 1930b). Sequential passage of the virus in mice led to an increase in its neurotropism,

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<sup>1</sup>In fact the first studies showing mice to be susceptible to herpes virus were done earlier by G Blanc and J CamioPetros (*Recherches experimentales sur herpes*. *CR Soc Biol* 1921; 84: 859).

suggesting that with continued passage the virus might eventually reach a stable stage of neurovirulence, i.e., become “fixed,” as had occurred with rabies virus after sequential passage in rabbit brain, with the fixed virus being less pathogenic in dogs. Theiler passed the French strain in mice in excess of 100 times, resulting in a “fixed” virus with a stable interval of 4 days between inoculation and animal death. This work provided the basis for a vaccine.

Neurotropism and viscerotropism of yellow fever were considered distinct viral properties. Theiler observed that rhesus monkeys, exquisitely sensitive to yellow fever virus, died of hepatitis – not encephalitis – when inoculated by the intracerebral route. In contrast the virus in which neurotropism in mice had been fixed by intracerebral passage had lost its capacity to produce hepatitis in monkeys after peripheral or intracerebral passage.

Theiler’s discovery, spurred by the medical impact of the disease in affected populations and the devastating effects of laboratory-acquired yellow fever, ushered in an intensive effort to develop a human vaccine based on virus attenuated via serial passage in mouse brain (Sawyer et al. 1931). Vaccine development would ultimately proceed as two essentially independent efforts by Theiler, who joined Sawyer at the Rockefeller Institute, and by Laigret’s group at the Institut Pasteur, Tunis in collaboration with Sellards at Harvard.

The Rockefeller group was the first to exploit Theiler’s mouse brain virus for human immunization using a vaccine comprising the French strain, fixed by intracerebral passage up to 176 times, and human immune serum – the latter added to safeguard against insufficiently attenuated virus (Sawyer et al. 1931). Indeed, laboratory-acquired cases of mild yellow fever due to “fixed” virus had occurred (Berry and Kitchen 1931), and monkeys inoculated intranasally, intracerebrally, intraspinally, or parenterally with the fixed virus occasionally developed encephalitis (Sellards 1931; Theiler 1951). Serum therapy, used during this period in human diphtheria and tetanus (refer to Chap. 7) and in veterinary medicine for rinderpest in cattle and hog cholera in swine, had previously shown efficacy in combination with virulent yellow fever virus in monkeys (Theiler and Sellards 1928), but the method had not been attempted in humans because of the potential for breakthrough disease. Rockefeller investigators would use a modification of this approach, “seroimmunization,” with the partially attenuated vaccine strains.

Their approach was meticulous and represented the safest methodology for live vaccine development of the era. Eight lots of neuroadapted, attenuated vaccine were prepared, differing principally in mouse passage levels. The vaccine was tested for bacterial sterility and for the presence of infectious vaccine virus following intracerebral inoculation of mice. However, a quantitative potency assay was not performed; the dose was defined by the weight of mouse brain represented in the vaccine rather than by actual virus titer. Human immune serum, added to the vaccine or administered at separate sites concurrently, was recognized as a potential source of adventitious infection but was inactivated with tricresol-ether, tested for sterility, and evaluated for potency by a mouse neutralization test developed by Theiler and by challenge of treated monkeys with virulent yellow fever virus. Preliminary studies

demonstrated that vaccine and serum produced no adverse reactions and induced solid immunity. In 1931, Rockefeller scientists performed human inoculations on inpatients at the Institute's research hospital (Sawyer et al. 1931, 1932). No significant adverse reactions were observed; mild adverse events including local tenderness and redness, fever, and arthralgias were attributed to the brain tissue component of the vaccine rather than infection with yellow fever virus. Attempts to recover infectious virus from patients' blood were unsuccessful.

Sero-vaccination with the mouse brain virus subsequently entered into standard use for the immunization of laboratory workers – a group at significant risk. By 1934, 56 persons had been immunized in New York, Brazil, Colombia, and Argentina (Sawyer 1934); similar studies had been conducted at the Wellcome Bureau of Scientific Research in London (Findlay 1934). However, the requirement for human immune serum was problematic; attempts to substitute animal antisera (Petit and Stefanopoulo 1933; Theiler and Smith 1936) introduced concerns over reactogenicity, adventitious animal agents, and cost. Additionally, technical difficulties in standardizing conditions and in the control of *in vitro* neutralization of virus-serum mixtures in vaccine preparation were major obstacles to its widespread use. Clearly, a better approach was warranted, motivating French workers to pursue the development of a neurotropic vaccine *without* the addition of serum. The Rockefeller group, believing the neurotropic virus to be too dangerous as a stand-alone vaccine, took a different approach, initiating a search for a less pathogenic variant and for improved methods of propagating the virus.

## 10.6 Development of the French Neurotropic Virus: 1932–1941

Sellards at Harvard and Laigret at the Institut Pasteur, Tunis were the first to inoculate humans in 1932 with the French strain without immune serum, using a 10% suspension of mouse brain infected with the neuro-adapted French strain at the 134th passage (Sellards and Laigret 1932). Five nonimmune subjects developed neutralizing antibodies, prompting inoculation of an additional seven with virus passed 143 times in mice (Laigret 1933). This was a different era with respect to the ethics of human experimentation; the subjects were patients with neurosyphilis, several of whom developed severe side effects, including nausea, vomiting, abdominal pain, hyperactivity, abnormal reflexes, hemoptysis, and albuminuria. Laigret considered a number of possible explanations for these adverse reactions, including superinfection with another agent, contamination of the vaccine with an adventitious virus, and the underlying syphilis, but ultimately concluded that an insufficiently attenuated neuro-adapted virus was responsible and therefore redirected his efforts to develop a non-reactogenic regimen by altering the formulation and lowering the dose.

The first approach to further attenuating the mouse brains was to “age” the vaccine for varying lengths of time, a process presumably borrowed from Pasteur's original approach to rabies vaccine (refer to Chap. 5). Humans were initially given the oldest

virus, in mouse brains incubated for 4 days, followed at 20-day intervals by virus treated for 2 days and 1 day, respectively. More than 3,000, mainly expatriates living in French West Africa were vaccinated by this method in 1934; approximately one-third reported febrile reactions, and there was one case each of meningitis and myelitis (Mathis et al. 1934). The formulation was subsequently modified to a single injection of mouse-brain virus that had been “attenuated” for 24 h at 20°C and coated with egg yolk or olive oil, believed to slow diffusion of the virus from the inoculation site. By 1939 more than 20,000 persons in West Africa had received one of the aforementioned vaccine regimens (Nicolle and Laigret 1935).

Thus, over a 7-year period the neurotropic vaccine had transitioned from initial testing in humans to large scale, albeit uncontrolled clinical trials. Initial concerns about safety and tolerability dissipated as larger numbers of people were vaccinated. Although careful follow-up studies were not conducted, there were reports of severe central nervous system reactions (Laigret 1936; Sorel 1936). Nevertheless, safety concerns received little attention as the risks associated with vaccination were considered much lower than the potential benefit of acquiring protection from wild-type yellow fever.

During this period efforts were made to simplify inoculation using scarification, as used for smallpox vaccination. Workers at the Institut Pasteur, Dakar, using mouse-brain vaccine at the 237th passage, showed that smallpox and the French neurotropic vaccines could be given simultaneously as a mixture (Peltier et al. 1939). In 1939 approximately 100,000 persons in Senegal were vaccinated with such a combined vaccine without untoward effects; 95.6% of 1,630 subjects tested developed neutralizing antibodies against yellow fever (Peltier 1947). Favorable results from subsequent trials of smallpox-yellow fever scarification, performed in Anglo-Egyptian Sudan and Côte d’Ivoire (Peltier 1941) paved the way for manufacturing refinements and a greatly expanded program of compulsory immunization across Francophone countries endemic for yellow fever.

## 10.7 Development of an Alternative Vaccine, 17D: 1931–1938

Sawyer, Theiler, and other Rockefeller Foundation scientists considered the French neurotropic vaccine too dangerous, reportedly capable of producing yellow fever encephalitis in monkeys and associated with neurological accidents in humans, the latter down-played by the French. Additionally, concern persisted regarding the potential of the vaccine virus to revert to virulence – residual viscerotropism – on repeated liver passage in monkeys (Findlay and Clarke 1935). A new method for serial passage of the wild-type virus and for selection of attenuated variants without adaptation in mouse brain was needed to mitigate the dangers of the neurotropic virus. Towards this end Theiler and colleagues would utilize recently described methods for the *in vitro* cultivation of minced mouse and chicken embryos, developed at the Rockefeller Institute by Alexis Carrell, the noted tissue culture expert, and Thomas Rivers, renowned virologist (refer to Chap. 9).

In 1932, Theiler and Eugen Haagen<sup>2</sup> demonstrated that the neurotropic French virus could also be propagated in chick embryo tissue cultures (Haagen and Theiler 1932) but attempts to grow unadapted virus strains failed. Their operational principle, that propagation of virus under conditions restrictive with respect to its host range would select for variants with altered phenotypic characteristics, appeared to explain a previous observation that viscerotropic yellow fever virus passaged in a transplantable mouse carcinoma had reduced virulence (Findlay and Stern 1935). The Asibi strain, maintained by monkey-mosquito passage without subinoculation into mouse brain, was used in lieu of the French virus for their studies.

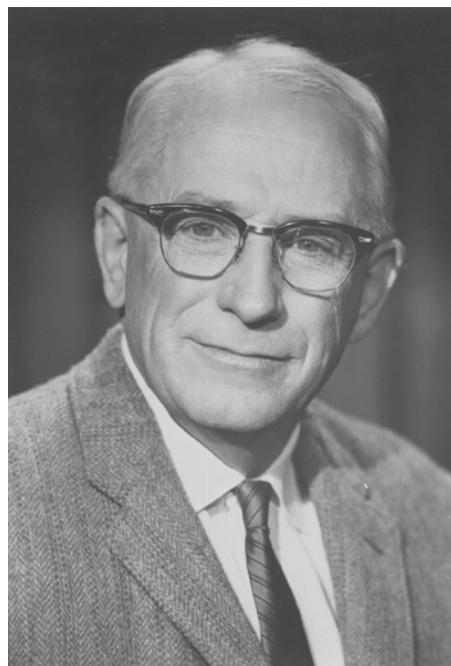
The first successful in vitro cultivation of the Asibi strain was accomplished in 1936; after 240 passages, the resultant virus – designated 17E – had lost viscerotropism for rhesus monkeys, although it retained its capacity to produce encephalitis after intracerebral inoculation (Lloyd et al. 1937). Deemed insufficiently attenuated for human inoculation without co-administration of immune serum based on its residual neurovirulence, the 17E virus replaced mouse brain virus in the sero-immunization regimen of laboratory workers (Lloyd 1935). Sawyer noted that “... a safer strain has supplanted the original neurotropic strain for use with immune serum in vaccination and it is confidently expected that a strain of virus safe for use without protective immune serum will finally be achieved” (Sawyer 1937).

Attempts to cultivate the Asibi virus in other substrates were unsuccessful. Additionally, despite the successful selection of more attenuated variants with regard to viscerotropism, viral neurotropism was not markedly diminished. Neural tissues present in the minced whole mouse embryos used in its initial propagation were believed to be responsible for maintaining neurotropism of the cultured virus, prompting Theiler to remove brain and spinal cord from the substrate prior to performing serial viral passages. The most important experimental passage series – designated 17D – used virus that had been subcultured eighteen times in whole mouse embryos, followed by 58 passages in whole minced chick embryo cultures, after which the virus was then passed in minced chick embryo depleted of nervous tissue. Each of the latter sub-passages was tested for neurotropism by intracerebral inoculation of mice. Hugh Smith (1902–1995) was responsible for oversight of the 17D lineage experiments.

After 100 serial passages in nervous tissue-depleted chick embryos (i.e., the 176th passage after initiating in vitro culture), Smith (Fig. 10.7) observed that challenged mice either resisted illness or developed nonlethal paralysis. The virus was also markedly attenuated for viscerotropism in rhesus monkeys, causing neither significant viremia nor hepatitis after subcutaneous inoculation (Theiler and Smith 1937). It was further determined that loss of neurovirulence for monkeys occurred between subcultures 89 and 114, and that neurovirulence for mice diminished between 114 and 176. Monkeys inoculated with the attenuated virus developed neutralizing antibodies and resisted lethal challenge with wild-type Asibi virus.

<sup>2</sup>Eugen Haagen became the Chief of the Medical Department of the Luftwaffe in Nazi Germany and apparently continued studies on yellow fever. Allegedly, he made new vaccines against yellow fever and tested them on inmates of the Nazi concentration camp at Natzweiler-Struthof.

**Fig. 10.7** Hugh Hollingsworth Smith  
(Rockefeller Archive Center)



The in vitro and pre-clinical data suggested that the 17D virus could be safely tested as a human vaccine without the addition of protective immune serum (Theiler and Smith 1937; Smith et al. 1938).

Based upon the breakthrough work of Goodpasture (refer to Chap. 9) earlier in the decade using embryonated hens' eggs to grow vaccinia virus, the Rockefeller scientists prepared clinical grade 17D vaccine from infected chick embryos macerated with normal human serum to stabilize the virus. Bacterial sterility was checked and potency testing performed by intracerebral inoculation of mice. Following the great traditions of early research on infectious diseases, the first two human subjects to be vaccinated were Theiler and Smith themselves, low-risk endeavors since both were immune: Theiler by virtue of an accidental laboratory infection while at Harvard in 1929, and Smith by immunization with the French neurotropic virus plus immune goat serum. After an additional two immune subjects were successfully vaccinated, five nonimmune individuals were given high doses of the attenuated virus. Despite a few minor febrile reactions, the product was well tolerated, and all subjects had increases in serum protective neutralizing antibody levels. Theiler and Smith had reached a critical milestone in yellow fever vaccine research.

Theiler unsuccessfully attempted to reverse the attenuated properties of 17D virus by passage in chick embryo brain (Theiler 1951), suggesting its phenotypic stability when maintained in this tissue. However, he did demonstrate the potential for its reversion to neurovirulence in monkeys following selective pressure in a mammalian host induced via sequential passages by intracerebral inoculation of

mice. Stabilizing the passage level of 17D during manufacture would later be recognized as a key requirement for maintaining viral phenotype. In this pre-molecular era, viral genotypic changes upon passage in different hosts could only be studied indirectly by following biological properties such as virulence. Despite attempts to reproduce the passage series that resulted in 17D's attenuation of viscerotropism and neurotropism,<sup>3</sup> Theiler could not explain the changes that occurred between the original passages 89 and 176. In general, virus attenuation by serial passage resulted in quite unpredictable changes in biological phenotype. The development of the 17D strain resulted from the systematic and meticulous application of the empirical process and keen, continuous observation by prepared minds. But they also had been extraordinarily lucky.

## 10.8 Field Trials in Brazil

The favorable results from preliminary clinical testing of 17D in New York were followed by its rapid transition into practical use in a country where yellow fever threatened public health – Brazil. Within 1 month of his arrival in Rio de Janeiro in January 1937 and despite ongoing efforts with the insufficiently attenuated 17E strain elsewhere in country, Smith initiated small-scale human trials. He first demonstrated tolerability and immunogenicity of the 17D vaccine in 24 non-immune mosquito control workers, although transient viremia was documented in about half the subjects (Smith et al. 1938). With Henrique de Azavedo Penna he established vaccine production capabilities in the local yellow fever laboratory, performed additional controlled immunizations, and within 6 months, field trials of the vaccine were broadened.

The selected site for vaccine evaluation was at Varginha in Mineas Gerais state in southeast Brazil, where coffee plantation workers were known to be at high risk of yellow fever. More than 2,800 subjects had been vaccinated by August 1937, without significant adverse reactions; by year's end 38,077 individuals had received the 17D vaccine. The vaccine appeared to be safe and immunogenic, with more than 95% of tested subjects developing a neutralizing antibody response. During that time, methods for vaccine manufacture in eggs were refined, production was scaled up at the Oswaldo Cruz Foundation laboratory in Rio de Janeiro,<sup>4</sup> and 17D vaccine was used to combat an outbreak.

A proposal by epidemiologist Fred Soper that the vaccine be used in a nationwide campaign to immunize millions against yellow fever elicited an accusation by

<sup>3</sup>Unfortunately, Theiler and Smith's passage series was not preserved and is not available for genetic studies, which would undoubtedly have shown which mutations were responsible for the phenotypic changes observed.

<sup>4</sup>In 1976 Bio-Manguinhos (Laboratorio de Technologia em Productos Biologicos de Manguinhos) was created as the manufacturing arm of the Oswaldo Cruz Foundation.

the famous entomologist Costa Lima of the Oswaldo Cruz Institute that the Americans were using Brazilians as human guinea pigs. He postulated that 17D-induced viremia could lead to infection of *Ae. aegypti* or jungle mosquito vectors and subsequent strain reversion to viscerotropism (Costa Lima 1938), triggering studies at Rockefeller that proved that 17D virus was incapable of oral infection of and transmission by *Ae. aegypti* (Whitman 1939). Soper's view prevailed and by the end of 1938, nearly one million Brazilians had received 17D vaccine (Soper and Smith 1938). A year later local manufacturing of the relatively inexpensive vaccine, estimated at \$0.10 per dose (equivalent to \$1.49 in 2008) including vaccine, vaccination paraphernalia, and the complicated cold chain required for field use, was initiated (Benchimol 2001).

## 10.9 Neurologic Accidents Caused by 17D

Several mishaps had occurred during the early use of the 17D vaccine in approximately two million individuals in South America, the U.S., England, and West Africa. Certain vaccine lots produced in Brazil in 1938–1939 were poorly antigenic, producing only 20% seroconversion rates. A 1940 outbreak of jaundice in Brazil was associated with 17D lots stabilized with normal human serum that had not been heat inactivated. In 1941 a Brazilian physician working in Mineas Gerais state noted more than 20 cases of encephalitis temporally associated with vaccination. An epidemiological investigation by the Yellow Fever Research Service, a joint enterprise of the Brazilian Ministry of Health and the Rockefeller Foundation, identified 273 (0.5%) unusually severe reactions, 199 (0.36%) complications involving the central nervous system, and one death with encephalitis among 55,073 vaccinees. Similar disease in the unvaccinated population was significantly less frequent. The highest incidence of encephalitis occurred in individuals who had received vaccine prepared from the NY104 substrain of 17D (Fox et al. 1942a).

It was apparent that the 17D vaccine, particularly certain lots, had the potential to cause post-vaccinal encephalitis (now called yellow fever vaccine associated neurotropic adverse events, YEL-AND). This signaled a change in the characteristics of the vaccine virus and was unexpected in the light of Theiler's difficulty in reverting 17D to neurovirulence by serial passage in chick embryo brain tissue. An extensive investigation of manufacturing procedures disclosed the use of various independent viral substrains, some more neurovirulent than others, with no controls on passage level in the preparation of vaccine batches (Fox and Penna 1943). Potential differences in neurovirulence had been disregarded for vaccine release based on three presumptions: the test dose inoculated intracerebrally in monkeys was high in relation to the dose given to humans by the subcutaneous route; it was believed viremia following subcutaneous inoculation in humans was insufficient to invade the brain; and no previous neurological adverse events had been noted during the field trials conducted prior to 1941.

It was now clear that even a small number of uncontrolled passages of yellow fever vaccine could have selected for variants or caused mutations that increased its

neurovirulence. Conversely, other contemporary studies showed that poor manufacturing controls led to the over-attenuation of some batches and lower, attendant seroconversion rates, causing primary vaccine failures and explaining the 1938 mishap (Fox et al. 1943). In response, workers at the Oswaldo Cruz Institute devised the “seed lot system” to stabilize the passage level and thus the characteristics of vaccine lots during 17D vaccine production. The seed lot system is now used to manufacture and control all live viral vaccines. The requirements for 17D vaccine were described in an international regulatory document in 1945 (United Nations Relief and Rehabilitation Administration 1945), but because implementation by vaccine manufacturers was not universal until a decade later, further neurological accidents occurred in conjunction with uncontrolled viral passage (Panthier 1956), leading the World Health Organization (WHO) to further refine the biological standards for vaccine production (WHO 1957).

The monkey neurovirulence test, first applied to the 17D vaccine, became a regulatory requirement for testing seed lots of all live viral vaccines developed subsequently.<sup>5</sup> It is noteworthy that the vaccine-related neurological accidents of 1941 in Brazil, their exhaustive public investigation, and the conclusions regarding virus-specific changes in neurovirulence did not deter French researchers who were introducing a more dangerous product – the French neurotropic vaccine – into widespread use despite reports of post-vaccinal encephalitis in field trials. This may be ascribed in part to the concurrent use of smallpox vaccine, a well known cause of the latter syndrome, in the French product.

After the institution of the seed lot system for manufacturing 17D vaccine in 1945, no cases of post-vaccinal encephalitis were reported for approximately 10 years (Stefanopoulo and Duvolon 1947; Stuart 1956). In 1952–1953, five cases occurred among 1,800 infants vaccinated at the Institut Pasteur, Paris (Stefanopoulo and Duvolon 1947), demonstrating that despite adherence to biological standards in its manufacture, 17D may provoke neurological accidents in infants. Fifteen other cases occurred during the 1950s in infants less than 7 months of age, leading to age restrictions contraindicating vaccine use in those less than 6 months of age (CDC 1969). From 1960 to 1991, only six cases of post-vaccinal encephalitis were reported, one of which occurred in a neonate in France, at a time when the age limitation was not universally practiced (Louis et al. 1981).<sup>6</sup>

<sup>5</sup> In 1945 the monkey neurovirulence test was introduced as a requirement for the control of yellow fever 17D vaccine. The monkey test for neurovirulence itself has undergone a number of refinements over the years. The original method involved only clinical observations of the animals. In the 1960s improved methods were developed by Nathanson et al. (Am J Epidemiol 1965; 82:359–381 and 1966; 84:524–540) who identified indicator centers (nuclei) in the brain that were sensitive to differences in virulence. In 1987, the methods were further developed by Levenbook et al. (J Biol Stand 1987; 15:305–313). They described an improved clinical scoring system and more discriminatory histopathological assessment of inflammation and neuronal damage.

<sup>6</sup> From 1990, when surveillance for vaccine associated adverse events was instituted in the US, through March 2006, a total of 29 additional cases of YEL-AND were reported worldwide. The cases since 1990 have been predominantly in adults (including elderly). Advanced age is now recognized as a risk factor. The incidence of YEL-AND is approximately 1 in 300,000 and the case fatality rate is probably 1–2%.

## 10.10 The French Neurotropic Vaccine in West Africa: 1942–1953

Based on the initial success of field trials with the French neurotropic vaccine in Francophone colonies in West Africa between 1937 and 1940, vaccination was made compulsory there in 1941. Over the next 6 years, approximately 14 million inhabitants of this vast, endemic area received the French vaccine (Peltier 1947) and by 1953, 56 million vaccinations had been performed, a number twice that of the region's population (Durieux 1956). This remarkable feat was possible because of the efficiency of the colonial health services, the simplicity of delivery by scarification, the thermostability of the vaccine under field conditions without refrigeration, and the low cost of manufacture of mouse brain vaccine – approximately 1,000 doses could be produced from a single mouse – at the Institut Pasteur, Dakar.

Following wide-scale vaccination, the incidence of yellow fever in the Francophone territory declined rapidly as the prevalence of immunity in the population grew from approximately 20% in surveys before compulsory vaccination to 86% in 1952–1953 (Bonnel and Deutschman 1957). Studies in selected villages indicated that vaccine-induced immunity was durable (Peltier 1947). “Natural controls” for the vaccination campaign were the adjacent English colonies of the Gold Coast (Ghana), Nigeria, and Sierra Leone where vaccinations were not practiced widely and where yellow fever epidemics, such as a large one in Nigeria in the early 1950s occurred at regular intervals, further highlighting the successes in French West Africa.

While American and English workers were reluctant to utilize the Dakar vaccine due to safety concerns, severe epidemics in eastern Nigeria and in Panama and Central America in the early 1950s presented formidable challenges regarding the deployment of thermolabile 17D virus and prompted emergency use of the French vaccine (Elton 1952; MacNamara 1953). The subsequent incidence of post-vaccinal encephalitis in Nigerian children was high – 3–4% – and associated with a high case-fatality rate (40%), indicating that cases of milder encephalitis were probably missed and serving to reinforce the conviction of public health authorities in Anglophone countries that the French vaccine was unsafe (MacNamara 1953).

Increasing recognition of central nervous system complications of the French vaccine led to a cessation of its routine use in children younger than 10 years of age (Bres et al. 1963) and to a major research effort to adapt the 17D vaccine for use in the tropics. Independence of the African colonies from France in 1960 compounded the change in vaccine policy; the dismantling of colonial administrations with their attendant infrastructure for compulsory vaccination negatively impacted immunization practices, resulting in the rapid accumulation of a non-immune population. Five years after the cessation of routine immunization, Senegal, which had not suffered a yellow fever outbreak since 1937, experienced one of the largest epidemics on record in Diourbel, east of Dakar, in 1965 (Chambon and Wone 1967). In response, mass vaccination of individuals 2 years and older was performed using the French vaccine, with the 17D vaccine, available in limited supply, used

to immunize younger children (Sankale et al. 1966). Among 498,887 persons vaccinated with the former, there were 231 documented cases of encephalitis, more than 90% in children aged two to eleven years, associated with a case fatality rate of 10% (Rey et al. 1966).

These data confirmed the need for a safer approach to yellow fever immunization, especially given the neurotropic vaccine's poor safety profile in children, its most important target population. In 1966, the Institut Pasteur, Dakar, with assistance from the WHO, established an expanded facility for the production of the 17D vaccine in eggs; shortly thereafter, a policy was established for its use in all persons less than 15 years of age (Ricosse and Albert 1971). By 1970, the distribution of 17D exceeded that of the French neurotropic vaccine and by 1982, production of the latter was discontinued altogether, closing an important chapter in yellow fever vaccinology. Although the French vaccine had saved many lives, it had taken a number too. As new methods of rapid, mass immunization were developed, such as the jet injector which reduced the relative value of scarification, and improvements made in the cold chain required for transport of the 17D virus, mouse brain vaccines were abandoned in their favor.

## 10.11 Adventitious Contamination of Yellow Fever Vaccines

Manufacture of 17D vaccine used pooled, heat inactivated and filtered human serum from yellow fever-naïve donors as a stabilizer during lyophilization. Cases of acute hepatitis, occurring 2–7 months after vaccination, were noted as early as 1937 (Findlay and MacCallum 1937); similar cases were reported in association with 17E serovaccination in Brazil (Benchimol 2001). The transmission of hepatitis by human blood had been described as early as 1885, involving smallpox vaccine prepared from human lymph (Lurman 1885); in the 1930s, outbreaks of jaundice were observed among recipients of human measles and mumps convalescent plasma (Anonymous 1937). Investigation into the occurrence of jaundice associated with specific lots of 17D vaccine used in Brazil in 1939–1940 excluded reversion to virulence of the vaccine virus (Fox et al. 1942b) and focused instead on an agent introduced with the normal human serum used in vaccine preparation. Thus, human serum was eliminated from 17D vaccine produced in Brazil (Smithburn 1956; Benchimol 2001).

Although little 17D vaccine was made in the U.S. before the 1940s due to its limited demand, manufacturing was dramatically scaled up at the Rockefeller Institute in anticipation of troop deployments with the advent of World War II. Ignoring the warnings about postvaccinal jaundice issuing from Brazil and under the mistaken notion that quality problems were limited to local manufacturing there (Benchimol 2001), pooled human serum was used as a stabilizer. In 1942, a massive outbreak of jaundice – 28,000 cases – occurred among U.S. military personnel vaccinated with domestically produced 17D; 62 deaths occurred in patients with fulminant hepatitis (Sawyer et al. 1944). Investigation of this outbreak, coordinated

by the Commission on Tropical Diseases of the Armed Forces Epidemiological Board under the direction of Wilbur Sawyer, confirmed the data from Brazil, and 17D was henceforward manufactured without serum in the U.S. (Hargett et al. 1943) with no further cases identified. In a later retrospective, serological study using stored specimens, hepatitis B virus was determined to be the etiologic agent of the outbreak (Seeff et al. 1987).

In 1966, secondary seeds lot of 17D, as well as measles vaccines produced in the U.K. were found to be contaminated with Rous sarcoma virus (Harris et al. 1966). Contamination of egg-based vaccines with adventitious agents had been well documented; simian virus 40 (SV40), another potentially oncogenic virus, had been previously discovered as a contaminant of primary rhesus monkey kidney cells used to produce polio vaccine (refer to Chap. 12) and would contemporaneously contaminate adenovirus vaccine seed lots (refer to Chap. 14). Avian leukosis viruses such as Rous sarcoma virus were known to be highly prevalent in commercial chicken flocks used to supply eggs for vaccine manufacture (Rubin et al. 1962), and 17D vaccines were confirmed to be contaminated with high titers (Piraino et al. 1967).

Human risk related to avian leukosis viruses remains unknown, although their oncogenic potential appropriately raised concerns in the 1960s. Despite the absence of antibody responses against these agents in recipients of egg-based vaccines, including 17D (Piraino et al. 1967; Richman et al. 1972), and the failure to demonstrate an increased risk of leukemia or other malignancies in veterans who had received the latter (Melnick 1968), the first avian leukosis virus-free vaccines were prepared in the late 1960s by the passage of seed lots in the presence of antibody to the adventitious agent (Draper 1967; Tauraso et al. 1968). All major manufacturers now use eggs from Special Pathogen Free (SPF) flocks.<sup>7</sup>

## 10.12 Vaccine Characteristics

Minimum vaccine potency specifications (United Nations Relief and Rehabilitation Administration 1945), 1,000 MLD<sub>50</sub> (50% lethal doses in mice), represented a significant excess of virus to allow for potency throughout a 24 month shelf life at storage temperatures of 2–8°C and to account for losses of virus during use in the field. Dose-response studies in humans confirmed that the vast majority of vaccinees seroconverted with these specifications (Fox et al. 1943). But 17D vaccine use in tropical countries was initially limited by its poor thermostability, both in the lyophilized state and after reconstitution.

Yellow fever vaccines produced without human serum as a stabilizer rapidly lost potency when exposed to temperatures in excess of 20°C (Burros and Hargett 1947),

<sup>7</sup> Nevertheless, vaccines manufactured in eggs, including yellow fever 17D, test positive by the product-enhanced reverse transcriptase (PERT) assay, reflecting the presence of defective particles containing endogenous avian leucosis or retrovirus sequences. No evidence has been found for infectious or inducible replication-competent retroviruses or for infection with avian leucosis or endogenous avian retrovirus in humans.

a major obstacle in the field, especially in areas without an established cold chain, such as Africa. Through the 1970s and 1980s, different formulations of stabilizing agents were systematically investigated to reduce losses of virus titer during both lyophilization and storage of dried vaccine (Robin et al. 1971; Burfoot et al. 1977; Barme and Bronnert 1984; WHO 1987), resulting in revised international thermo-stability requirements of 17D vaccine lots (WHO 1988).

Although never formally tested in a controlled fashion, the efficacy of 17D yellow fever vaccine, as measured by protection against disease, is accepted based on many years of experience. A large body of pre-clinical data in nonhuman primates demonstrated its protective activity against lethal challenge with wild-type virus (Smithburn 1956; Mason et al. 1973) and showed that neutralizing antibodies, detectable within 7 days (Smithburn 1956), are strongly correlated with protection, the latter also demonstrated by passive transfer studies before or shortly after challenge with virulent virus (Bauer 1931; Davis 1934). A significant level of protection appears to be present 1–2 days before the appearance of detectable neutralizing antibodies (Smithburn and Mahaffy 1945; Smithburn 1956). Human 17D immunization is also followed by the rapid appearance of neutralizing antibodies (Courtois 1956). Their minimum protective level was established in the 1970s as a  $\log_{10}$  neutralization index of 0.7 (Mason et al. 1973),<sup>8</sup> a level later accepted as an immune correlate of protection and used as the cut-off to evaluate seroconversion in modern vaccine trials (Monath et al. 2002), in which the effectiveness of the 17D vaccine-based neutralizing antibody response was 99% in adults vaccinated in the U.S. (Monath et al. 2002) and more than 95% in infants vaccinated in Peru (Belmusto-Worn et al. 2005).

Epidemiologic observations attest to the vaccine's effectiveness. Laboratory infections with yellow fever were commonplace prior to routine immunization but disappeared thereafter. Additionally, observations over 50 years show that jungle yellow fever in South American countries occurs only in unimmunized persons and that immunization during outbreaks results in rapid disappearance of cases (Soper 1938). Finally, yellow fever in Francophone Africa virtually disappeared after the institution of mandatory immunization with the French vaccine, despite continued human exposure to the enzootic cycle and epidemics in neighboring Anglophone countries in which routine vaccination was not practiced.

According to the International Health Regulations, the yellow fever immunization certificate for international travel is valid for 10 years, but actual vaccine immunity appears to last several decades if not for life. The 10-year revaccination requirement derives from studies showing that neutralizing antibodies were present in 92–97% of individuals up to 19 years after vaccination (Groot and Riberiro 1962; Rosenzweig et al. 1963). However, a more recent analysis of Navy and Air Corps veterans, 30–35 years after vaccination with 17D revealed persistent seropositivity in 97% (Poland et al. 1981).

<sup>8</sup>Determined in a constant serum-virus dilution test, the  $\log_{10}$  neutralization index being the difference in  $\log_{10}$  titer of virus with and without serum containing neutralizing antibody.

Yellow fever 17D vaccine was long considered to be among the safest live vaccines; the neurotropic adverse events were rare, confined largely to infants, and the safety profile further improved when vaccination of infants younger than 6 months of age was terminated in 1960<sup>9</sup>. However, in 2001 seven cases - six of them fatal - of multi-organ system failure were described in association with vaccines manufactured in Brazil (17DD substrain), France, and the U.S. (17D-204 substrain) (CDC 2001; Chan et al. 2001; Martin et al. 2001; Vasconcelos et al. 2001). The signs and symptoms of the syndrome resembled those of wild-type yellow fever: rapid onset of high fever, malaise, and myalgias within 2–5 days of vaccination followed by jaundice, oliguria, cardiovascular instability, and diffuse hemorrhage. Large amounts of 17D viral antigens were present in liver, heart, and other affected organs. A retrospective analysis of viral isolates from patients in Brazil suggested that the occurrence of vaccine-associated viscerotropic adverse events had been occurring for more than 30 years but went unrecognized as such, attributed instead to natural infection (Engel et al. 2006).

By 2008, 40 case reports of vaccine-associated viscerotropic adverse events (YEL-AVD) had been reported, caused by both 17D and 17DD vaccines manufactured in multiple countries, with a case fatality rate of 50% (Monath et al. 2007; Lindsey et al. 2008). Several risk factors were identified, most notably advanced age and a history of thymectomy, implicating acquired immunological deficits in the pathogenesis of this overwhelming infection due to a highly attenuated virus. However, this does not readily explain the occurrence of cases in young, otherwise healthy vaccinees, and it is suspected that inherited susceptibility factors also play an important role. The incidence of YEL-AVD has been estimated to be approximately 0.4 per 100,000 (Monath et al. 2007; Lindsey et al. 2008), although a recent mass immunization campaign in Peru was associated with an unexplained incidence of 7.9 per 100,000 (Pan American Health Organization 2008).

That such an unforeseen, severe adverse event would come to light after such a long track record of 17D vaccine use serves as a reminder that biological organisms frequently challenge dogmas and historically accepted assumptions. Multiple, as yet unproven hypotheses for the recent recognition of viscerotropic disease have been promulgated, including the loss of general “sero-immunization” protection related to the cessation of immune serum globulin use for hepatitis A prophylaxis (Kaplan et al. 1984); more sensitive surveillance; and increasing immunization of persons at risk, particularly the elderly. Genomic analyses, including those involving the Peruvian cases, confirmed that neither the 17D and 17DD lots used for vaccination nor the viral sequences isolated from affected organs contained mutations consistent with a reversion to virulence; phenotypic studies in monkeys confirmed that the virus isolates were nonpathogenic (Galler et al. 2001; Lindsey et al. 2008). Thus, host factors, both acquired and inherited, are believed to be responsible for the increased susceptibility to the vaccine virus.

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<sup>9</sup>The age cut-off was later changed to 9 months, which is the age at which infants are routinely immunized in the Expanded Programme for Immunization in endemic countries.

## 10.13 Current Status and Lessons Learned

Yellow fever remains an epidemic threat where vaccine coverage is low, and there is the potential for introduction outside of endemic areas to regions with high human population density that are permissive to the urban vector *Ae. aegypti*, such as coastal regions of South America, the Caribbean, and areas of Africa comprising large urban centers. Approximately sixty million doses of 17D vaccine are used annually, the vast majority for routine immunization in endemic countries and mass campaigns for catch-up, revaccination, and epidemic control. Much smaller numbers are used for the immunization of travelers and military – in the U.S., about 300,000 doses annually.

Yellow fever immunization has been implemented for decades, with high coverage rates, in all eleven countries with endemic yellow fever in South America, with vaccination of infants now a component of the WHO Expanded Programme of Immunization (EPI). By 1998, half of the 33 African countries endemic for yellow fever, a combined population of 508 million, had incorporated vaccine into the EPI, although coverage remained low. The WHO Yellow Fever Initiative was started in 2006 to achieve higher coverage rates in West and Central Africa.

The story of yellow fever vaccine development is remarkable, in part, because it spans multiple eras of vaccine history. Its beginning, in the late nineteenth century, coincided with a time when concepts of vaccination, driven by revelations in microbiology and immunology, were evolving rapidly. Because of the medical and public health impact of yellow fever, the public and the scientific community were impelled to embrace potential preventive measures, some of which proved ineffective or dangerous. During the process, a number of lessons were learned that have become part of vaccine liturgy: the importance of controlled safety evaluations; quantitative potency assays; thermostability assessments, especially for diseases of tropical climates; standardization and control of viral passages during manufacture; sensitive markers of toxicity, both neuro- and viscerotropism; control of adventitious viruses; and surveillance for rare adverse events.

The future of yellow fever vaccine commenced with the elucidation of the complete consensus nucleotide sequence and genomic structure of the 17D virus in 1985 (Rice et al. 1985), followed shortly thereafter by the nucleotide sequence of the parental Asibi strain, which revealed a large number of nucleotide changes resulting in 32 amino acid differences between the two viruses (Hahn et al. 1987). A decade later description of the three-dimensional crystallographic structure of the flaviviral envelope protein (Rey et al. 1995) allowed the localization and structural correlation of mutations suspected to underlie the attenuation of the Asibi virus in the 17D vaccine, generating hypotheses that could be tested using infectious clone technology and site directed mutagenesis. Molecular technology also made it feasible to harness the immunogenicity of the 17D virus as a live vector of foreign genes (Rice et al. 1989), an approach used to fashion vaccines against cancer, malaria epitopes, and, most importantly, envelope proteins of heterologous flaviviruses, including dengue, Japanese encephalitis, and West Nile

(Lai and Monath 2003; Pugachev et al. 2008). Key figures involved in the development of 17D as a vector for novel, live, attenuated, chimeric vaccines have included Charles Rice, Thomas Chambers, Konstantin Pugachev, Farshad Guirakhoo, and the author. The biotechnology company, Acambis plc, developed the chimeric products; Sanofi Pasteur, who acquired Acambis in 2008, is in the process of commercializing them.

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# Chapter 11

## Influenza

Andrew W. Artenstein

### 11.1 A Brief History of Influenza and Early Virology

In attempting to understand the history of specific infectious diseases, it is appropriate to remember the aphorism that “the credibility of historical records is, in general, inversely proportional to their antiquity” (Kilbourne 1987). Because of the nonspecific respiratory symptoms and lack of pathognomonic features of influenza, it is exceedingly difficult to discern the history of this disease against the background of other common communicable maladies that impacted humans prior to the advent of modern scientific methods.

Influenza was likely present in ancient civilizations, probably resulting from the close proximity of early humans with domesticated animal reservoirs. As urbanization evolved, epidemics of acute respiratory disease consistent with classic clinical descriptions of influenza appear to have occurred in frequent, irregular intervals and with varying severity in humans from the sixteenth through the nineteenth centuries (Wright and Webster 2001; Cunha 2004). In many cases, historical accounts note contemporaneous outbreaks of acute respiratory tract diseases in livestock (Kilbourne 1987), a feature that would prove to be of vital importance to understanding the epidemiologic dynamics of influenza and the interdependence of various animal species in viral pathogenesis.

Classically, influenza has been defined by its sudden onset of constitutional symptoms which include a prominent, “violent” cough (Major 1932). It was undoubtedly one of the causes of “catarrhal fevers”, implicated in numerous human epidemics over the past 700 years (Kilbourne 1987). However, it was not until the early twentieth century that epidemiological data and clinical descriptions were documented completely enough to allow for the systematic study of influenza outbreaks. Explanations for the seasonal recurrence of multifocal influenza outbreaks of varying severity and the periodic occurrence of global outbreaks – pandemics – would have to await

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**Fig. 11.1** Richard Shope  
(Albert and Mary Lasker  
Foundation)



additional advances in influenza virology, discoveries that would derive from the study of diseases of animals.

Influenza, a member of the orthomyxovirus family, was first isolated from a mammalian host in 1931 by Richard E. Shope (1901–1966) working with swine specimens obtained while investigating an epizootic outbreak of hog cholera in Iowa in 1928 (Shope 1931a). Shope (Fig. 11.1), a research physician working in the Department of Animal Pathology at The Rockefeller Institute's Princeton, New Jersey laboratories, had already established himself as an innovative, young virologist with his elegant studies describing the filterable agent of “mad itch” disease in cattle, its transmissible nature and host range, and its identity to pseudorabies (Shope 1931b). He would later describe the etiologic relationship between myxoma viruses, fibromas, and papillomas in wild cottontail rabbits (Corner 1964; Andrewes 1979), a major contribution to the pathogenesis of cancer and one that bolstered the controversial theories of another Rockefeller Institute scientist, Peyton Rous (refer to Chap. 9). Shope came by animal research honestly, being reared the son of a respected physician-farmer in Iowa; his experiences growing up on the family farm informed his knowledge of animal diseases, his scientific independence, and his love of nature (Andrewes 1979).

A prevailing, albeit disputed, hypothesis of the early twentieth century was that influenza was caused by a bacterium. A fastidious organism, *Bacillus influenzae*, had been isolated from nasal secretions of infected patients by the German bacteriologist Richard Pfeiffer in 1892 while working under Robert Koch in Berlin (Brock 1999; Van Epps 2006). Because of the extant supremacy of Koch’s recently described postulates at that time, it was accepted as the etiologic agent of human influenza

(van Helvoort 1993). Shope, expanding on the inconclusive findings of others at The Rockefeller Institute and some at the United States Department of Agriculture laboratories immersed in the burgeoning discipline of filterable agents of the 1920s, embarked on a series of investigations that would definitively address the etiology and microbiologic characteristics of swine influenza and have the unintended effect of leading directly to effective human vaccines.

In his laboratory in Princeton, Shope induced the clinical syndrome of swine influenza in healthy pigs through the intranasal instillation of respiratory secretions with or without suspensions of infected lung and lymph node tissue; demonstrated the maintenance of infection in serial passages in animals (Shope 1931c); and with his laboratory chief, Paul Lewis, isolated *Hemophilus influenzae suis*, a bacterium similar to the Pfeiffer bacillus, from the respiratory tracts of infected pigs (Lewis and Shope 1931). However, animals inoculated intranasally with pure cultures of this organism developed neither clinical nor pathologic signs of influenza, thus failing to fulfill Koch's postulates of causality (Shope 1931d). Additional experiments revealed a filterable agent that induced only a mild, in some cases subclinical syndrome in the animals characterized by apathy, anorexia, and leukopenia, not typical for swine influenza but with the stereotypical pathologic changes and transmissibility characteristics of the disease (Shope 1931d). When the filtrate was inoculated in combination with *H. influenzae suis* the hogs developed the classic clinical features and pathologic lesions of swine influenza (Shope 1931d).

From his experimental findings, Shope deduced that the two agents somehow acted synergistically to produce the severe infection in swine. He appeared to favor the hypothesis that the virus damaged the respiratory tract of the host creating a milieu in which the bacterium could exert further pathogenic effects (Shope 1931d). Perhaps most important, however, was the recognition of the similarities between the clinical and pathological presentations of epidemic influenza in humans and swine.

The implications of his work became apparent in short order. In 1933, Christopher Andrewes, Wilson Smith, and Patrick Laidlaw, from the British National Institute for Medical Research, reported the isolation of a virus from filtrate of throat washings derived from infected humans using Shope's methods (Smith et al. 1933). Laidlaw and the veterinary surgeon George Dunkin had begun work on animal distemper disease at the National Institute at Mill Hill in 1923, funded through the donations of concerned dog owners (Parish 1965). They had isolated the virus and developed an effective vaccine. In 1932, Laidlaw's team was actively engaged in further characterization of its distemper vaccine when serendipity intervened (Carver and Skehel 2000). While investigating the immune responses to the vaccine among ferrets, a host particularly susceptible to distemper virus, the scientists had noted that some of their animals were exhibiting similar symptoms to those of the research personnel who were ill with influenza during a seasonal outbreak and with whom they were in close contact. Following healthy animal challenge with the filtrates containing human influenza virus, Laidlaw noted that "the ferrets were sneezing" (Fenner 1985); this seemingly innocuous observation not only signaled the successful isolation and transmission of influenza virus from humans but also heralded the ferret as a preferred animal model for its study.

## 11.2 Towards a Vaccine

On the heels of the British scientists' discovery, investigators from multiple other continents produced confirmatory results (van Helvoort 1993). With the viral etiology of human influenza firmly established, a series of incremental yet critical advances followed in relatively rapid succession: the detection of serum neutralizing antibody and its utility as a diagnostic assay as well as a protective marker in animals; the identification of the antigenically distinct influenza virus B (Francis 1940), as the original 1933 isolate later became known as influenza A; and the recognition that influenza viruses induced agglutination of red blood cells (Hirst 1941). The latter finding led to the description of the surface proteins hemagglutinin (H) and neuraminidase (N), providing accessible markers for the study of influenza virus both *in vitro* and *in vivo* and leading to a more complete understanding of the biological behavior of the pathogen.

The recognition of antigenic variation among influenza viruses proved to be a significant development, as it illuminated the biological basis for the epidemiologic observation of recurrent epidemic and pandemic disease and elucidated considerations of importance to the development of vaccines. Genetic mutations in the two viral surface proteins underlie the phenomena of antigenic drift and antigenic shift. In the former process, point mutations within the genes encoding the H and N glycoproteins accumulate over time, causing the virus to vary enough from previous strains to escape from the human immune system's protective repertoire (Kilbourne 1996). These relatively minor variations account for seasonal recurrences of epidemic influenza. The process of antigenic shift involves major genetic variations in the surface glycoproteins resulting from replacement of gene segments either by reassortment between avian and human viruses or by direct transfer of an animal influenza virus to humans (Wright and Webster 2001). Antigenic shift essentially creates a novel virus relative to immune recognition and has the potential to result in pandemic disease.

In addition to its frequent recurrences as epidemics throughout history, influenza was recognized for its propensity to periodically recur in pandemic form. Although influenza pandemics were likely to have occurred prior to the twentieth century, these preceded the modern era of microbiology and immunology; hence, there are scant scientific data from these reports (Potter 2001). The pandemic of 1889–1890 was severe and may have presaged the 1918 pandemic (Shope 1958). The influenza pandemic of 1918 (Fig. 11.2) though was the first that could be subjected to careful clinical and scientific scrutiny, although not until the revelations of Shope and others more than a decade later. Occurring amidst the backdrop of World War I, an epidemiologic event that was a clear determinant of its transmission dynamics, the 1918 pandemic had resulted in global devastation of a pace and scale never before witnessed in human history – more than 50 million people dead of influenza worldwide over a period of less than 1 year (Taubenberger and Morens 2006).

As basic understanding of influenza viruses evolved rapidly after their isolation in 1933, laboratory techniques were contemporaneously introduced that paved the



**Fig. 11.2** Makeshift influenza ward, Army hospital, 1918 (Otis Historical Archives, National Museum of Health and Medicine, AFIP, Washington DC)

way for the development of vaccines against these pathogens. In the early 1930s, Goodpasture reported the successful cultivation of fowlpox virus and vaccinia in the chorioallantoic membranes of chick embryos (refer to Chap. 9). A young Australian physician, Frank Macfarlane Burnet (1899–1985), had been working in Andrewes' laboratory at the British National Institute for Medical Research attempting to extend Goodpasture's technique to other viruses at the time that influenza was initially isolated there from ferrets. Upon returning to his own laboratory at the Hall Institute in Melbourne, Burnet (Fig. 11.3) began working on influenza, culminating in the successful cultivation of influenza A virus in the amniotic sacs of chick embryos in 1940 (Burnet 1941, 1973).

The cultivation of influenza in chick embryos facilitated investigations into its biology and made it possible to produce large amounts of virus for the development of diagnostic assays and vaccines. Burnet's career would have been remarkable had it been solely based on his discoveries in microbiology; he made important contributions to the understanding of bacteriophages and microbial genetics, animal viruses, laboratory microbiology, Q fever, and of course, influenza (Fenner 1985). However, at age 57, he made a deliberate decision to completely shift the focus of his efforts to immunology, a prescient strategy that led to his formulation of the

**Fig. 11.3** Sir F. MacFarlane Burnet  
(Albert and Mary Lasker Foundation)



clonal selection theory to correctly explain antibody diversity (refer to Chap. 3) and to the Nobel Prize he shared with Peter Medawar in 1960 for the discovery of the phenomenon of immunologic tolerance.

### 11.3 The Commission on Influenza and Vaccine Development

Even before the events at Pearl Harbor in December of 1941 that precipitated U.S. entry into World War II, military planners expressed concerns regarding the potential re-emergence of pandemic influenza in association with the massive troop movements that were involved in a preamble to war. After all, the most recent and catastrophic global outbreak of 1918 was inextricably linked to war and disproportionately impacted military populations (Hoyt 2006). While influenza was not the only infectious disease that preoccupied American strategists, as fears of typhus, biological weapons, and other disease threats were also heightened, concern was such that the U.S. Secretary of War created a specific scientific advisory body in 1941 to address it – the Board for Investigation and Control of Influenza and Other Epidemic Diseases in the Army. By 1949 the entity had come to involve all military branches and became known as the Armed Forces Epidemiological Board (AFEB).

The AFEB and its 11 Commissions represented an innovative collaboration between the most accomplished civilian academic scientists of the time and military

researchers. The system was empowered to recommend, direct, and conduct basic science and applied research on infectious diseases of military importance, including acute respiratory diseases, meningococcal meningitis, enteric infections, and rickettsial diseases (Woodward 1994). Specifically, the AFEB administered the Commissions whose missions involved describing the pathophysiology of infections, delineating disease epidemiology, designing diagnostic assays, discovering novel treatments, and developing vaccines. During its more than 30-year existence, the AFEB made a number of major contributions of public health import. Among them, the first use of gamma globulin to prevent infectious hepatitis; the identification of adenoviruses as the major cause of acute respiratory disease in recruits, and the development of an effective vaccine for the two prominent serotypes; the development of vaccines to prevent pneumococcal disease; antimicrobial prophylaxis for rheumatic fever; and effective antimicrobial treatments for scrub typhus and typhoid fever (Woodward 1994).

The Commission on Influenza was a part of the initial AFEB charter in 1941 (Fig. 11.4) and its inaugural director was Thomas Francis Jr. (1900–1969). Francis (Fig. 11.5), a Yale-educated physician, had began working with influenza at the Rockefeller Institute in the 1930s where he isolated the virus, discovered antigenically distinct influenza subtypes, and performed early vaccine work (Woodward 1994). He would later become widely known for designing and executing the definitive, massive, national clinical trial of the inactivated polio vaccine developed by his former student, Jonas Salk in 1954. But in 1941, he was focused on another



**Fig. 11.4** Original members of the Board for the Investigation and Control of Influenza and Other Epidemic Diseases, 1942 (History of the Armed Forces Epidemiological Board, Borden Institute)

**Fig. 11.5** Thomas Francis Jr  
(Albert and Mary Lasker  
Foundation)



virus, and a primary goal of the Commission on Influenza under his leadership was to study candidate protective vaccines for this pathogen.

The development of an influenza vaccine was facilitated by the scientific knowledge accrued over the preceding decade: the etiologic agent of influenza had been isolated in Britain in 1933; Burnet had successfully cultivated the organism in chick embryos; other influenza types had been recognized; neutralizing antibodies had been identified and quantitated; and viral surface glycoproteins, H and N had been described. Thus, many of the key preliminary steps toward a vaccine had already occurred, in contrast to the circumstances that befell earlier viral vaccines, such as rabies, in which Pasteur's work was undertaken in the absence of an identified organism or established cultivation methods (refer to Chap. 5).

Investigations led by Francis while he was still at Rockefeller demonstrated that healthy medical students who were vaccinated with live, virulent influenza A virus grown in embryonated eggs developed serum neutralizing antibody titers and kinetics analogous to those of natural infection without clinical manifestations of disease (Francis and Magill 1937). However, because further studies using influenza B virus raised doubts about both the safety and efficacy of live vaccines, the Army Surgeon General directed that experimental work proceed only with a formalin-inactivated influenza vaccine (Meiklejohn 1994).

A persistent problem in the planning and execution of influenza vaccine field trials during the World War II years related to the unpredictable occurrence of epidemics and its impact on the determination of efficacy in vaccine trials; a threshold incidence of the disease under study was required in order to definitively

demonstrate efficacy of any vaccine. The state of knowledge in the early 1940s was that formalin-inactivated influenza vaccines demonstrated not only immunogenicity but also a significantly higher rate of local and systemic reactions than controls; their efficacy was not known at the time (Meiklejohn 1994).

A vaccine efficacy study, performed among personnel at the Army Student Training Program at Cornell University and other military posts across the country in October 1943 and begun, fortuitously, in advance of a large-scale influenza outbreak during that winter, resulted in the Commission's recommendation for mass vaccination within the military (Members of the Commission on Influenza, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army 1944; Eaton and Meiklejohn 1945; Salk et al. 1945). Among 12,474 subjects, half of who were vaccinated with an inactivated, bivalent influenza A and B product, efficacy was approximately 69% (Members of the Commission on Influenza, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army 1944), although serologic data confirmed that protection was inversely correlated with variation in the prevalent infecting strains, all of them influenza A, in different locations. Protection was therefore incomplete, with 140 cases of influenza in the vaccinated population. The reasons for breakthrough appeared to be multi-factorial and included low serologic responses in some vaccinated subjects and the timing of vaccination relative to the early appearance of influenza in some locations. The vaccine was generally well tolerated.

Over the next 18 months, various technical modifications to the bivalent inactivated influenza vaccine were introduced, culminating with the implementation in late 1945 of mass immunization within the U.S. Army. The incidence of influenza B, which had not been assessed during the vaccine efficacy trials because of the lack of viral circulation, began increasing concurrent with the start of the program; the vaccine was observed to protect immunized Army but not unvaccinated Navy personnel during that period (Meiklejohn 1994).

## 11.4 Immunology and Epidemiology Inform Vaccines

By the late 1940s it had become obvious that variants of influenza A virus, initially termed “A-prime,” with different biological and immunologic behaviors were becoming widespread; their emergence compromised the effectiveness of the inactivated vaccine (Kilbourne et al. 2002). Investigations by the Commission continued over the next two decades and contributed to a greater understanding, although not complete, of such strain variation, or antigenic drift, among influenza viruses and led to altered formulations of vaccine in order to optimize the correlation between vaccine antigens and circulating influenza viruses and thus, vaccine efficacy.

As knowledge of influenza accumulated in parallel with the maturation of modern virology in the mid-twentieth century, it became clear that the “A-prime” strain was a drifted variant of the 1918 strain of influenza. The H1N1 subtype designation was used to describe the specific, antigenic configuration of its surface glycoproteins

(Kilbourne 1987). On the basis of retrospective serotyping, the 1918 pandemic virus was also noted to be of the general H1N1 configuration. A contributing rationale for the explosive nature of the pandemic in 1918 lay with the revelation that at the time, the virus represented a novel antigenic form of influenza, one that had undergone antigenic shift, and with which the population was immunologically inexperienced and thus highly susceptible to infection. The outbreaks of “A-prime” influenza in 1946–1947 were also due to H1N1-type viruses, but variants that were antigenically distinct enough from their progenitor strains that immune recognition was only partial – providing sufficient cross-protection to avert a pandemic but rendering vaccination ineffective.

The year 1957 was marked by the appearance of a new pandemic – “Asian influenza” – that probably originated in southwestern China and rapidly spread throughout Asia, reaching North America within 3 months (Shope 1958). It was the first influenza pandemic to occur in concert with the availability of modern laboratory techniques for virologic analysis and was shown to be caused by a H2N2 subtype of virus, an antigenic variant not known to circulate in humans since the severe pandemic of 1889–1890 (Kilbourne 1987). Because the virus was again the result of antigenic shift, immunity in the population was low and confined largely to those who had lived through its earlier iteration; hence, morbidity and mortality were excessive, though much less so than in 1918. A similar circumstance was encountered in the 1968 “Hong Kong influenza” pandemic, due to a novel H3N2 subtype. In the latter case some level of partial immunity existed in the population due to ongoing circulation of the neuraminidase antigen (N2) type from the 1957 strain.

The mutability of the influenza viral genome with its attendant antigenic drift necessitates annual reassessments and reformulations of the influenza vaccine to approximate predicted circulating viral antigens during periods of seasonal, epidemic influenza. The use of inactivated vaccines comprising heterologous strains of similar subtype may compromise vaccine effectiveness, as was shown with the use of “Hong Kong influenza” vaccines in preventing outbreaks caused by a variant strain in 1972 (Hoskins et al. 1973; Stiver et al. 1973) and more recently in a controlled trial in which efficacy was no different than placebo, yet significantly different from the 86% level engendered by well-matched vaccine antigens (Bridges et al. 2000). However, this is not invariably the case, as “mismatched” vaccine antigens may nonetheless provide adequate levels of cross-reactive protection (Sugaya et al. 1994; Carrat and Flahault 2007). This vaccine dilemma acquires increased significance during pandemic periods, when a shift of viral antigens would be expected to abrogate cross-protective immunity to a substantial if not complete extent, as was hypothesized in the 1918 and 1957 pandemics and theoretically illustrated in the events in the U.S. in 1976.

A relationship between influenza in animals and influenza in humans was postulated based on early descriptions of disease epidemiology and was demonstrated in the laboratory by Shope’s investigations of the disease in swine in the early 1930s (Shope 1931a; Shope and Francis 1936). In the winter of 1976 13 army recruits undergoing basic training at Ft. Dix, New Jersey became infected with a novel strain of influenza A of the H1N1 genotype comprising H glycoproteins

derived from swine; one soldier died of viral pneumonia (Goldfield et al. 1977; Kendal et al. 1977). The H1N1 subtype of influenza had not been noted to circulate since the introduction during the 1957 pandemic of H2N2 strains, and this particular virus bore resemblance to that causing the 1918 pandemic, triggering widespread concern of another incipient pandemic. The origins of this novel virus remain speculative; hypotheses include a random occurrence of a zoonotic anomaly facilitated by the epidemiologic predisposition to influenza in a wintry, crowded, basic training environment, or a genetic reassortment event between circulating human influenza A viruses and animal viruses.

Despite the fact that detailed epidemiologic study found no evidence of exposure to pigs in the infected individuals and surveillance in the surrounding communities disclosed solely the expected H3N2 strains but no additional cases of “swine influenza” in humans (Dowdle and Hattwick 1977; Hodder et al. 1977), serologic testing suggested limited person-to-person spread at Ft. Dix, raising the specter of a potential pandemic strain. This prompted a recommendation from the U.S. Public Health Service for the mass immunization of susceptible, high risk individuals against the novel form of H1N1 influenza (Sencer and Millar 2006). What followed was a controversial federal program that vaccinated nearly 45 million Americans in a span of 10 weeks. By the end of the year, with no evidence of further transmission of the novel H1N1 virus, and with a possible association between vaccination and Guillain-Barré syndrome, an autoimmune neurological illness, the program was terminated.

Similar concerns to those prevalent among health officials three decades ago with swine influenza are again present in the context of novel avian influenza variants circulating in Asia since 1997 and novel H1N1 viruses circulating in humans throughout the world since early 2009. It has become evident that despite the relative effectiveness of current vaccines, they are suboptimal, providing adequate protection largely against strains closely matched to those included in the vaccine formulation or at the least to those of homologous subtype. Improved products are needed that furnish enhanced protection against variant strains of influenza, including pandemic ones.

## 11.5 Current Vaccines

Inactivated vaccines, introduced in the mid-1940s, continue to be the mainstay of seasonal, epidemic influenza prevention. Their Achilles’ heel, though, remains their inconsistent protective efficacy against variant or drifted strains. Additionally, due to production issues related to embryonated eggs, they are not felt to be a nimble platform for a rapid and efficient large-scale response to potential novel viruses that may pose a pandemic threat. In the former regard various maneuvers have been employed to improve the level and breadth of antigenicity (Hilleman 1969).

Surveillance systems designed for the early, global detection of novel strains may provide an opportunity to formulate vaccines based on these emergent, potential threats. The use of adjuvants to improve immunologic performance was initiated by Jonas Salk, later of polio vaccine fame, as a member of the Commission on

Influenza in the mid-1940s. He continued this line of investigation in the years immediately preceding his landmark work with polio, demonstrating the favorable effects of these immune enhancers on the magnitude and duration of antibody titers following vaccination and on the breadth of humoral immunity against heterologous strains (Salk et al. 1952a, b).

The use of reassortant viruses comprising genetic recombinations of wild-type, contemporary influenza isolates from humans with high-yield, laboratory-manipulated strains in current inactivated vaccines has improved immunogenicity (Kilbourne et al. 1971). Viral strains in modern inactivated vaccines are generally treated with organic solvents or detergent after purification in order to release the surface glycoproteins; these “split” vaccines are less reactogenic, especially in children, but are somewhat less immunogenic than whole inactivated virion vaccines in unprimed subjects (Kilbourne 1987). Current subunit vaccines consist of enriched H and N antigens and also represent less reactogenic forms of inactivated vaccines. In general split or subunit vaccines are 60–90% protective against viruses with similar antigenic characteristics.

Inactivated influenza vaccines, though, are associated with the induction of relatively poor mucosal and cell-mediated immunity, and because of their unreliable cross-protection against drifted variants, they must be reformulated annually to provide the best opportunity for seasonal protection. Investigators, from an early stage, have been drawn toward the prospect of broad immunity that might be engendered by the use of live viruses. A live attenuated vaccine approach was proposed by Burnet after his successful adaptation of the influenza virus to chick embryos in the early 1940s (Burnet 1943), but he abandoned this strategy once it appeared that inactivated vaccines developed under the aegis of the Commission on Influenza provided protection. However, live attenuated influenza vaccines were used in the former Soviet Union and in China, albeit in uncontrolled fashion since the late 1930s (Kilbourne 1987).

Investigations into live influenza vaccines were slowed for years by the inability to develop a reliable, effective method of attenuation that resulted in a stable vaccine virus capable of infecting the respiratory tract and stimulating both arms of the immune system while not causing illness in humans. After experimentation with various methods of attenuating virus, cold-adapted strains proved to be the most robust. Serial passage of influenza A and B viruses at 25°C in tissue culture and eggs introduces point mutations in the viral genes that result in cold-adapted, temperature-sensitive, attenuated strains, which are then subjected to reassortment in the laboratory with strains related to those predicted to circulate in the community in a given season (Wareing and Tannock 2001). This live attenuated vaccine is administered intranasally and in a similar manner to natural influenza infection, induces both arms of the immune system, including mucosal responses, and has demonstrated high rates of protective efficacy in children (Palese 2006). Additionally, there is evidence, at least in children, that it may be more reliably effective and protect against antigenically drifted variants to a significantly greater degree than inactivated vaccines (Belshe et al. 2000, 2007), although the evidence in adults infected with drifted variants is discordant (Ohmit et al. 2006).

## 11.6 Future Outlook

Because variants of H1N1 and H3N2 subtypes of influenza remain the predominant cause of epidemic human infection throughout the world, current vaccines are formulated to contain representatives of these basic genotypes, as well as an influenza B virus, that are predicted to circulate in the next influenza season. The process of vaccine formulation and production is labor intensive, time-consuming and such predictions are accompanied by an intrinsic level of risk; in some years, vaccine mismatches with viruses circulating in the community have resulted in excess influenza morbidity and mortality (Palese 2006). Live attenuated vaccines have not materially changed the aforementioned fundamental process, which renders currently available influenza vaccines suboptimal platforms for protection from pandemics.

The history of influenza vaccines therefore remains to be fully written. Novel approaches to vaccine prevention against this pathogen are in development, applying innovative molecular technologies to the problems of antigenic drift and shift that characterize influenza viruses and necessitate annual vaccine reformulations. The holy grail of influenza, one that has remained elusive, is the development of a universal, cross-protective vaccine that would be useful against all strains in both interpan-demic periods as well as pandemics. Because of the inherent mutability of influenza viruses, such a vaccine would likely have to be based on conserved antigens and would have to be augmented through the use of adjuvants or other methods to enhance the immunologic response.

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# Chapter 12

## Polio

David Oshinsky

On April 12, 1955, a killed-virus polio vaccine developed by Jonas Salk, a University-of-Pittsburgh researcher, was found to be safe, potent, and effective. The news set off a national celebration. Schools and factories closed for the day. From Boston to Seattle, people poured into the streets, weeping openly with relief. Banner headlines read: POLIO IS CONQUERED.

Salk became an instant hero, the nation's first true celebrity-scientist. Hollywood studios fought for the exclusive rights to his life story. *Newsweek* insisted that Salk's achievement matched those of medicine's greatest minds – "Jenner, Pasteur, Schick, and Lister." The U.S. House and Senate awarded Salk a Congressional Gold Medal, the nation's highest civilian award, putting him in the company of Thomas Edison, Charles Lindbergh, and Walter Reed. On April 22, Salk and his family were honored at a White House ceremony, where President Dwight D. Eisenhower, his voice trembling with emotion, lauded the 40-year-old researcher for saving the children of the world. "I have no words to thank you," the president said. "I am very, very happy" (Oshinsky 2005a).

In truth, of course, the story of the polio vaccine does not begin – or end – with Jonas Salk. Dozens of researchers were involved in this life-saving process – at Harvard and Yale, at Johns Hopkins and the Rockefeller Institute, at the Universities of Cincinnati, Michigan, and Pittsburgh, and at laboratories throughout the world. Most of these men and women have been lost to history, their contributions ignored by the media's intense focus upon Salk and his bitter rival, Albert Sabin, in the race for a vaccine. Who were these polio researchers, and what did they do? How was the money raised to fund their vital work? What role, if any, did the federal government play? Why did so many Americans embrace the controversial polio vaccine field trials of 1954 – the largest public health experiment in U.S. history? And where does the world stand today in terms of "conquering" this insidious paralytic disease? These are the key questions to be considered.

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## 12.1 Epidemic Disease

The word “poliomyelitis”, a combination of the Greek words “polios” (gray) and “myelos” (marrow), and the Latin suffix “itis,” describing inflammation, was shortened to “polio” by American newspapers following World War II, as the disease cropped up regularly in the headlines. Polio is an intestinal infection, spread from person to person via the fecal-oral route. Humans are the only natural hosts, though the infection can be induced in primates and certain types of rodents. The agent is a virus that enters the body through the mouth, travels down the digestive tract, and is excreted in the stools.

Although some replication occurs in the throat and tonsils, the main breeding ground for poliovirus is the small intestine. In most cases, the infection it produces is mild, with symptoms such as a headache, slight fever, and nausea. In rare instances – estimated to be 1 in 150 – the virus enters the bloodstream to invade the brain stem and central nervous system, destroying the nerve cells, or motor neurons, that stimulate the muscle fibers to contract. At its worst, the infection causes irreversible paralysis and can be fatal. Most deaths occur when the muscles of respiration are involved, a condition known as bulbar polio, in which the brain stem is compromised.

Once known as infantile paralysis, polio has several defining traits: its victims are mainly children and young adults; it strikes hardest in the warm summer months, and the worst outbreaks have occurred in places where standards of cleanliness and personal hygiene are on the rise. Indeed, in strictly epidemic form, polio appears to be a twentieth century disease of the more developed nations, with the greatest tolls occurring in Australia, Canada, Western Europe, and, especially, the United States. Better hygiene meant that fewer Americans were being exposed to poliovirus as infants, when the infection is milder and maternal antibodies offer temporary protection against the disease.

The first major polio epidemic on American shores occurred in New York City in 1916, with foreigners shouldering the blame. New York’s population had exploded in the late nineteenth century, as waves of impoverished immigrants – mainly from central and southern Europe – overwhelmed the city’s housing stock and social services. Living in wretched, overcrowded conditions, they were blamed for each new epidemic that appeared – cholera, diphtheria, tuberculosis, and typhoid fever, among them. In the summer of 1916, Italian immigrants were accused of infecting the city with polio, a disease few New Yorkers had ever heard of. Baffled by the crisis, medical authorities relied on methods that worked successfully against epidemic disease in the past, such as scrubbing sidewalks, screening windows, collecting garbage, closing theaters, quarantining the sick, and rounding up stray animals – “72,000 Cats Killed in Paralysis Fear,” read one of that summer’s more bizarre headlines (Gould 1995; Rogers 1992; NY Times 1916).

The epidemic spread throughout the Northeast. Children leaving New York City were required by the Health Department to get a “travel certificate” stating they were “polio free.” Meanwhile, neighboring towns posted armed guards along roads

and rail lines to keep out the fleeing New Yorkers. Before ending in October, the epidemic had claimed 6,000 lives. New York City reported 8,900 polio cases and 2,400 deaths, 80% being children under five. Those studying the outbreak were alarmed by what they found. Quarantine had not worked, leading some to conclude that there were asymptomatic carriers of poliovirus. Furthermore, polio appeared to strike rural and affluent neighborhoods with even greater force than the teeming immigrant slums. At first, health officials assumed that polio had been brought into the better neighborhoods by immigrants who worked there as cooks and maids, or by innocent middle-class folk who rode the city subways “reeking with billions of germs caused by the filthy foreign element constantly among them.” Before long, however, these officials were wondering aloud whether slum dwellers had been naturally immunized by exposure to poliovirus at an early age. The “menace for the future,” said one expert, “is very real” (Rogers 1997).

Polio was still a curiosity to most Americans when it struck down one of the nation’s leading political figures, Franklin Delano Roosevelt, in 1921. The news was unsettling; at 39 years of age, robust and aristocratic, Roosevelt seemed an unlikely target for a disease that largely victimized children. Yet polio would almost kill the future president, leaving both legs paralyzed for the rest of his life. In 1926, before returning to politics, Roosevelt purchased a failing resort in Warm Springs, Georgia, which would soon become the nation’s premier polio rehabilitation center. In the 1930s, while president, he helped organize the National Foundation for Infantile Paralysis, better known as the March of Dimes, to find a cure for polio while providing the best treatment for those already afflicted. Roosevelt hand-picked his former law partner, Basil O’Connor, to be the first director (Walker 1953; Lippman 1977; Gallagher 1985; Ward 1989).

It proved a brilliant choice. Stubborn and single-minded, a workaholic with enormous managerial skills, O’Connor revolutionized the way philanthropies raised money, recruited volunteers, and advertise their cause. It was the National Foundation that invented the poster child, the telethon, and the mothers’ marches that other charities would imitate. It was the National Foundation that first used celebrities – from Eddie Cantor to Grace Kelly to Elvis Presley – to grab public attention. It was the National Foundation that created a new model for “giving” in America, with donors promised the ultimate personal reward: the conquest of a disease (Oshinsky 2005a).

Polio reached its peak in the 1940s and early 1950s. No disease struck more terror, and for good reason. Polio hit without warning. There was no protection, no cure. Each summer, like clockwork, came photos of paralyzed youngsters in jam-packed polio wards, with iron lungs lining the walls (Fig. 12.1). Beaches and movie theaters would be deserted, as people avoided crowds. Children were warned not to jump into puddles, play too hard, or share a friend’s ice cream cone. Parents panicked at the first signs of fever, nausea, stiffness, an aching limb. Some gave their children a daily “polio test.” Did the neck swivel? Did the toes wiggle? Could the chin reach the chest?

In truth, however, polio was never the raging epidemic portrayed in the media, not even at its height. Tens times as many children would be killed in accidents in



**Fig. 12.1** Children in iron lungs (Children's Hospital Boston Archives)

these years, and three times as many would die of cancer. Polio's special status was due, in large part, to the efforts of the National Foundation, which helped turn a horrific but relatively uncommon disease into the most feared affliction of its time.

## 12.2 Towards a Vaccine

Basil O'Connor knew little about basic science. What he did understand, however, was that progress on the polio front had made little headway since 1908, when Karl Landsteiner first isolated the virus in his Vienna laboratory. With abundant resources on hand, O'Connor convinced Thomas Rivers, a pioneering virologist of the time and a scientific director at the Rockefeller Institute, to form a committee on scientific research within the National Foundation. Then he hired Harry Weaver, a superb administrator, to supervise the committee's agenda. Weaver saw polio research as applied science – seeking a specific solution to a particular problem. For him, and for Rivers, that solution lay in a vaccine.

This was hardly surprising. Vaccines had proved successful against other viruses, smallpox and rabies being notable examples. And a vaccine against polio seemed especially promising, since humans appeared to be the only natural hosts. What was missing, at this point, were the answers to three basic questions: How many different types of poliovirus existed? How could one produce a safe and steady supply of each virus type for use in a vaccine? And what, exactly, was the pathogenesis of polio – its route to the central nervous system where the damage was done?

The first question proved the most tedious. A successful vaccine would have to protect against every strain of poliovirus in the world, and no one knew how many there were. The logistics were daunting. Hundreds of strains would have to be located, transported to special laboratories, and studied in numbing detail. As Weaver himself admitted: “I know of no problem in all the medical sciences that was more uninteresting to solve. The solution necessitated the monotonous repetition of exactly the same technical procedures on virus after virus, seven days a week, 52 weeks a year, for three solid years” (Lee 1953; Oshinsky 2005a).

There were benefits, however. The typing project welcomed young, ambitious scientists anxious to win funding from the grant-rich March of Dimes; among those it attracted was Pittsburgh’s Jonas Salk. In all, 196 virus strains were tested, and all fit neatly into three distinct types. Type I, named “Brunhilde” after a chimpanzee from the laboratory of Johns Hopkins researcher David Bodian, comprised 82% of the strains; Type II, dubbed “Lansing” in memory of a deceased polio victim from that Michigan city, 10%; Type III, called “Leon” for a Los Angeles boy who had also died from the disease, 8%. The poliovirus family proved remarkably, conveniently, small (Paul 1971).

The next step – the growing of safe and plentiful poliovirus for the vaccine – combined scientific intuition with plain good luck. In the 1930s, polio researcher Albert Sabin had demonstrated that poliovirus could be grown in test tube cultures – a huge step forward. But Sabin also encountered a problem that had plagued fellow researchers for years: he could only get the virus to survive in nervous tissue, which was known to cause encephalomyelitis, an inflammation of the brain and spinal cord, when injected into human beings. If this were true – if the virus could only be grown in the dangerous nerve tissue of animals – then it could not be safely harvested for use in a vaccine (Sabin and Olitsky 1936).

By the 1940s, the art of in vitro cultivation was rapidly progressing. “Wonder drugs,” antibiotics such as penicillin and streptomycin, made it simpler to maintain sterile cultures, while new techniques, such as the roller tube device developed by George Gey at Johns Hopkins, were used to enhance yields by exposing the tissue to proper amounts of air and fluid. Researchers also discovered that tissue cultures would survive longer if the nutrient medium was changed at regular intervals, about every 4 days.

The big breakthrough, discussed in depth in Chap. 9, came in the laboratory of John Enders at the Children’s Hospital of Boston in 1948. Working with Frederick Robbins and Thomas Weller, Enders suggested that poliovirus be injected into cultures containing both nerve and non-nerve embryonic tissue, using the latest techniques. “It was in the back of my mind,” he remembered, “that, if so much

poliovirus could be found in the gastrointestinal tract, then it must grow somewhere besides nervous tissue" (Henig 1996; Robbins 1997; Simmons 2002).

His instincts were sound. The poliovirus grew easily in bits of skin, muscle, and kidney tissue. At last, a safe reservoir of virus had been created, free from the contaminating effects of animal nerve tissue. And that made possible the mass production of a polio vaccine. "I'll tell you one thing," Tom Rivers recalled, "that report sure as hell captured everyone's attention... It was like hearing a canon go off" (Benison 1967). In 1954, a brief 5 years after their monumental discovery, Enders, Robbins, and Weller were awarded the Nobel Prize in Medicine – the only polio researchers to be so honored.

But a key question remained unanswered: how did poliovirus invade the central nervous system? A generation of polio researchers, mentored by Simon Flexner at the Rockefeller Institute, had been trained to believe that poliovirus entered the body through the nose and traveled to the nervous system via the brain, without ever entering the bloodstream. If this were true, it meant that a vaccine designed to stimulate antibodies in the blood, the natural defenses against infection, would do no good.

Working independently, two polio researchers – Dorothy Horstmann at Yale and David Bodian at Johns Hopkins – tried a new approach. After blocking the nasal passages of several chimpanzees, Horstmann and Bodian fed them poliovirus orally. The primates fell quickly to the disease, showing the mouth – not the nose – to be the point of entry. Even more significant were the traces of poliovirus found in their blood. Why had previous researchers failed to discover this? The answer was deceptively simple: they had waited too long to look. When poliovirus enters the blood, it creates the very antibodies that will soon destroy it. Indeed, the virus can only be found there during a brief period of incubation before these antibodies have marshaled their forces (Oshinsky 2005b). For the most part, the puzzle had been solved. Researchers now knew how many types of poliovirus there were, how to produce safe virus for a human vaccine, and how that virus traveled through the body. They were confident, at last, that polio might be tamed.

Much of the drama in the polio story centered on the race for a vaccine. The two major competitors, Jonas Salk and Albert Sabin, came from similar backgrounds – a fact that may have intensified their bitter lifelong feud. Both were Jewish, both came of age in the education-driven culture of America's urban immigrant communities, and both attended medical school at New York University – one of the few places in the 1920s and 1930s that did not have a "quota system" limiting the number of Jews. By 1950, Salk and Sabin were the most favored recipients of March of Dimes grant money, each receiving more than \$100,000 per year (Oshinsky 2005a).

But there were marked differences as well. Though 8 years younger than his rival, Salk (1914–1995) took a more traditional – some said old-fashioned – approach to vaccination. His research skills had been honed in the laboratory of the highly respected epidemiologist Thomas Francis at the University of Michigan. Working there on an influenza vaccine, Salk (Fig. 12.2) had become a devotee of his mentor's killed-virus approach, which assumed that immunity to disease did not require a natural infection. If properly prepared, Salk believed, a killed-virus vaccine could trick the immune system into believing that the body was under attack by

**Fig. 12.2** Jonas Salk  
(Albert and Mary  
Lasker Foundation)



enemy invaders. The key was to fully inactivate the virus without destroying its ability to stimulate protective antibodies – a delicate balancing act.

Sabin (1906–1993) disagreed. His position, supported by the vast majority of vaccine researchers, was that powerful and lasting immunity to disease depended on the body's response to a natural infection. This followed in the vaccine tradition of Jenner, Pasteur, and Koch. Sabin (Fig. 12.3) scoffed at the idea that a killed-virus vaccine could produce antibody levels that were high enough or durable enough to protect against polio. Only a live-virus vaccine could do this, he insisted, and anything less would be a dangerous waste of time and money (Oshinsky 2005a).

In truth, there were advantages to both methods. Salk's was quicker and simpler. If developed properly, a killed-virus vaccine could not revert to virulence, removing the danger of vaccine-induced polio. Moreover, Salk could rightly assume that the formaldehyde agent needed to inactivate the most powerful of the three virus types would be strong enough to inactivate the other two. Speed and safety appeared to be on his side.

Sabin faced a harder road. Live viruses require more attention, because they continue to multiply inside the body. Each strain must be strong enough to produce a mild infection, yet docile enough to do no further harm. Properly attenuating three strains for one vaccine, Sabin recalled, “was no job for someone in a hurry” (Sabin 1965; Chanock 1996; Blume and Geesink 2000).

Still, a live-virus polio vaccine had numerous advantages. Given by mouth, as opposed to injection, it simulated the pathophysiology of wild-type poliovirus,

**Fig. 12.3** Albert Sabin  
(Albert and Mary  
Lasker Foundation)



thus producing a high – and lasting – antibody response. There was no need for needles or for “booster shots” down the road. A live-virus vaccine also appeared to work more quickly, within a matter of days, not weeks, which meant that it could halt an epidemic already in progress. Furthermore, it offered the prospect of “passive vaccination” to the general public, since those who ingested the vaccine shed the weakened virus back into the environment through their feces, thereby potentially immunizing large portions of the unvaccinated population. As a result, a live-virus vaccine, if safely developed, had the potential to eradicate polio completely.

As expected, Salk was ready first. Surrounded by a superb group of researchers and technicians at the University of Pittsburgh, and using the building blocks provided by Thomas Francis, John Enders, David Bodian, Dorothy Horstmann, and others, Salk successfully tested his killed-virus vaccine on primates. In 1952, with the full knowledge and support of Basil O’Connor from the National Foundation, he quietly expanded the testing to include children in the Pittsburgh area. In keeping with tradition among vaccine researchers, Salk immediately vaccinated himself and his three young sons. At a time when “informed consent” was little more than a nebulous concept, he tested his vaccine in places like the Polk School for the Retarded and Feeble-Minded by obtaining blanket permission from the institution’s superintendent. Salk gave most of the injections himself. “When you inoculate children with a polio vaccine,” he said later, “you don’t sleep well” (Carter 1966).

The results were impressive. The vaccine not only proved safe, it also stimulated a high level of antibody responses against all three types of poliovirus. And it came

against the backdrop of truly alarming news; 1952 would be the worst polio year on record, with more than 57,000 cases nationwide. Close to half would suffer permanent paralysis, and about 3,000 would die. There was good reason to hurry.

In 1953, the National Foundation prepared for a major field trial of the Salk vaccine. Was the product safe? Would it work well enough to justify the enormous effort involved? To Harry Weaver, the Foundation's research director, there was only one way to find out. "These questions," he said, "can only be determined after injecting a relatively large number of human beings" (Benison 1967; Paul 1971; Oshinsky 2005a).

How large, exactly? Considering the incidence of polio among American children – around 50 per 100,000 – the sample, to be convincing, had to be enormous. Numbers mattered, and so, too, did the selection of volunteers. Studies showed that 5-year-olds had the highest rate of polio, making them logical candidates for vaccination. The problem, however, was that the Foundation hoped to run the experiment through the local school systems, where good record keeping and regular attendance were guaranteed. This meant using older children, with an emphasis on grades 1–3. The planners also wanted the participating counties to be spread widely across the nation, representing all regions, classes, races, and ethnicities. Finally, because these trials needed strong grass-roots support, the Foundation focused on places where its local chapters were well-entrenched. All else being equal, it hoped to maximize what were euphemistically called "advantageous situations" (Oshinsky 2005a).

The trials were designed to include three categories: one group would receive the real vaccine; a second group would receive a look-alike placebo; and a third group would act as "observed" controls. The experiment would be "double-blind," meaning that neither the child receiving the inoculation nor the person giving it would know which formulation – the real vaccine or the placebo – was being used. There would be three separate vaccinations, spaced over several weeks. All the information would be carefully coded, known only to those who ran the trials and recorded the results.

Salk initially opposed the double-blind model. He thought it morally unconscionable to deny his vaccine to anyone participating in the study. "I feel that every child who [gets] a placebo and becomes paralyzed will do so at my hands," he wrote O'Connor, adding: "It is enough to make a humanitarian shudder [and] Hippocrates turn over in his grave" (Carter 1966). But the Foundation refused to budge. And the scientist it chose to evaluate the field trials, Salk's mentor Tom Francis, convinced Salk that scientific rigor was paramount in an experiment of such extraordinary importance.

By the spring of 1954, everything was in place. The vaccine, produced by Parke-Davis and Eli Lilly, had been triple-tested for safety; first in Salk's laboratory, next by the manufacturer, and finally by National Institutes of Health. An army of helpers stood ready, including "14,000 school principals, 50,000 classroom teachers, 20,000 physicians, 40,000 nurses [and] somewhere between 200,000 and 250,000 active non-professional volunteers." In a letter to the nation's parents, O'Connor shrewdly described participation in the vaccine trials as both a moral act, benefiting children everywhere, and a privilege bestowed upon those special enough to be

called “polio pioneers.” Indeed, the parental consent form was changed from the standard “I give my permission” to the more exclusive “I hereby request,” implying that not every child would be fortunate enough to be picked (Glasser 1954; O’Connor 1954).

On April 26, 1954, at an elementary school in Virginia, a 6-year-old named Randy Kerr, sporting a crew cut and a smile, rolled up his left sleeve so that Dr. Richard Mulvaney could give him an injection. “I could hardly feel it,” boasted America’s first polio pioneer. “It hurt less than a penicillin shot” (Time 1954).

In the coming weeks, more than 600,000 children would be vaccinated at least once with the Salk vaccine or the placebo. Another 725,000 would act as observed controls. In perhaps the most telling statistic, 95% of these “polio pioneers” would return for all three vaccinations – a sign of the public’s intense fear of polio, its extraordinary faith in the March of Dimes, and its devotion to this passionate crusade. As expected, national attention was riveted on these trials, with news coverage rivaling other big stories from that remarkable spring: *Brown vs. Board of Education*, the Army-McCarthy hearings, and the fall of Dien Bien Phu in French Indochina. A Gallup poll showed more Americans aware of the field trials than knew “the full name of the President of the United States” (Francis et al. 1955; Meier 1989; Meldrum 1998; Monto 1999).

The field trials ended in late spring, just as polio season began. All eyes now turned to the Vaccine Evaluation Center in Ann Arbor, Michigan, where Francis and his staff of 120 collected, processed, and evaluated a mountain of coded material. Most of the data were entered by hand; some were sent to an IBM office in Detroit, which tabulated the results on a “decibel drum memory machine” that used a new programming language soon to become known as FORTRAN. Francis held no press conferences, provided no updates, and tolerated no leaks from the staff. The job, he said, would be “finished when it’s finished” (March of Dimes Foundation 1954; Thomas Francis Diary 1954).

It took an entire year. On April 12, 1955, the tenth anniversary of Franklin D. Roosevelt’s death, Francis announced the results at a raucous press conference on the University of Michigan campus. The raw numbers were as follows:

	Vaccinated areas		Observed areas	
	Vaccinated	Placebo	Vaccinated	Observed
Number of children	200,745	201,229	221,988	725,173
Number of paralytic cases	33	115	38	330

The vaccine, Francis declared, was extremely safe and “80–90% effective against paralytic poliomyelitis.” When Jonas Salk followed him to the podium, the audience responded with a standing ovation. But Salk’s speech that day would haunt him for the rest of his life. The problem was one of recognition. Salk applauded Francis, Basil O’Connor, and the March of Dimes. He thanked the parents of America and noted the various deans and trustees from the University of Pittsburgh. He seemed to acknowledge everybody – except, that is, the people in his own lab.

This group, seated proudly together in the packed auditorium, would feel painfully snubbed (Oshinsky 2004, 2005a).

They were not alone. A number of top polio researchers blamed the Foundation for creating a “celebrity scientist” and Salk for acting the part. Some criticized Salk for elevating himself at the expense of those who had done the pioneering research. What had *he* done to deserve so much attention? Who was *he* to reap all these rewards? Others complained about the carnival atmosphere surrounding the Ann Arbor announcement, and the fact that the trial results had been released at a press conference as opposed to a scientific conclave or in a scholarly journal, where an experiment of this sort was normally judged. “I am deeply concerned, as are many others, with all the hysterical publicity – Polio is licked, Salk the miracle man stuff,” wrote one scientist. “I am tempted to get out my sharpest pen and stick it into the balloon as far as I can” (Clark 1955; Carter 1966; Paul 1971).

But then a glitch appeared, warning of big trouble ahead. On April 24, 1955, just 2 days after Salk’s White House visit, a first-grader in Idaho contracted polio following an injection of the Salk vaccine. Three days later, she was dead. Similar cases piled up: one in Chicago, two in San Diego, others in Oregon, Washington, and across the Far West. What had gone wrong? In 1954, as the Salk trials were getting underway, Basil O’Connor made a fateful decision. Believing that the polio vaccine would prove successful, he had privately agreed to pay six drug companies more than 9 million dollars of Foundation money to manufacture it, so that stockpiles would be available the following year. This made perfect sense. Parents would be clamoring for the vaccine if it worked. And the drug companies, sensing a potential windfall, quickly signed on (Offit 2005; Oshinsky 2005a).

Within hours of the announcement at Ann Arbor, the Department of Health, Education, and Welfare (HEW), led by Secretary Oveta Culp Hobby, moved to license Salk’s vaccine. Millions of polio shots were now ready for distribution. The problem, however, was that the vaccine produced by these six drug companies had not been tested as thoroughly as the vaccine used in the 1954 trials. And one company, in particular – Cutter Laboratories of Berkeley, California – had experienced serious problems in trying to fully inactivate the poliovirus in its vaccine. Indeed, clumps of live virus had turned up repeatedly in its testing process. Cutter’s response had been to discard these contaminated vaccine lots without informing HEW. This practice was deceptive but not illegal. Cutter was under no obligation, under the loose guidelines then in place, to submit the protocols for the vaccine lots it did not intend to market (Engel 1955; U.S. Depart of Health, Education and Welfare, Public Health Service 1955; Nathanson and Langmuir 1963).

Acting quickly, Surgeon General Leonard Scheele, a career public health officer, dispatched several scientists from the fledgling Epidemic Intelligence Service (EIS) to find the source of the problem. Formed during the Korean War as a defense against biological attack, the EIS was an arm of the Communicable Disease Center in Atlanta, now the Centers for Disease Control and Prevention (CDC). Within a week, a pattern was discovered: all the cases involved children inoculated with Cutter vaccine. At this point, more than 400,000 youngsters, mostly on the West Coast, had already taken the Cutter vaccine, and another 400,000 doses were in the

hands of distributors. Scheele asked Cutter to recall all the unused doses, and the company agreed.

A national panic ensued. Dozens of cases of polio were reported from the Cutter vaccine, as rumors flew about the safety of the Wyeth and Lilly vaccines as well. Sabin went public with his view that all Salk vaccinations should be suspended, and he was not alone. On May 8, in a dramatic television address, Scheele ordered a halt to further vaccinations pending a review of all six manufacturers. “Turmoil Over Salk Shots,” blared *The New York Times*, which claimed that “the air of victory” surrounding the Salk vaccine had become a stench of “confusion, conflict, and doubt” (NY times 1955; Williams 1955).

In the end, more than 200 polio cases were attributed to the Cutter vaccine. Most victims were severely paralyzed; 11 died. Even worse, the suspension of polio vaccinations in 1955 led to thousands of cases among unvaccinated children. Public confidence had clearly been shaken.

But the Cutter incident brought progress as well. The success of the EIS raised the profile of federal agencies in fighting disease. The rules for producing polio vaccine were quickly amended. Better safety measures were introduced, and record keeping was upgraded to prevent the burying of mistakes. Vaccine testing now became a major function of the National Institutes of Health (NIH), whose budget swelled from \$81 million to \$400 million between 1955 and 1960, accelerating the pace of federal support that had begun in earnest following World War II. As one writer noted, “the testing of the Salk vaccine, the largest public health experiment ever [in the United States] was also in all likelihood the last such trial that could ever be managed in its entirety by a private organization” (Strickland 1972; Starr 1982; Smith 1990).

Through all this controversy, Sabin was making strides on his live-virus polio vaccine, a process he began in 1951. But Sabin was not the only scientist working in this regard. Hillary Koprowski, a brilliant virologist working in private industry for Lederle Laboratories, had already tested an oral, live-virus polio vaccine on chimpanzees and then on institutionalized children, with promising results. Known as a risk-taker, the pioneering Koprowski would face a recurring problem throughout his career in polio research. His various vaccines, critics charged, had a tendency to revert to virulence during their passage through the human body, raising the specter of vaccine-induced polio (Vaughan 2000).

By 1956, Sabin had made great progress in choosing his virus strains. Since he could not perform large trials in the United States, given the large number of children already vaccinated with the Salk vaccine, Sabin accepted an invitation to experiment inside the Soviet Union and other Iron Curtain countries. The Sabin trials of 1959 were a world apart from the Salk trials of 1954. Aside from choosing the oral polio vaccine, the Russians rejected the “double-blind model” in favor of “humanitarian testing” that contained no placebos or control groups: in short, the very model that Salk had wanted for his own trials. In all, more than 77 million children and adolescents received the Sabin vaccine (Benison 1982; Horstmann 1991).

The results were exceptional, some thought too good to be believed. Sabin was credited by the Russians with virtually wiping out polio in the Soviet Union using

a vaccine that revealed no safety problems at all. This was welcome news for most American vaccine researchers, who had long favored Sabin's approach over that of Salk. In August 1960, the Surgeon General approved Sabin's OPV vaccine for trial manufacture in the United States, the first step in the licensing process. Widely seen as the more effective product, it soon became the vaccine of choice in the Western Hemisphere and most of the world. In 1961, the last year in which the Salk vaccine was exclusively administered in the United States, the number of reported polio cases dropped below 1,000, the lowest total ever. Within a decade, the Sabin vaccine would bring that figure down into double digits, almost wiping polio off the American map.

There was one drawback, however. After the disastrous Cutter incident of 1955, not a single case of polio in the United States had been attributed to the Salk vaccine. No one could question its safety, if properly prepared. Unfortunately, the same could not be said of the Sabin vaccine, which, new studies revealed, was responsible for causing a small number of polio cases – about one per each million doses administered – with those at special risk being infants with weakened immune systems. Put simply, the Sabin vaccine had clear advantages over the Salk vaccine yet one glaring fault. Almost everyone could see this except Sabin, who refused to give an inch. As Joseph Melnick, a friend and virologist colleague of Sabin's put it: "He was so strong-willed, he thought he could will it away" (Sabin 1985; Schmeck 1993).

## 12.3 Aftermath

Albert Sabin died of heart failure in 1993, at the age of 86. Jonas Salk died of similar causes 2 years later, at the age of 81. Salk remained the people's hero, the man who had won the initial vaccine race, while Sabin seemed the ultimate victor, the one whose vaccine had triumphed in the end. Yet neither man could have seen what lay ahead. In the late 1990s, the American medical establishment, led by the CDC Advisory Committee on Immunization Practices, reversed course completely, recommending a return to a more powerful version of the Salk killed-virus polio vaccine in the United States. The logic was clear: the Sabin vaccine, so successful in disrupting the life cycle of wild-type poliovirus, had become the final obstacle to fully eradicating the disease (Zimmerman and Span 1999).

So much has changed since that celebratory moment at Ann Arbor in 1955. Polio has become a distant memory in the United States, though it still haunts distant corners of the world. The enormous effort of the March of Dimes in the domestic polio crusade has been taken up, globally, by groups like the Rotary International, the Bill and Melinda Gates Foundation, the World Health Organization (WHO), and the CDC. Through these efforts, the number of paralytic polio cases in the world – centered largely in Afghanistan, India, Nigeria, and Pakistan – has dropped to fewer than 2,000 per year. While favoring the Sabin vaccine at this point for practical reasons, the WHO has urged polio-free nations to begin phasing it out

in favor of the Salk vaccine, noting that “the continued use of OPV for routine immunization could compromise the goal of eradicating all paralytic disease due to circulating polioviruses.” The hope now, says the WHO, is that polio will go the way of smallpox by the year 2010, providing a future in which “no child will ever again know the crippling effects of this devastating disease” (WHO 2004).

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# **Chapter 13**

## **Measles, Mumps, and Rubella**

**Kathleen M. Gallagher, Stanley A. Plotkin, Samuel L. Katz,  
and Walter A. Orenstein**

Following the landmark discovery of the Enders lab that led directly to the successful development and deployment of polio vaccines, investigators turned their attention to addressing the other, common viral diseases of childhood. Over the next two decades, these efforts would yield vaccine dividends against three of the most important causes of viral disease and would dramatically and favorably alter the global landscape of infectious diseases.

### **13.1 Measles**

#### **13.1.1 Background**

Measles, one of the most common of all childhood diseases at one time, was recognized as early as the seventh century by the Hebrew physician, Al Yehudi (Babbott and Gordon 1954; Black 1989). However, credit for the first written description of measles is usually given to the tenth century Persian physician, Rhazes

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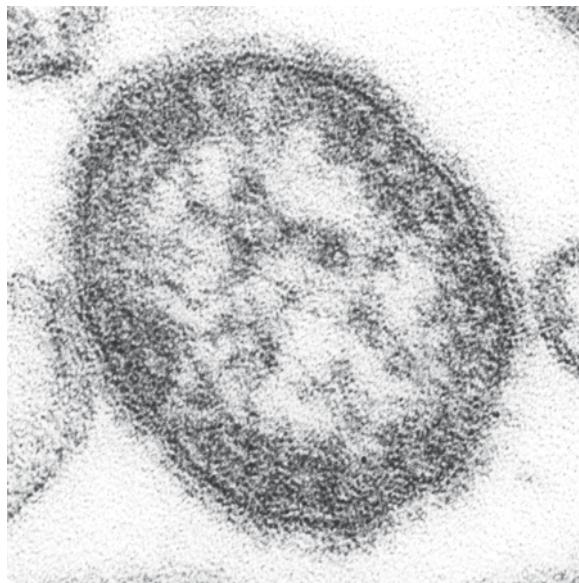
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(Abu Beqr 1747; Wilson 1962), who referred to the disease as *hasbah*, the Arabic word for “eruption.” Rhazes was also the first to note a distinction between measles and another serious, common illness of the time, smallpox; he considered measles “more to be dreaded than smallpox” (Black 1989). Despite his early insights into the disease, Rhazes did not believe that measles was an infectious disease (Black 1989). The term “Rubeola,” a Latin word, was first used to describe the disease in the Middle Ages.

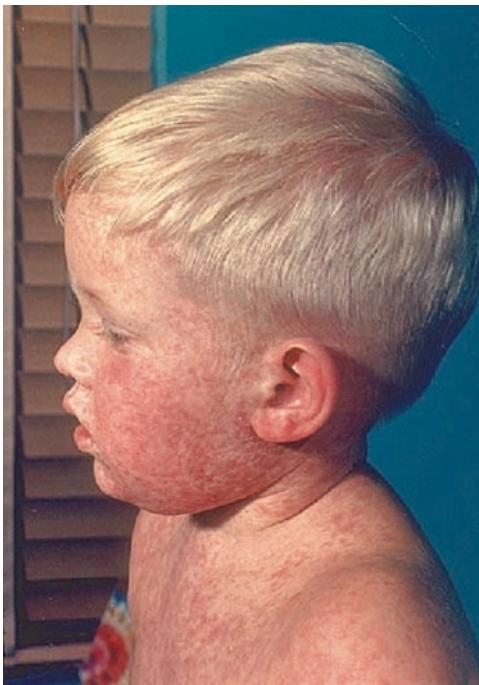
By the beginning of the seventeenth century, annual bills of mortality in London were recording smallpox and measles deaths separately, suggesting that by then a clear distinction between the two diseases was recognized (Wilson 1962). Around this time, Thomas Sydenham, “the father of English medicine,” described the clinical characteristics of measles. Both he and Francis Home, a Scottish physician who played prominently in the first attempts at vaccination against measles in a manner akin to that of smallpox, believed measles to be infectious. The Danish physician Peter Panum confirmed this during his investigation of an outbreak on the isolated Faroe Islands in 1846; additionally, he documented the 2-week interval between exposure to an infectious case and the development of rash, the excess in case fatality rates (CFRs) at the extremes of age, and the durable immunity afforded to persons who acquired natural infection with measles (Panum 1939).

Infection with the measles virus, an RNA virus from the genus Morbillivirus in the Paramyxoviridae family (Fig. 13.1), causes an acute illness characterized by a prodrome of fever and malaise, cough, coryza, and conjunctivitis, followed by a



**Fig. 13.1** Measles virus, electron micrograph (Courtesy of C. Goldsmith, Centers for Disease Control and Prevention, Atlanta, GA)

**Fig. 13.2** Child with measles  
(Photo Courtesy of Centers for  
Disease Control and Prevention,  
Atlanta, GA)



maculopapular rash (Fig. 13.2). Koplik's spots, small bluish-white dots surrounded by a reddish zone that appear on the mucous membrane of the oral mucosa, named after the American pediatrician Henry Koplik who first described them, are considered pathognomonic for the disease (Koplik 1896). Measles is generally mild or moderately severe in healthy children, but complications such as diarrhea, otitis media, croup, pneumonia, and encephalitis may occur, especially in young children. Acute encephalitis occurs in about one in every 1,000 cases and can result in permanent brain damage (Miller et al. 1956; Cherry 1998). The most severe, long-term sequela of measles virus infection, subacute sclerosing panencephalitis (SSPE), is a fatal disease of the central nervous system which results in behavioral and intellectual deterioration and seizures, generally within 7–10 years of primary infection.

With improved nutrition, decreased crowding, older age of infection, and the availability of treatment for secondary pneumonia, CFRs in developed countries declined precipitously, even before the introduction of the measles vaccine. In the U.S., CFRs for measles fell from approximately 25 per 1,000 cases early in the twentieth century (US Public Health Service 1914, 1947) to one per 1,000 cases in 1962 (CDC 1962). In developing countries, CFRs are significantly higher as a result of crowding, younger age of infection, malnutrition and vitamin A deficiency, malaria, underlying immunodeficiencies, and limited access to medical care.

Measles is one of the most contagious diseases of humans; attack rates in susceptible close contacts exceed 90% (Katz et al. 1962). Throughout history, therefore, large epidemics have been described. Measles outbreaks in the New World were first noted shortly after the onset of European colonization; the first epidemic in what is now the U.S. was reported in 1765 (Hinman et al. 2004). The magnitude and frequency of the epidemics were determined by the size of the population, the frequency of contact between infectious patients and susceptible individuals, and the rate of entry into the population of new susceptibles through either birth or migration (Black 1966).

Prior to vaccination programs, measles was considered ubiquitous in most geographic settings, with disease affecting nearly every person in the population by adolescence. In developed countries, the highest risk for infection and the largest proportion of cases occurred in school-aged children (Babbott and Gordon 1954; Langmuir 1962; Black 1989). During the 1940s, epidemics of measles occurred every second year in England and Wales with cases first being noted in large urban areas such as London, Manchester, and Liverpool, with subsequent spread to towns and rural areas (Cliff et al. 1993; Grenfell et al. 2001). Similarly, in the U.S. major outbreaks occurred every 2–3 years with peak incidence in the late winter or early spring (Hedric 1930; Babbott and Gordon 1954; London and Yorke 1973; Yorke and London 1973; Black 1989). Studies by Langmuir in the 1960s suggested that virtually all Americans had been infected with measles by age fifteen (Langmuir 1962); in developing countries, the average age of infection in the pre-vaccine era was thought to be substantially lower (Morley et al. 1963; Morley 1969; Scheifele and Forbes 1973; Assaad 1983; Black 1989). Up to 50% of 2-year-olds and 100% of 4-year-olds in certain African countries have serologic evidence of measles infection (WHO 1977).

The public health impact of measles undoubtedly played a role in the development of the vaccine. In the U.S. in the pre-vaccine era, approximately 500,000 measles cases and 500 deaths were reported annually but in reality, it is likely that three to four million people, an entire birth cohort, were acquiring infection each year (Orenstein et al. 2004). As recently as 2000, measles was still a leading cause of vaccine-preventable mortality in children worldwide, associated with an estimated 800,000 deaths, more than half of these in Africa (Murray et al. 2001).

### **13.1.2 History of Vaccine Development**

Even before receiving the Nobel Prize in Physiology or Medicine in 1954 with colleagues Robbins and Weller for their cultivation of the poliovirus in cell culture (refer to Chap. 9), John Enders had returned to his long-standing interest in isolating and propagating the measles virus. In 1954, he dispatched Thomas Peebles, a pediatric fellow in his lab, to obtain throat swabs and blood specimens from children attending a suburban Boston school where an outbreak of measles was underway. Using human kidney cells, measles virus was successfully cultivated and

passaged several times (Enders and Peebles 1954). This strain of virus was referred to as the Edmonston strain in honor of 13-year-old David Edmonston, the young student from whom the virus was initially isolated. Further passage of the Edmonston strain 24 times in primary kidney cells, 28 times in primary human amnion cells, adaption to chick embryos (six passages) and finally passage in chick embryo cells, all at 35–36°C, resulted in the virus that was to ultimately be included in the live virus vaccine licensed in 1963 (Katz et al. 1958; Enders 1962a, b).

Unlike viruses derived from earlier passages in human kidney cells, inoculation of measles-susceptible cynomolgus monkeys with this further-attenuated virus did not result in the detection of viremia or rash. The lack of pathologic changes in the central nervous systems of these monkeys paved the way for human trials. Measles-specific antibodies developed in all vaccine recipients. Although high rates of fever and rash were reported in vaccine recipients – the former only detected because of routinely scheduled temperature observations – an absence of concomitant disability or malaise was notable (Enders 1962b). The rash did not resemble that of natural measles; it was diminished in both extent and duration (Katz et al. 1963). These adverse events were mitigated by the simultaneous administration of very small doses of immune globulin (IG) (Krugman et al. 1963).

A formalin-inactivated measles virus vaccine derived from the Edmonston strain was also licensed in the US in 1963 (Feldman 1962). However, use of this vaccine was discontinued in 1967 after it was determined that the immunity conferred was short-lived and incomplete, and that it placed many recipients at risk for atypical measles infection, characterized by obtundation, atypical exanthema, and nodular pneumonia (CDC 1967a).

Since 1963, over 20 further attenuated measles vaccines have been developed and are in use throughout the world (Strebel et al. 2008). Most of these derive from the original Edmonston strain of virus. Passage of the Edmonston Strain an additional 85 or 40 times in chick embryo cells at a lower temperature (32°C) than the original passages resulted in the development and licensure of the Schwarz vaccine in 1965 (Andelman et al. 1963; Schwarz 1964), and the Moraten vaccine in 1968 (Hilleman et al. 1968), respectively. Further attenuations resulted in a dramatic decrease in the frequency and severity of adverse events, particularly fever and rash, in recipients of these newer vaccines when compared with those who had received the original Edmonston B vaccine. These new vaccines were used without the administration of IG (CDC 1965).

In the U.S., the Moraten Vaccine (Attenuvax, Merck) is now the only vaccine currently in use; in other parts of the world, the Schwarz vaccine is commonly used. In Japan, several different further-attenuated measles vaccines, including the AIK-C, Schwarz F88, CAM-70, and TD97, have been developed and are in use. The Leningrad-16 strain of vaccine, originally developed by Smorodinstev, was introduced in Russia in 1967 and has been the primary vaccine strain used in Eastern Europe (Peradze and Smorodinstev 1983). Along with the CAM-70 and TD-97 vaccines used in parts of Asia, and Shanghai 191 vaccine used in China, this vaccine is among the few not derived from the Edmonston Strain (Strebel et al. 2008). Most of these measles vaccines have been attenuated and produced in chick

embryo fibroblasts. Several of the vaccines currently in use internationally have been attenuated via passage in human diploid cells. These include the Edmonston-Zagreb vaccine, used extensively in the former Yugoslavia since 1969 and now the predominant measles-only vaccine procured by UNICEF for developing countries, and vaccines used in Iran and China.

### ***13.1.3 Implementation of Vaccination Programs and their Impact on Disease Burden***

The U.S. was one of the first countries to recommend the routine use of measles vaccine, originally in children 9 months of age, after its licensure in 1963. Within a few years of widespread vaccine use, the incidence of measles had declined substantially; the greatest declines were seen in children less than 10 years of age (CDC 1998). Attempts at elimination were initiated three times in the U.S. The first, in 1966, was based on a strategy of routine single-dose measles vaccination for infants at 12 months of age (Sencer et al. 1967). In 1978, a second call for elimination by 1982 was based on a strategy of achieving high population immunity to measles with a single dose of vaccine, enhanced surveillance for disease, and aggressive outbreak control (CDC 1978; Hinman et al. 1979). Despite dramatic reductions in the incidence of measles with each of these efforts, “elimination,” defined as the absence of endemic transmission of the disease, was not achieved.

A major resurgence of measles during 1989–1991, associated with outbreaks of measles among college students who had previously received only one dose of vaccine, resulted in the recommendation for routine immunization of children with two doses of measles-containing vaccine to reduce the 5% rate of primary vaccine failure (i.e., failure to make a protective immune response following the first dose). However, the major cause of the measles resurgence was low measles vaccine coverage among pre-school aged children, leading to major efforts to improve immunization of children in the first 2 years of life for all recommended vaccines, including measles. A third and final attempt at measles elimination was called for in 1993 (CDC 1994) by the Childhood Immunization Initiative; similarly, in 1994, the Pan American Health Organization (PAHO) called for the elimination of measles in the Americas (PAHO 1994). Measles was declared eliminated in the US in 2000 (Katz and Hinman 2004) and in the remainder of the Western Hemisphere in 2002 (de Quadros et al. 2004).

Measles vaccine has been included in the World Health Organization’s (WHO) Expanded Programme on Immunization (EPI) since 1983, and measles vaccine coverage in less industrialized countries increased from 18% in the early 1980s to 76% by 1990 (Cutts and Oliver 1999; Strebel et al. 2008). In February 2001, the American Red Cross convened a meeting with other global healthcare organizations including the CDC, UNICEF, the United Nation’s Foundation, and the WHO to discuss the growing problem of measles in Africa. From this meeting the Measles Initiative was launched to establish a long-term commitment and partnership among

leaders in public health in support of the goal of reducing measles deaths globally by 90% by 2010. Between 2000 and 2007, worldwide coverage with one dose of measles-containing vaccine increased from 72 to 82%, and measles deaths decreased from an estimated 750,000 in 2000 to 197,000 in 2007, a 74% change. The African region has made remarkable strides over this period with an 89% reduction in measles-associated mortality (CDC 2008a).

## 13.2 Mumps

### 13.2.1 Background

The origin of the word “mumps” is obscure but is believed to derive from an Old English verb meaning “grimace, grin, or mumble.” The clinical manifestations of mumps were described much earlier in the fifth century BC by Hippocrates in Book 1 of *His Book of Epidemics*. He described an illness characterized by the swelling of one or both ears and in some cases, the swelling, sometimes painful, of one or both testicles. In 1790, Hamilton, a Scottish physician, presented evidence of involvement of the central nervous system in some patients and emphasized the importance of orchitis in males with mumps (Hamilton 1790).

Historical records from as far back as the eighteenth century suggest that epidemics of mumps were occurring worldwide in all regions of the globe. Epidemiological characterization of reported outbreaks of parotitis during the first half of the nineteenth century revealed a predilection for spread within crowded environments such as prisons, orphanages, schools, and ships (Hirsch 1886). Large epidemics of mumps occurred among U.S. military recruits during the Civil War and during World War I. During the latter, mumps was the major cause of loss of active duty days among U.S. troops in France; only influenza and gonorrhea had higher rates of hospitalization (Stokes 1958; Feldman 1990). The prevalence of the disease in this setting was directly related to the size of the camp, with more densely populated camps experiencing larger outbreaks. Attack rates for the disease were also higher in camps in which a larger proportion of recruits came from rural, non-endemic areas of the U.S.

Outbreaks of mumps continued to occur in the military during World War II, although the incidence rate was reduced almost tenfold. In 1940, the U.S. Surgeon General stated that “next to venereal diseases, mumps was the most disabling of acute infections among US military recruits” (Parran 1940). Karl Habel, a surgeon in the U.S. Public Health Service, suggested that one possible solution to this problem would be the identification of susceptible individuals and their subsequent immunization with a mumps vaccine (Habel 1946).

Mumps is an acute viral illness that is the only known cause of epidemic parotitis in humans. The occurrence of unilateral or bilateral parotitis (Fig. 13.3) is often preceded by non-specific prodromal symptoms such as fever, headache, malaise, anorexia, and myalgia. Thirty percent of persons with mumps may be either

**Fig. 13.3** Child with mumps  
(Photo Courtesy of Centers for  
Disease Control and Prevention,  
Atlanta, GA)



asymptomatic or have more generalized symptoms making the recognition and diagnosis of disease incomplete. Although generally a mild, self-limited illness, serious sequelae can occur in patients with or without the presence of parotitis.

Involvement of the central nervous system is seen commonly in persons with mumps. Aseptic meningitis can occur in up to 10% of cases but is generally benign; encephalitis, the leading cause of viral encephalitis in the U.S. in the pre-vaccine era, has been reported in up to 0.3% of symptomatic mumps infections and is the primary cause of death in fatal cases (Russell and Donald 1958; Koskineni et al. 1983; Koskineni and Vaheri 1989). Mumps is the most common cause of unilateral, acquired, sensorineural hearing loss in children. Although deafness may be sudden in onset, bilateral, and permanent in rare cases (Hall and Richards 1987), transient, high frequency deafness is a well-described complication of mumps, occurring in up to 4% of cases (Strattin et al. 1994).

While approximately 25% of post pubertal males with mumps will develop orchitis, permanent sterility rarely ensues (Beard et al. 1977). However, an increased risk of testicular cancer has been reported in patients with a history of mumps orchitis (Beard et al. 1977; Swerdlow et al. 1987; Brown et al. 1987). One study observed mastitis in 31% of post pubertal females with mumps (Philip et al. 1959); oophoritis has also been reported. Despite the absence of an increased incidence of congenital anomalies in pregnancies complicated by infection, an association between maternal mumps during pregnancy and spontaneous abortion or intrauterine death has been reported (Kurtz et al. 1982). Mumps is considered to be a relatively benign infection of neonates (Sterner and Grandien 1990).

Serosurveys to assess the presence of mumps antibodies prior to the initiation of vaccination programs suggest that the average age of mumps infection worldwide

varied considerably. Studies conducted in England, Wales, the Netherlands, and Singapore showed that the majority of children less than 4 years of age were immunologically naïve (Wagenvoort et al. 1980; Morgan-Capner et al. 1988; Cox et al. 1989). The percentage of children susceptible to mumps varied inversely with age; half of the children between the ages of four and six were susceptible, but by the age of 15, only about 10% lacked antibodies to mumps. Contrasting surveys performed in Poland and Saudi Arabia have demonstrated that a large proportion of adults remained susceptible (Imbs et al. 1984; Bakir et al. 1988). Possible explanations for these differences include real differences in transmission rates for different strains of mumps viruses; varying lengths of inter-epidemic periods; or differences in clinical sampling or laboratory techniques (Galaska et al. 1999).

An inter-epidemic period of 3 years has been suggested for mumps based on the analysis of epidemiologic data in specific locations (Anderson et al. 1987; Nokes et al. 1990). Prior to implementation of routine mumps vaccination, the peak incidence of disease in temperate climates generally occurred in the winter and early spring with a nadir in the summer months. In tropical climates no such seasonality has been observed. Before widespread vaccine use, 200,000 cases of mumps associated with 20–30 deaths were reported annually in the U.S.

### ***13.2.2 History of Vaccine Development***

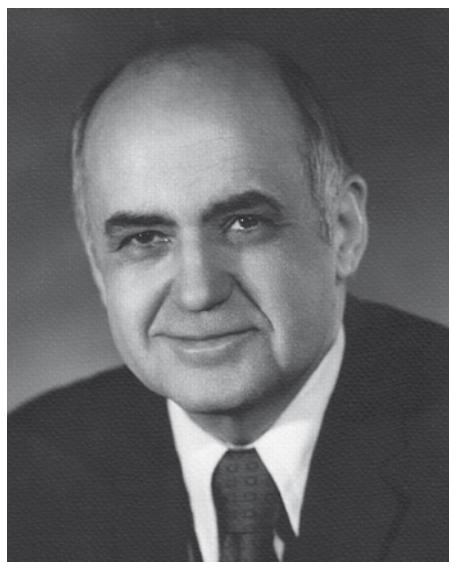
The debate regarding whether the microbial etiology of mumps was a bacterium, a spirochete, or a filterable virus was finally resolved in 1934 when Claud Johnson and Ernest Goodpasture (refer to Chap. 9) demonstrated that a virus obtained from the saliva of patients with epidemic parotitis caused parotitis in monkeys (Johnson and Goodpasture 1934). Because of the well recognized importance of mumps in military settings, the U.S. Public Health Service devoted a considerable amount of investigative effort on devising methods to determine susceptibility to mumps and to develop a prophylactic vaccine. The virus was cultivated in developing chick embryos in 1945 concurrently by Karl Habel at the U.S. National Institutes of Health (NIH) (Habel 1946) and Enders at Harvard Medical School in Boston (Enders 1946). Soon afterwards, attempts at producing a vaccine using a formalin-inactivated virus were begun by these two investigative teams (Stokes et al. 1946; Habel 1951).

With support of the Commission on Measles and Mumps of the Office of the U.S. Surgeon General, trials of formalin-inactivated mumps vaccine in humans were started in 1945 by Joseph Stokes and his colleagues at the Children's Hospital of Philadelphia and by Enders. Parental consent was obtained to vaccinate a total of 42 mentally-impaired children from three separate institutions. Although only half of the immunized children developed evidence of “increased resistance” to mumps, the experiment was considered a success because it demonstrated that it was possible to induce a protective immune response to mumps in humans by the parenteral inoculation of inactivated viruses (Stokes et al. 1946).

In May 1946, Habel at NIH began field testing his inactivated vaccine in Florida in a group of migrant field-workers who had recently arrived from the Bahamas. This population was selected because of ongoing annual mumps outbreaks among groups of West Indian sugar plantation workers in the U.S. since the end of World War II. Habel's vaccine, administered to over 1,300 individuals, reduced the incidence of mumps in the vaccinated group and the severity of disease in vaccinated subjects who became infected (Habel 1951). Despite their partial protective effects, the use of formalin inactivated mumps vaccines in the U.S. was abandoned in the 1950s due to the limited duration of immunity that they provided (Hilleman 1992, 1996).

Work on the development of live attenuated mumps vaccines began in 1959 in the laboratory of noted vaccinologist Maurice Hilleman and colleagues at Merck. Hilleman (1919–2005), whose early career plans as a manager at the local J.C. Penney store in Montana had been supplanted by a scholarship to college and a doctoral degree, had joined the company after an 8-year stint at the Walter Reed Army Institute of Research (WRAIR) where he co-discovered adenoviruses in the early 1950s (refer to Chap. 14). Hilleman (Fig. 13.4) would subsequently spend nearly 50 years at Merck and be credited with lead roles in the development of numerous effective vaccines against human and animal pathogens (Artenstein et al. 2005).

When his 5-year-old daughter, Jeryl Lynn, developed mumps in March 1963, Hilleman collected a throat swab from her and isolated the virus from amniotic fluid after inoculation into embryonated hen's eggs (Buynak and Hilleman 1966). The virus was further attenuated by 15 passages in embryonated hen's eggs followed by four passages in chick whole embryos. A protective efficacy of 100%



**Fig. 13.4** Maurice Hilleman  
(Courtesy of the Hilleman family)

of the attenuated Jeryl Lynn virus was demonstrated in a controlled trial among 867 children near Philadelphia (Hilleman 1996). It was this virus that ultimately became the basis for the vaccine that was licensed in the U.S. in 1967. A clone of the “Jeryl Lynn” virus is still used today in formulations of the vaccine produced in the U.S. and throughout much of the world. As of 2007, more than 500 million doses of mumps vaccine made from the Jeryl Lynn strain had been administered worldwide (WHO 2007).

Numerous vaccine strains of mumps virus have been developed and used with varying degrees of success and distribution since the early 1960s. Coincident with Hilleman’s work, Smorodintsev and colleagues at the Academy of Medical Sciences in Leningrad began their development of an attenuated mumps strain, now dubbed “Leningrad -3,” via 15 passages through guinea pig kidney cells and seven passages through Japanese quail embryo cells (Smorodintsev et al. 1995). This vaccine has been used in the former Soviet Union and other countries since 1974. Further attenuation via four additional passages through chick embryo fibroblasts resulted in yet another new strain, the Leningrad-Zagreb strain that has been widely produced in Croatia and India.

Live mumps vaccine made from the Urabe Am9 strain of virus was developed by the Biken Institute in Japan and licensed there in 1979. The strain was attenuated by two passages in human embryonic kidney cells, one passage in African green monkey kidney cells (AGMK), six passages in embryonated hen’s eggs, and two passages in quail embryo fibroblast cell culture. It was subsequently used in Italy, France, and Belgium. An increased risk of vaccine-associated adverse events, specifically aseptic meningitis at a rate of one case per 11,000–14,000 doses, has been reported in persons receiving vaccines containing the Urabe strain (Miller et al. 1993; Dourado et al. 2000) as compared to no cases among persons who received the Jeryl Lynn strain. As a result of these and other reports several vaccines derived from this strain have been removed from distribution.

Virus isolated from the urine of Carlos Rubini, a young boy diagnosed with mumps in Switzerland in 1974 was used to develop a vaccine that was originally licensed in Switzerland in 1985. However, subsequent to licensure, several studies demonstrated that this vaccine was inferior with regard to immunogenicity and effectiveness when compared with those made from the Jeryl Lynn and Urabe Am9 strains (Toscani et al. 1996; Chamot et al. 1998; Schlegel et al. 1999; Ong et al. 2005). For this reason, the WHO no longer recommends that mumps vaccine made from the Rubini strain be used in national immunization programs.

Currently, there are at least 15 different mumps vaccines used worldwide; these are produced from at least 13 different strains of virus (Plotkin and Rubin 2008). With the exception of vaccines made from the Rubini strain, all have demonstrated seroconversion rates and/or protective efficacy in excess of 90% during clinical trials, although calculated vaccine effectiveness is considerably lower when assessed in outbreak settings where attack rates of vaccinated and unvaccinated children are compared. Two doses of mumps vaccine are recommended to reduce the impact of primary vaccine failure and to achieve maximum protection against disease.

### ***13.2.3 Implementation of Vaccination Programs and Impact on Burden of Disease***

Initial recommendations for use of the newly licensed mumps vaccine in 1967 were limited because of concerns about duration of immunity, competition with other vaccine preventable disease efforts, and the perceived benignity of mumps. The Advisory Committee on Immunization Practices (ACIP) originally recommended the vaccine be targeted for use in children approaching puberty, children living in institutional settings, and adolescents and adults, especially males, without a history of mumps. The ACIP also suggested that routine use of the vaccine in younger children be deferred until additional information about the durability of immunity was available (CDC 1967b). Nevertheless, on the basis of data suggesting that the highest incidence rate of mumps was in children less than the age of nine and that these children were the likely source of most infections in older children and adults, some local public health programs choose to expand their immunization efforts to younger children.

In Massachusetts, a 3-year program established in 1968 initially targeted children in the first and second grades, followed by all other school-aged and pre-school children, and finally, all remaining susceptibles (Fiumara 1968). Within a year, a 50% reduction in the number of reported mumps cases had been observed in the Commonwealth (De Maria 2008). Another program in 1968 offered mumps vaccine to school-aged children through public health clinics in Washington and demonstrated that demand for the vaccine was highest in younger children; adults and older adolescents had not sought the vaccine in significant numbers (Jones 1969). By November of 1968 the ACIP had modified its recommendations for mumps vaccine to include “consideration” for use in all susceptible children beyond 1 year of age (CDC 1968).

Use of mumps vaccine increased substantially after the licensure of the combined measles/mumps/rubella (MMR) vaccine in 1971; by 1974 in the U.S. nearly 40% of children less than 10 years of age had been vaccinated (Modlin et al. 1975). Investigation of mumps outbreaks among school-aged children in the U.S. during the 1980s revealed that they were more commonly seen in states that lacked laws mandating immunization against mumps as a requirement for school entry. As a result, as of 2005, 45 states required that children be vaccinated against mumps to attend school (CDC 2009). Mumps vaccine, given as part of MMR vaccine has been shown to be cost effective, reducing the costs associated with mumps by up to 85% (Koplan and Preblud 1982).

Although not part of EPI, the WHO recommends a routine two-dose mumps vaccination schedule in countries that already have well-established childhood immunization programs and the ability to maintain measles and rubella vaccine coverage in excess of 80%. As of August 2008, mumps immunization had been incorporated into the routine national immunization program of 115 countries, including most developed countries, countries with transitional economies, and a substantial proportion of developing countries; almost all (114/115) use trivalent

MMR vaccine as the preferred way to deliver mumps vaccine (WHO 2008). However, none of the 50 countries categorized as the least developed, including most in Africa and Southeast Asia, use mumps vaccine on a routine basis.

Dramatic declines in the incidence of mumps have occurred in many countries coincident with the implementation of large-scale immunization programs. Between vaccine licensure in 1967 and 1985 in the U.S., there was a 98% reduction in the number of reported mumps cases. Reductions in mumps incidence ranging from 79 to 95% have been reported within 2 years following the introduction of mumps vaccine in the U.K. (Jones et al. 1991), Finland (Peltola et al. 1986), Croatia (Beck et al. 1989), and Israel (Slater et al. 1990).

The recent occurrence of several large outbreaks in countries that had high levels of vaccine coverage for many years has raised concern about long-term vaccine effectiveness and waning immunity for mumps vaccine (Cohen et al. 2007; Dayan et al. 2008). In 2006, the U.S. experienced a major resurgence of mumps with the greatest number of cases reported since 1987. The outbreak primarily affected college students in the Midwest, many of whom had received two doses of mumps vaccine, suggesting that waning immunity appeared to play some role. However, since 2006 incidence has decreased raising questions about how important waning immunity is to sustaining transmission of the virus. At this time, there are no recommendations for a third dose of vaccine.

### 13.3 Rubella

#### 13.3.1 *Background*

It all started with an astute observation by an Australian ophthalmologist. Norman McAlister Gregg noticed in the winter of 1941 that he was seeing many infants with congenital cataracts (Gregg 1941; Forbes 1969). It was said that he overheard mothers in his office talking about the rubella they had suffered while pregnant. Gregg inferred a causal relationship between rubella and the cataracts and went on to publish the first article on the subject. Until then rubella, described first in the medical literature at the end of the eighteenth century, had been regarded as a minor rash disease in children and young adults caused by a filterable virus (Hiro and Tasaka 1938).

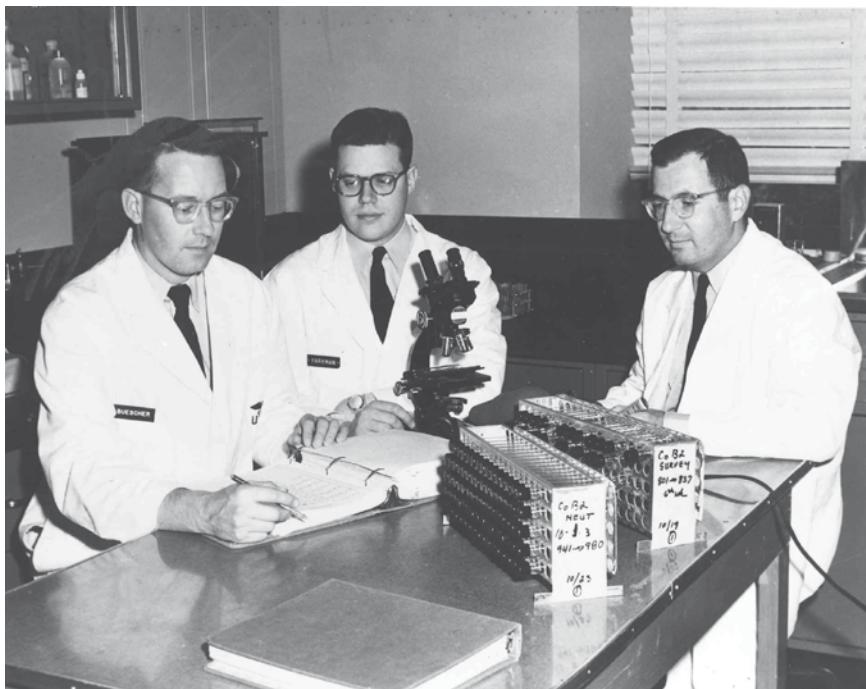
Subsequent epidemiological studies conducted in many different countries confirmed this observation, and it became well known that rubella was a threat to pregnant women and their offspring. Moreover, rubella in pregnancy resulted in more than congenital cataracts; subsequent studies defined a syndrome with multiple abnormalities (Table 13.1), predominantly ocular, aural, and cardiac. Deafness as well as cataracts became hallmarks of congenital rubella syndrome (CRS) (Alford and Griffiths 1983).

About 20 years after Gregg's initial observations, two groups concurrently succeeded in cultivating the rubella virus: Thomas Weller and Franklin Neva at

**Table 13.1** Prominent clinical findings in congenital rubella syndrome

Encephalitis	Intrauterine growth retardation
Microcephaly	
Mental retardation	
Autism	Metaphysical rarefactions
Patent ductus arteriosus	
Peripheral pulmonic artery stenosis	Hepatosplenomegaly
Cochlear deafness	Thrombocytopenic purpura
Central auditory imperception	Interstitial pneumonitis
	Diabetes
	Hypothyroidism
Retinitis	
Cataracts	
Microphthalmia	
Glaucoma	

(From Alford CA, Griffiths PD. In: Remington JS, Klein JO, (eds.) Infectious Diseases of the Fetus and Newborn infant. WB Saunders, Philadelphia PA

**Fig. 13.5** Edward Buescher, Paul Parkman, Malcolm Artenstein (l-r)

Harvard Medical School used human amniotic cells to detect a cytopathic effect; and Paul Parkman, Malcolm Artenstein, and Edward Buescher at the WRAIR (Fig. 13.5) detected the virus by interference with an enterovirus in cultures of AGMK cells (Weller and Neva 1962; Parkman et al. 1962). These discoveries were

almost contemporaneous with a huge outbreak of rubella that started in Europe in 1961 and spread across the Atlantic Ocean to the U.S. in 1962 and 1963. In the U.S. alone the epidemic left in its wake thousands of cases of congenital anomalies, spontaneous abortions, and fetal stillbirths, while causing thousands of women to choose therapeutic abortion because of laboratory- or clinically- confirmed rubella (Alford and Griffiths 1983).

### ***13.3.2 History of Vaccine Development***

The 1962–1963 epidemic erased any doubt about the need for a vaccine against rubella that would protect women and their fetuses during pregnancy. Numerous laboratories set to work, most prominently that of Parkman and Meyer at the Food and Drug Administration (FDA) in Bethesda and Plotkin's laboratory at the Wistar Institute in Philadelphia, together with workers at Merck, Glaxo SmithKline, and Pitman-Moore pharmaceutical companies (Parkman et al. 1966; Meyer et al. 1966; Peetermans and Huygelen 1967; Buynak et al. 1968). Although inactivated whole virus vaccines were investigated (Sever et al. 1963), they did not produce sufficient immune responses, and to this day efforts at subunit vaccines have not produced encouraging results. Accordingly, almost all efforts were directed at a live attenuated vaccine, particularly with the then recent example of a successful, attenuated measles vaccine.

In the early 1960s rubella virus was adapted to many different cell types of primary cells, including AGMK, dog kidney, rabbit kidney, and duck embryo. Passage of each of these cells was thought to provide the correct level of attenuation, although in each case care had to be taken to eliminate any possibility of undetected adventitious agents being present in the cells cultivated from organs of the respective species.

In 1963 Paul Parkman left WRAIR to join Harry Meyer at the NIH Division of Biologics Standards, a forerunner to the Center for Biologics Evaluation and Research. The two colleagues subsequently developed the first live attenuated rubella vaccine using the 77th AGMK cell passage of a strain derived from Army recruits. Their high passage virus strain, HPV-77, was safe and immunogenic in clinical trials and was provided to Hilleman at Merck who adapted it by five additional passages in duck embryo cell culture, proved its effectiveness, and included it in the initial MMR vaccine preparation that was used in the 1970s in the U.S. (NIH 2005).

One of the authors (SP, Fig. 13.6) chose an alternative approach, using WI-38 fetal human lung cells. This decision was not taken lightly. The WI-38 cells were derived from frozen ampoules laid down by Leonard Hayflick and Paul Moorhead at the Wistar Institute. They had discovered that human fetal cells could be cultivated and passaged *in vitro* to produce enormous numbers of cells, but that the resulting cell strain had a finite number of passages before becoming senescent and ceasing replication (Hayflick 1968; Plotkin et al. 1969a; Plotkin 1971).

Thus, these cells were intermediate between primary cells and continuous cell lines. The former often contained unknown latent agents; whereas the latter were suspected of carrying oncogenic viruses or genetic material. However, human cell strains had normal diploid chromosomes and were free of contaminants, as long as they came from healthy donors. Hayflick and Moorhead had developed a large number of such cell strains, including WI-38, and in discussion with Hilary Koprowski, who had earlier developed the first live, oral polio vaccine, and the author, the idea of using those cells for vaccine production was formulated. Since then, human diploid cells have been used for the development of vaccines against rabies, varicella, and hepatitis A. However, at the time, this was an idea that was not accepted in scientific circles, as described below.

The author had ideas about developing a rubella vaccine even before the epidemic, having spent a year during 1961 and 1962 as a Registrar in Pediatrics at the Hospital for Sick Children on Great Ormond St. in London, where Alastair Dudgeon was then Consultant in Microbiology with interest in congenital infections. Spare time in his laboratory was used to investigate rubella virus, facilitated by the reports of its successful cultivation in 1961 (Plotkin et al. 1963; Plotkin 1996). Thus, upon return to Wistar at the end of 1962, the author was prepared to take on the project of rubella vaccine development.

Koprowski provided a laboratory, and with the acquisition of some small grants, work began. The disastrous epidemic of 1962–1963 was paradoxically providential in that the lab became involved in diagnosis of both infections during pregnancy and confirmation of fetal infection after abortion. Among the many rubella abortuses received, the 27th proved to be particularly important. Tiny bits of many organs from this fetus were placed into plastic culture vessels, hoping to see



**Fig. 13.6** Stanley Plotkin

infected cells attach and produce the virus. The third culture – RA 27/3 – which happened to be of fetal kidney, produced significant amounts of virus and was therefore passaged onto WI-38 cells to start the process of attenuation.

From work in Koprowski's laboratory on the development of attenuated polioviruses, the author had learned that passage at lower than physiological temperature, i.e., below 37°C, resulted in selection of attenuated virus populations. Therefore, rubella virus was adapted to passage in this fashion; the eventual lowest temperature at which good viral growth occurred was 30°C. However, any such process requires a measure of attenuation, so as to reach the right level without overly weakening the virus. Fortunately, there were two ways in which this could be done: by observing changes in *in vitro* markers – in this case plaque size – in rabbit kidney 13 cells, growth in baby hamster kidney cells, and growth at low temperature; alternatively, human clinical trials could directly assess attenuation.

The latter was possible owing to connections with St. Vincent's Catholic orphanage. Institutions such as the orphanage were subject to epidemics of communicable diseases, including those of rubella. Moreover, it was known that rubella was generally well tolerated in children, and that a single infection was liable to engender lifelong immunity. After obtaining permission from the Catholic authorities, a series of clinical trials with different levels of passage of the RA 27/3 virus was begun (Plotkin et al. 1965, 1967, 1968, 1969b; Plotkin 1968).

Both a change in *in vitro* markers of the virus and a diminution of virulence were noted with passage. This appeared to occur more rapidly than in rubella vaccine strains developed in monkey kidney (77 passages), duck embryo (82 passages), rabbit kidney (51 passages), or dog kidney (89 passages) cell substrates, but it was probably the adaptation to growth in the cold rather than the cell substrate that accounted for the rapid attenuation.

Although promising, the use of human diploid cell substrates for vaccines was controversial. At a 1968 meeting at the National Institutes of Health in which all of the groups developing rubella vaccines presented their results, sage virologist Albert Sabin made some invited remarks that included a denunciation of the RA 27/3, because in his view human diploid cell substrates were dangerous (Plotkin et al. 1969b; Plotkin 1971). Despite Sabin's formidable prestige, the author argued that all the experimental data were against his assertion, and that his opinion was strictly *ex cathedra* and without foundation in fact. This was probably impertinent for a young researcher, but the speech was greeted with thunderous applause, as the audience concurred (Sabin and Plotkin 1969).

Nevertheless, the situation was highly competitive; in 1969, all of the rubella vaccine strains mentioned above were licensed in the U.S. except for the RA 27/3 human diploid cell strain. However, the following year the Wellcome Labs in the U.K. licensed RA 27/3. This came about because the British regulatory head, Frank Perkins, was an early advocate of human diploid cell strains as a safer substrate.

Thus, during the 1970s several rubella vaccine strains were in use. It gradually became evident that the dog kidney vaccine was too reactogenic and that the rabbit kidney and duck embryo vaccines were too attenuated. The latter fact was ascertained by studies of superinfection after exposure to rubella virus,

most notably the work of Dorothy Horstmann of Yale University (Horstmann 1979). Additionally, the experience with RA 27/3 in Europe, where it was produced by a French manufacturer (Institut Mérieux) as well as Wellcome Labs, was successful.

To his everlasting credit, Maurice Hilleman at Merck laboratories realized that the RA 27/3 was superior to the vaccine Merck had been producing. The Wistar Institute subsequently granted Merck, as well as Glaxo SmithKline and other manufacturers overseas the rights to the virus; by the late 1970s, RA 27/3 was the only rubella vaccine used outside of Japan.

Although admittedly the authors may be biased, RA 27/3 was preferred because of its high immunogenicity, durability of antibody, induction of resistance to reinfection at the mucosal level, ability to immunize intranasally as well as parenterally, and acceptable level of attenuation, including harmlessness for the fetuses of inadvertently vaccinated pregnant women (Beasley et al. 1969; Dudgeon et al. 1969; Furukawa et al. 1969; Plotkin et al. 1973; Fogel et al. 1978; Polk et al. 1982). Whatever the case, rubella and the congenital rubella syndrome have all but disappeared in North and South America and Scandinavia using RA 27/3 (Cochi et al. 1989; Paunio et al. 1991; Plotkin 2006; CDC 2008b). Efforts are being made to eliminate rubella from all of Europe and from the Western Pacific region. Incorporation of rubella vaccine with measles or measles and mumps vaccines has facilitated its use in virtually all developed and mid-level developing countries, and efforts are being made to use it in poor countries where congenital disease still occurs. In these efforts men as well as women are being immunized in order to maximize herd immunity. As long as routine vaccination of infants is combined with vaccination of seronegative women of child-bearing age, success in controlling rubella is guaranteed.

### ***13.3.3 Implementation of Vaccination Programs and Impact on Burden of Disease***

The initial strategies for use of rubella vaccines varied. Fears about the potential teratogenicity of the vaccine viruses precluded vaccination of susceptible pregnant females, the group which most needed to be protected to prevent infection of their developing fetuses. The epidemiology of rubella showed that the major transmitters of infection were children, who presumably were the source for most of the infections of pregnant women.

Based on the epidemiology, the U.S. adopted a universal vaccination program of both girls and boys through school-age. Because epidemics had been occurring at 6–9-year intervals, with the last one during 1964–1965, there was concern that another epidemic was imminent at the time vaccines were licensed in 1969. Thus in that year, mass immunization campaigns of school-aged children were undertaken, followed by the institution of routine immunization, along with measles vaccine, during the second year of life. Vaccination of children was associated with

a marked decrease in reports of acquired rubella cases; the predicted epidemic of rubella and CRS never materialized.

In contrast, in the U.K. there was great concern that immunity induced by rubella vaccines might be short-lived and that maintaining circulation of the wild virus among children, with the long-term immunity that wild virus infections induced, was important to controlling the disease and preventing infection during pregnancy. Instead of vaccinating young children, a strategy of vaccination of pre-adolescent girls was chosen to induce protection in those who had not yet been infected by wild virus (Preblud et al. 1980; Dudgeon 1985). The U.K. strategy was designed to fill in the immunity gaps left by persons not infected by wild virus and thus, to develop a highly immune, post-pubertal female population.

Both strategies had their successes and failures. While the U.S. focus on childhood vaccination clearly impacted rubella by inducing herd immunity, cases of CRS continued to occur, albeit at low levels, presumably through adult-to-adult transmission. Thus, over time efforts were made to immunize susceptible women of child-bearing age through programs such as prenatal antibody screening and vaccination of identified seronegatives during the post-partum period. Implementation of the U.S. strategy led to a 2004 declaration that rubella was no longer endemic in the country (Reef and Cochi 2006).

In the U.K. problems continued with failures to achieve high coverage among pre-adolescent girls, continued circulation of the wild virus associated with cases of CRS, and multiple “scares” when pregnant women of unknown immunity status were exposed to rubella. Thus, in 1988, as part of the introduction of MMR vaccine into the U.K., the country adopted a strategy to terminate transmission through herd immunity, similar to that adopted by the U.S. (Hutchinson 1988). In essence, both countries developed programs that included components of the other’s strategy.

Currently, rubella vaccine is incorporated into the vaccine schedule in 130 countries worldwide (WHO 2008). In addition to developed countries, most middle income countries in the Middle East and Asia are vaccinating against rubella. PAHO established a goal to eliminate rubella and CRS in their region by 2010; by 2008, all 38 countries and territories there, with the exception of Haiti, had introduced MMR in their routine immunization schedules (CDC 2008b).

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# Chapter 14

## Diseases of Military Importance

Alan Cross and Phil Russell

### 14.1 Introduction

Beginning with George Washington's decision to immunize his army against smallpox during the War of the American Revolution, vaccines have traditionally been used as a primary strategy for the prevention of infectious diseases in military settings (Artenstein et al. 2005). This chapter will highlight two vaccine histories involving diseases of military importance. The history of epidemic typhus, caused by rickettsial organisms, is a story that weaves vaccine approaches together with other strategies that eventually controlled the disease and supplanted the need for a vaccine; another rickettsial disease, scrub typhus, continues to be of military concern, and protective vaccines remain elusive. In contrast, the history of adenovirus vaccines presents a dramatic success story but also a cautionary tale regarding the fragility of disease control in selected circumstances.

### 14.2 Typhus

#### 14.2.1 Background

Epidemic or classic louse-borne typhus is an acute, systemic febrile illness with rash caused by *Rickettsia prowazekii* that spreads from human to human via the body louse vector, *Pediculus corporis humanus*. Infection is typically acquired following

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the self-inoculation of a bite with the infected feces deposited at the site by the louse. Mortality rates from untreated typhus generally exceed 20% (Snyder 1965), but may be higher in the setting of other, comorbid illnesses. The disease may recrudesce as Brill-Zinsser disease years after infection due to loss of immunity. *R. prowazekii* has been listed as a category B threat agent of bioterrorism because of its potential for aerosol transmission and its weaponization by the former Soviet Union in the 1930s (Martin et al. 2007).

Perhaps more than any other organism, epidemic typhus has had a dramatic impact on wars throughout history. It may have affected the ancients, although it is difficult to delineate typhus in the absence of consistent case definitions from the other systemic, exanthematous, communicable illnesses that were prevalent at that time. However, it is evident that epidemic typhus was known in Europe by the fifteenth century and shortly thereafter landed in the New World along with the Spanish explorers and other infectious diseases (Weiss 1988). Outbreaks contributed to massive mortality among armies and their impacted civilian populations in preindustrial Europe. Typhus was a major factor in Napoleon's unsuccessful invasion of Russia in 1812, although it concurrently ravaged the Russian army as well. Epidemics were noted along the east coast of the U.S. throughout the mid-nineteenth century (Woodward 1973); the last outbreak, 484 cases, occurred in the tenements of the urban poor in New York City in 1892–1893.

While the prevalence of epidemic typhus in civilian risk groups decreased as a result of the implementation of improvements in sanitation and other public health interventions in the eighteenth and nineteenth centuries, it remained a prominent cause of noncombat morbidity and mortality during wars of the twentieth century. The disease was associated with crowded, poorly hygienic conditions, especially in cold weather environments, that extended beyond armies to involve the social disruptions engendered by war - refugees and displaced populations - and affect other subgroups, such as the poor, the homeless, the incarcerated, and other confined populations.

Epidemic typhus was rampant in parts of Europe during World War I. Approximately, 25 million cases with a resultant 3 million deaths due to typhus occurred in Russia in the years spanning World War I and its aftermath (Moe and Pederson 1980). In contrast to the high prevalence of louse-borne typhus on the Eastern front, it was a rarity on the Western front, where a total of only 42 cases were reported. Typhus was an important cause of disease in North Africa in the early part of U.S. involvement there, with more than 200,000 cases among the civilian population during World War II; the incidence markedly diminished with the institution of various preventive measures in that region. While only a single case of louse-borne typhus was reported among U.S. troops engaged in the Korean conflict, there were more than 30,000 cases among the South Korean soldiers and civilians associated with nearly 20% mortality (Kelly et al. 2002). Within the last two decades there have been intermittent outbreaks of epidemic typhus associated with famine, civil wars, and other forms of social disruption in Africa, Latin America, Eastern Europe, and Asia.

In 1909, Charles Nicolle, Director of the Pasteur Institute in Tunis, observed that once the hospitalized patients were bathed and their clothes changed, they were no

longer contagious. He then demonstrated that the uninfected lice were able to transmit typhus from infected monkeys to uninfected monkeys. The discovery of the role of body lice in the transmission of typhus earned Nicolle the Nobel Prize in 1928. He was the first to develop an experimental animal model of infection, the guinea pig. Later in 1909, Howard Ricketts, who had earlier discovered the etiologic agent of Rocky Mountain spotted fever (RMSF) that was subsequently named after him, identified an organism in the blood of ill typhus patients; work performed in Poland under the auspices of the International Red Cross in the 1920s provided definitive details regarding the etiology and vector of typhus (Weiss 1988).

### 14.2.2 Vaccines

Rudolf Weigl, a Polish biologist, produced the first effective typhus vaccine by feeding infected lice on volunteers, allowing *R. prowazekii* to propagate in the louse gut, and then using phenol-buffered suspensions of the typhus-engorged intestines as a vaccine (Murgatroyd 1940). He apparently vaccinated thousands of individuals during the 1930s, including European missionaries to China and Ethiopia (Wincewicz et al. 2007) and demonstrated a protective effect, although controlled studies were never performed (Weiss 1988). Early tissue culture methods of growing the agent of epidemic typhus (Nigg and Landsteiner 1932; Kligler and Aschner 1934) were supplanted by the more efficient techniques developed by Goodpasture in the early 1930s, who initially demonstrated the cultivation of poxviruses in chorioallantoic membranes of chick embryos (Chap. 9). In 1938, Herald Cox (1907–1986), a bacteriologist working for the U.S. Public Health Service at the Rocky Mountain Laboratory in Montana, cultivated rickettsiae in the yolk sacs of chick embryos and by doing so laid the foundation for advanced typhus vaccine development.

Although vaccines created from suspensions of louse feces or from killed, typhus-infected vectors were developed prior to the work of Cox, their methods were labor intensive and inefficient for large-scale production (Kelly et al. 2002). Cox (Fig. 14.1) developed the first rickettsial vaccine using a formalin-killed suspension of yolk sac-derived *R. rickettsiae* and through the chick embryo culture system, was able to efficiently produce large enough quantities of antigen for commercial production (Cox 1941). Experience using this “Cox-type” approach in millions of immunized troops and civilians during World War II subsequently suggested that such an inactivated vaccine prevented or ameliorated the manifestations of epidemic typhus, even though controlled studies were never performed (Snyder 1965).

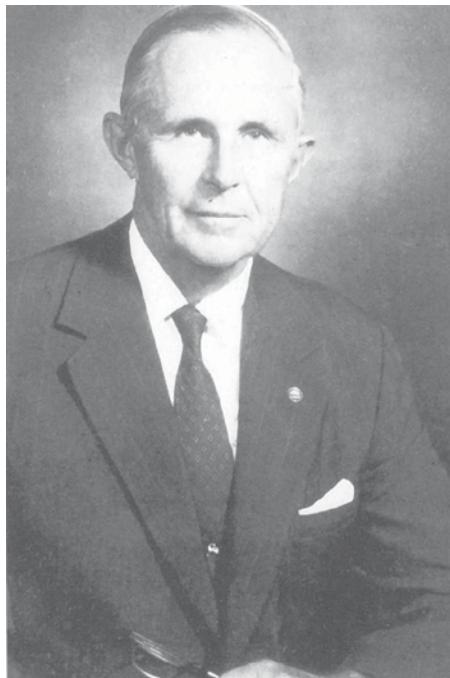
Shortly after the Japanese attack on Pearl Harbor in 1941, members of the U.S. military and the U.S. Public Health Service recommended the use of inactivated vaccine in U.S. forces deployed to combat theaters in order to prevent outbreaks of louse-borne typhus. The U.S. Typhus Commission, a joint service working group created by Presidential Executive Order in 1942, was instrumental in devising comprehensive, effective control strategies that included vaccination.



**Fig. 14.1** Herald Cox (Wellcome Library)

A sharp increase in typhus cases was noted in North Africa and Iran, sites of Allied force deployment during the war, due in part, to the arrival of 28,000 Polish refugees to Iran from Russia. The Commission recommended vaccine administration to U.S. military personnel deployed to southern Europe and North Africa. Despite the high incidence of epidemic typhus in military and civilian populations during World War II, there were only 64 cases and no deaths among American forces (Sadusk 1949), a fact attributed largely to a combination of the use of dichlorodiphenyl-trichloroethylene (DDT) as a vector control agent, command-enforced hygiene, and immunization with inactivated typhus vaccine.

In 1943, Theodore Woodward (1914–2005), a young Army medical officer and member of the Typhus Commission assigned to North Africa, tested an inactivated vaccine strain of *R. prowazekii* grown in chick embryos (i.e., “Cox-type”) against a strain harvested from rat lungs and used by the French, in a small group of healthy incarcerated volunteers in Morocco (Woodward 1986). In one of the few experimental studies on inactivated vaccine, albeit uncontrolled, and in data published more than 40 years after the study was performed because of their “classified” nature, Woodward (Fig. 14.2) demonstrated that subjects who had received the Cox-type vaccine developed adequate immune responses, no serious vaccine-associated reactions,



**Fig. 14.2** Theodore Woodward (History of the Armed Forces Epidemiological Board, Borden Institute)

and protection from disease after live organism challenge lasting 6 months. Following his military service, Woodward spent the rest of his career as a clinician, educator, and researcher at the University of Maryland School of Medicine, much of it devoted to the study of rickettsial diseases.

Several strategies for the control of typhus were developed by the Typhus Commission during the later years of the war. The inactivated vaccine, given to the physicians and laboratory personnel at the Cairo Fever Hospital in Egypt, was again shown to be effective in reducing the severity of typhus and preventing fatalities (Bayne-Jones 1943; Gilliam 1946; Snyder 1965). Vaccination, when used as a component of a comprehensive intervention strategy, controlled the disease. In the war ravaged city of Naples, experiencing a burgeoning outbreak in 1943 with 371 cases recognized in December alone, the Typhus Commission initiated a highly organized program of case and contact finding and immediate control measures of delousing and vaccination, thereby successfully halting a winter typhus epidemic for the first time in history (Wheeler 1946). Lessons learned from the successful anti-typhus campaign in Italy were applied to the control of louse-borne typhus in German concentration camps, in Japan, and later in Korea.

A second form of typhus vaccine was investigated in the 1950s. In his initial studies concerning rickettsial growth in chick embryo tissues, Cox had observed the attenuation of *R. rickettsiae*, the causative agent of RMSF, after serial passage in

eggs and noted that guinea pigs and rhesus macaques vaccinated with these strains were immune to challenge with high doses of virulent ones (Cox 1941). The concept of a live, attenuated typhus vaccine offered certain theoretical advantages over inactivated forms, most notably, the possibility of a prolonged duration of immunity with a single vaccine dose. The development of avirulent, *R. prowazekii* strain E vaccine, based on more than 260 serial passages in eggs represented one such approach (Fox 1956). Strain E vaccine underwent clinical testing in the early 1950s among volunteers in the Mississippi State Prison system and in extensive field trials in Peru (Fox et al. 1957). Although found to induce high levels of humoral immunity and protection from virulent challenge for more than 5 years, the live, attenuated vaccine was associated with significant adverse reactions that ultimately limited its acceptability (Wisseman 1972).

The relatively crude, but effective inactivated typhus vaccine was never approved by the U.S. government, yet it remained available into the 1960s. It was recommended for use by scientific investigators, medical personnel caring for typhus patients, and laboratory workers but not for routine civilian use (ACIP 1968). U.S. Army recruits received a two-dose series as recently as 1964 (Barker et al. 1967). After World War II, with the implementation of effective public health measures, vector control, and improved standards of living in many areas of the world, louse infestation and subsequently, epidemic typhus declined steadily until a vaccine approach was no longer needed. This could potentially change if the agent of epidemic typhus was to be employed in aerosol form as an agent of bioterrorism.

### **14.3 Scrub Typhus (Mite-Borne Typhus or *Tsutsugamushi* Disease)**

In contrast to epidemic typhus, scrub typhus continued to be a concern following the end of World War II. First described in the nineteenth century, the disease clinically manifests as an acute febrile, systemic illness with rash and frequently an eschar at the site of the vector bite; infection can lead to pneumonia, meningitis, disseminated intravascular coagulation, and vascular collapse. The etiologic agent, *Orientia tsutsugamushi*, is transmitted by the bite of a larval mite vector and is associated with three antigenically distinct prototype strains and more than 30 additional, antigenically distinct serotypes.

During World War II, scrub typhus was the most important rickettsial disease affecting American troops (Kelly et al. 2002). There were approximately 16,000 cases among Allied forces in the Pacific theater; more than 7,000 of these occurred in U.S. troops, associated with in excess of 300 deaths (Bavaro et al. 2005). Despite the absence of specific therapy or preventive vaccines, the use of repellents to destroy vectors and the clearance of bush surrounding camp areas eventually provided adequate preventive measures. Mission-compromising scrub typhus infections were common in Viet Nam and while there were no reported deaths, up to 30% of non-malarial, undefined fevers were attributable to this disease. Outbreaks have occurred within the past decade among U.S. forces in a variety of Asian Pacific settings.

**Fig. 14.3** Joseph Smadel  
(Albert and Mary Lasker  
Foundation)



Antimicrobial therapy for scrub typhus was pioneered by Joseph Smadel (1907–1963), Head of the Rickettsia Laboratory at the Walter Reed Army Institute of Research (WRAIR). Smadel (Fig. 14.3) directed the field trials, performed under the auspices of the Armed Forces Epidemiological Board with Woodward in Malaysia in 1948, that definitively demonstrated the curative potential of chloramphenicol in scrub typhus and showed that the drug also effectively treated murine typhus and typhoid fever (Smadel et al. 1948, 1949; Woodward 1994). Smadel and Woodward were nominated for the Nobel Prize in 1948 for their efforts toward the control of typhus and typhoid fever. Revelations of antibiotic resistance in up to 15% of scrub typhus isolates in Southeast Asia in the 1990s (Bavaro et al. 2005) provided further impetus to pursue vaccine strategies.

### **14.3.1 Vaccines**

Efforts to develop a protective vaccine against scrub typhus began in the 1940s using rickettsiae grown in chick embryos, analogous to the method applied to epidemic typhus vaccine, and continued in the latter twentieth century using cell culture-derived organisms. Neither of these approaches yielded a vaccine capable of providing long-term protection against scrub typhus, likely a function of the antigenic diversity of the causative agent.

Various vaccine strategies have been investigated; none have demonstrated long-term protection against heterologous strains of *O. tsutsugamushi*. A single serotype, formalin-killed vaccine was tested in 15,000 troops in the mid-1940s and showed no differences in the incidence of infection, morbidity, or death between vaccinated and unvaccinated subjects (Berge et al. 1949). Experiences with both live and irradiated vaccines have similarly demonstrated poor long-term protection against heterologous strains and poor tolerance with the former; however, irradiated vaccines induced a cellular immune response that may be important in protection.

Recent research has focused on the use of subunit vaccine strategies against *O. tsutsugamushi*. At least two protein antigens have been identified that were shown to induce humoral and cellular immune responses and in the case of one highly conserved gene containing both group-reactive and strain-specific B cell epitopes, protected animals from both homologous and heterologous challenge (Bavaro et al. 2005). Until a vaccine strategy is optimized for durable, broad protection, disease control must be based on preventive public health measures to limit exposure to vectors, and on prompt recognition and treatment of suspected cases.

## 14.4 Adenovirus

### 14.4.1 *Discovery of the Viruses and Association with Disease*

Respiratory diseases have been a significant problem for military forces throughout the history of modern warfare. Conditions of military life, including the crowded living conditions and physical stressors lend themselves to the spread of communicable respiratory illnesses (Gray et al. 2000), resulting in epidemics of viral and bacterial respiratory infections that have adversely affected military training and operations in every major human conflict. The most dramatic illustration of this, the devastating influenza pandemic of 1918, emerged during the final year of World War I, and global transmission was clearly facilitated by troop movements and military environments (Barry 2005).

At the beginning of World War II, the threat of communicable respiratory diseases to military operations was of such concern that the Army Surgeon General formed a Board for the Investigation and Control of Influenza and other Epidemic Diseases in the Army (refer to Chap. 11) comprising, among others, a Commission on Air-Borne Infections (Woodward 1994). This combined civilian and military advisory body later became the Commission on Acute Respiratory Diseases (CARD) under the auspices of the Armed Forces Epidemiologic Board. The CARD carried out its research agenda on the causes and prevention of respiratory disease in the military at several civilian academic laboratories as well as at WRAIR and the National Institutes of Health (NIH) (Jordan 1994). The work in civilian laboratories was funded through Army contracts; coordination was accomplished through semiannual meetings of the Commission with civilian and military scientists.

Detailed epidemiologic studies and human volunteer experiments conducted by the CARD defined an acute respiratory disease syndrome (ARD) that was prevalent in military training bases (Jordan 1994). ARD was differentiated from other prevalent respiratory diseases, common colds and influenza, by several lines of clinical and epidemiologic evidence, including observations that the institution of preventive measures and antibiotics developed during and immediately following World War II had largely controlled outbreaks of bacterial pneumonias and streptococcal diseases in military populations, and that annual vaccination with recently developed influenza vaccines had greatly reduced morbidity due to these viruses (Meiklejohn 1994).

However, seasonal epidemics of ARD continued and were recognized as a major cause of hospitalizations and loss of training time in Army and Navy recruit training facilities and to a lesser extent, in Air Force training facilities in the years following the War (Jordan 1994). These epidemics were especially severe in large northern bases such as Fort Dix, New Jersey (Top 1975), and the Great Lakes Naval Training Center (Rosenbaum et al. 1965) in North Chicago, Illinois. High disease attack rates in recruits during their first several weeks in camp during the annual winter epidemics of ARD severely disrupted the training activities (Buescher 1967). Military training bases were required to maintain large hospitals to care for the serious cases. During peak weeks of illness in the larger camps, attack rates of up to 80% were noted with as many as 20% requiring hospitalization (Dudding et al. 1973). The latter rate translated into hundreds of weekly admissions at some large recruiting centers. Additionally, 10–25% of ARD cases developed viral pneumonia, leading to additional and major losses of training time and rare deaths (Hilleman and Werner 1954; Jordan 1994).

In the early 1950s, scientists at two U.S. government virus research laboratories, using the newly described tissue culture methods of Weller, Robbins, and Enders (refer to Chap. 9), discovered the viral etiology of ARD. In 1953, Wallace Rowe, working to culture common cold viruses in Robert Huebner's Laboratory of Infectious Diseases at NIH, reported the recovery of three strains of viruses from adenoidal tissue that were initially designated adenoid degenerative agents (Rowe et al. 1953). Huebner, a virologist who later made seminal contributions to theories of viral oncogenesis, subsequently reported on 143 isolates and used the term adenoidal pharyngeal conjunctival (APC) viruses to describe the group (Huebner et al. 1954). Maurice Hilleman, a microbiology research scientist assigned as Chief of the Department of Respiratory Diseases at WRAIR, reported the isolation of a novel virus, RI-67, from the throats of military recruits suffering from ARD at Fort Leonard Wood, Missouri in the winter of 1952–1953 (Hilleman and Werner 1954). Hilleman referred to these agents as respiratory illness (RI) or ARD viruses.

The organisms from the NIH and WRAIR groups were found to be biologically related and represented a new family of viruses, variably designated in the literature of the time as Adenoid Degenerative viruses, APC viruses, RI viruses, or ARD viruses. At a scientific session in 1956 chaired by the venerable John Enders, consensus was reached on the term “adenovirus” to denote these pathogens (Enders et al. 1956); Hilleman's RI-67 virus became adenovirus type 4. Subsequent studies

showed that two strains of adenoviruses, types 4 and 7, caused most of the ARD cases in Army recruits. Later experience showed that other adenovirus serotypes including types 3, 14, and 21 also caused ARD in recruits but were much less prevalent (Gaydos and Gaydos 2004).

#### ***14.4.2 Inactivated Vaccines***

The two competing government laboratories proceeded to develop formalin-inactivated vaccines from viruses grown in primary rhesus monkey kidney cell cultures. Within 3 years of the identification of the infectious agent, Hilleman's group had developed a bivalent type 4 and 7 adenovirus vaccine which was shown to produce neutralizing antibodies in volunteers when administered parenterally. Field trials among recruits at Fort Dix and Fort Leonard Wood in 1957 and 1958 demonstrated more than 90% effectiveness of the vaccine in preventing ARD and correlation between virus neutralizing antibody in vaccinated volunteers and protection (Hilleman et al. 1958). The vaccine was licensed in 1958. For Hilleman (Fig. 13.4), who left WRAIR in 1957 to become the Director of Virus and Cell Biology at Merck Research Laboratories where he would flourish for the next 47 years, this would represent only the first in a long string of vaccine successes (Offit 2007).

Huebner's group developed an inactivated trivalent vaccine containing serotypes 3, 4, and 7, and it was also shown to be effective in initial trials. Later studies indicated significant variation in potency of these vaccines (Buescher 1967). The discovery of the oncogenic simian vacuolating virus, SV-40, that also contaminated oral and inactivated polio vaccines of the era, in primary rhesus monkey kidney cells and in the vaccine's seed viruses led to a suspension in the use of these vaccines by the military; their licenses were later revoked. Although never associated with human disease, SV-40 virus was shown to be oncogenic in animals and capable of transforming human cell cultures. Incorporation of SV-40 genes into type 7 adenovirus produced a potentially oncogenic hybrid virus (Huebner et al. 1964), an additional significant cause for concern. These events provided impetus for the isolation of vaccine virus strains that were free of adventitious agents.

#### ***14.4.3 Live Virus Vaccines***

The initial idea that unmodified, wild-type adenoviruses could be given orally in an enteric-coated formulation to selectively replicate in the intestinal tract and generate a protective immune response without causing disease resulted from discussions between Huebner and infectious diseases researchers Robert Chanock and Robert Couch at the National Institute of Allergy and Infectious Diseases (NIAID) (Chanock 2008). This novel concept was tested using low-passage type 4 and type 7 adenoviruses isolated from patients in human embryonic kidney (HEK) cells.

Viruses given to adult volunteers in enteric-coated capsules proved to be safe and highly immunogenic. Viral replication in the intestinal tract resulted in the excretion of the virus in stool and the generation of neutralizing antibody levels similar to those observed in natural infections. The two serotypes did not interfere when given simultaneously, and viral transmission to unvaccinated volunteers or close contacts was not observed (Couch et al. 1963).

The development of live, oral adenovirus vaccines was the result of a close collaboration between two physician scientists: Robert Chanock (1924–), who in 1968 became Huebner's successor as Chief of the Laboratory of Infectious Diseases at NIAID, a position he held for more than 30 years, and then Lieutenant Colonel Edward Buescher (1925–1989), Chief of the Department of Virus Diseases at WRAIR. The two were close friends, both had been fellows and were mentored in Albert Sabin's laboratory at Children's Hospital in Cincinnati, and both had served in the Army's 406th General Medical Laboratory in Japan conducting viral research. Between them they overcame the institutional rivalries of the past and in collaboration with Wyeth Laboratories provided the leadership that resulted in the successful development of the type 4 and type 7 oral adenovirus vaccines.

Chanock (Fig. 14.4) and coworkers tested adenovirus type 4 strains that had been isolated and passaged in WI-38 human diploid cell culture and formulated in enteric-coated capsules (Chanock et al. 1966). The vaccines proved to be safe and immunogenic; a small field trial in Marine trainees at Parris Island demonstrated a specific



**Fig. 14.4** Robert Chanock  
(Courtesy of the Chanock family)

protective effect (Edmondson et al. 1966). Studies of the transmissibility of the vaccine strain from vaccinees to family members were conducted by George Gee Jackson at the University of Illinois and indicated that infection of family members was rare and even when it occurred did not produce illness (Mueller et al. 1969).

Buescher (Fig. 13.5), at WRAIR, took over further development of the live adenovirus vaccines. Wyeth Laboratories received the vaccine strains from Chanock's laboratory and produced the vaccines for the Army. After preliminary safety, immunogenicity, and dose-ranging studies of a type 4 vaccine (Smith et al. 1970), a large-scale field trial in recruits at Fort Dix in 1965 demonstrated a 95% reduction of ARD due to type 4 adenovirus and an overall decrease in respiratory disease hospitalization by two-thirds. However, a second large vaccine trial in 1966 showed initial effectiveness that was followed by a resurgence of ARD to previous levels, attributed to the epidemiologic replacement of the previously dominant type 4 by type 7 adenoviruses due to vaccine-induced suppression of the former. This phenomenon greatly limited the military utility of a monovalent type 4 vaccine; it became readily apparent that a bivalent vaccine was required (Buescher 1967).

Further development of live, oral, type 7 adenovirus vaccines had been suspended after the demonstration of the oncogenicity of certain adenovirus serotypes in suckling hamsters; type 7 viruses were observed to have moderate oncogenic potential. Concern about human oncogenicity prevented further vaccine development until extensive studies by Maurice Green at St. Louis University, in which he searched for evidence of oncogenic adenoviruses in human tumors proved negative, and further understanding of the oncogenicity of adenoviruses in rodents allayed fears that these agents might be associated with human cancers (Wold et al. 1979).

Franklin Top Jr, working in Buescher's Department at WRAIR became the principal investigator in developing and testing type 7 adenovirus vaccines. He field tested the two vaccines administered simultaneously and found that when used together at the beginning of training, the vaccines were extremely effective in suppressing ARD epidemics in recruits (Top et al. 1971a, b, c). An efficient formulation of lyophilized virus in enteric-coated tablets developed at Wyeth replaced the capsules, and although a loss of potency occurred in some early lots during storage, the problem was traced to solvent trapped in the tablet during the manufacturing process and was solved by a relatively trivial modification.

In 1971, the Army began vaccinating all incoming recruits with both type 4 and type 7 products during the winter months. A comprehensive surveillance system that monitored ARD rates, determined the viruses involved, and rapidly provided the information to Army preventive medicine authorities was implemented in training camps. This surveillance system enabled Army scientists to track vaccine effectiveness (Dudding et al. 1973). From 1971 until 1980, the vaccine was used in recruit training installations under an investigational new drug (IND) status. The impressive safety record developed then enabled Wyeth Laboratories to obtain licensure in 1980. Routine use of type 4 and type 7 adenovirus vaccines effectively controlled ARD in military recruits for 25 years with only a few lapses due to delayed vaccine production (Top 1975; Gaydos and Gaydos 2004). The large hospital wards at recruit camps formerly used to treat ARD cases were closed. Sporadic, small outbreaks of disease due to adenovirus type 21 led to initial clinical

studies of a live, oral type 21 vaccine (Dudding et al. 1972; Scott et al. 1972), but it was shown to interfere with the type 7 vaccine when given simultaneously and when development ceased (Takafuji et al. 1979).

In 1984, Wyeth Laboratories asked the Department of Defense (DoD) for funds to upgrade their manufacturing facilities to meet increasingly stringent regulatory requirements. The DoD refused the request for additional funds and after years of fruitless negotiations, Wyeth ceased manufacturing adenovirus vaccines in 1995. Despite a program of reduced use, vaccine stocks were exhausted by 1999. The result of the shortsighted policy decisions was the re-emergence of adenovirus respiratory disease outbreaks in recruit training bases (Barraza et al. 1999). A population-based surveillance system, established in 1996 under the leadership of Gregory Gray at the Naval Health Research Center, had documented the resurgence of adenovirus disease in four military training facilities (Ryan et al. 2002). The disease patterns were similar to those observed in the 1960s, with adenovirus types 4 and 7 predominating (Gray et al. 1999, 2000); two deaths due to adenovirus pneumonia occurred in naval recruits (CDC 2001). Unvaccinated personnel with ARD were significantly more likely to be infected with adenovirus than were their vaccinated counterparts.

#### ***14.4.4 Redevelopment of the Vaccine***

An analysis of cost effectiveness published 2 years after the initial adenovirus vaccine program's implementation was favorable (Collis et al. 1973); another study, requested by the DoD, nearly 30 years later again showed impressive cost effectiveness of the vaccines in addition to their important health benefits (Hyer et al. 2000). Following strong recommendations from the Institute of Medicine (Lemon et al. 2001) and the Armed Forces Epidemiological Board, the DoD contracted with Barr Laboratories in 2001 to redevelop live, oral, adenovirus type 4 and 7 vaccines using technology and virus strains obtained from Wyeth. Apart from minor manufacturing and formulation modifications, the vaccines are the same as the original products. A phase three clinical trial in Army and Navy training installations was begun in 2007; regulatory approval of the vaccine is expected in 2009.

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# **Chapter 15**

## **Varicella and Zoster**

**Anne A. Gershon**

### **15.1 Introduction**

It has been more than 30 years since Michiaki Takahashi of Osaka, Japan, attenuated the varicella–zoster virus (VZV) to produce the Oka vaccine strain of live, attenuated varicella vaccine. The initial trials of safety, immunogenicity, and efficacy were carried out in Japan in the early 1970s. Subsequently, large-scale confirmatory studies of immunogenicity and safety were performed mainly in the United States. Initially, these involved immunocompromised children, but later healthy children were immunized, and there was greater interest in vaccinating healthy populations than immunocompromised ones. These clinical trials culminated in the licensure of a single dose of varicella vaccine for all American children in 1995. Currently, the vaccine is used to prevent chickenpox worldwide and has been licensed for universal vaccination in many countries including Australia, Canada, Germany, Israel, Qatar, Sicily, South Korea, Taiwan, and Uruguay (Gershon et al. 2008). While it has not achieved general acceptance in Japan, interest in its use there is increasing. While it took many years to gain general acceptance in Japan, the vaccine is now being used more and more in that country. The latest exciting advance regarding the Oka vaccine is that a high-titered formulation is now being used as a therapeutic vaccine to boost immunity to VZV in the elderly with latent infection in order to prevent zoster. This chapter discusses the almost 40-year saga of the development of vaccines against VZV.

### **15.2 Disease History**

The dermatomal eruption of zoster was probably first described by the ancient Greeks. The history of varicella was confounded by its clinical similarities to smallpox, and until the late eighteenth century, it was commonly thought to represent an

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altered, milder form of that disease. Epidemiologic evidence in the latter part of the nineteenth century suggested the co-identities of the microbiologic etiologies of varicella, also called chickenpox for uncertain reasons, and zoster (Wood 2000). In one of the multiple futile attempts to isolate varicella in the late 1940s, Thomas Weller in the Enders laboratory fortuitously observed the successful cultivation of poliovirus in tissue culture explants in 1948; the report revolutionized virology and vaccinology, as discussed in Chap. 9, and facilitated the cultivation of other organisms. By 1952, once poliovirus investigations had concluded in the Enders laboratory, Weller returned to the investigation of other viruses and successfully cultivated varicella from vesicular fluid of individuals with either varicella or zoster, thus also establishing their viral identity (Weller and Stoddard 1952; Weller 1992).

### 15.3 Vaccine Development

Attenuation of VZV by Takahashi (1928–) and his colleagues in 1974 was truly a remarkable advance (Takahashi et al. 1974). Takahashi (Fig. 15.1), then Professor of Virology at the Research Institute for Microbial Diseases at Osaka University, had graduated from Osaka University Medical School in 1954 and completed the Graduate Course of Medical Sciences in 1959, majoring in poxvirus virology. He was then appointed Research Assistant in the Department of Virology at the Research



**Fig. 15.1** Michiaki Takahashi  
(Courtesy of Dr. Michiaki  
Takahashi)

Institute where he studied attenuated measles and polio vaccines. Subsequently, he performed serial 1-year stints between 1963 and 1965 at Baylor Medical College in Texas as a research fellow studying adenoviruses and at the Fels Research Institute of Temple University in Philadelphia researching bacteriophage genetics.

While in Houston, his son developed severe chickenpox, and Takahashi was inspired to try to develop a live, attenuated varicella vaccine. Between 1970 and 1985, he mentored what became a world-class group of virologists and physician-scientists, many of whom such as Koichi Yamanishi, Yoshizo Asano, Takao Ozaki, and Hitoshi Kamiya remain leaders of Medical Departments and/or Research Institutions in Japan. Takahashi, for his part, received many awards for varicella vaccine development, including the Kojima Saburo Memorial Award (1975), the Asahi Award (1985), and the Scientific Achievement Award from the VZV Research Foundation (1997).

As Weller had learned, VZV is a notoriously difficult virus to propagate; the latter property, of course, is a pre-requisite for vaccine development. Yields of VZV in culture tend to be low, and the virus is highly cell-associated and does not reach the culture media in an infectious form. Moreover, there was (and is) no animal model to test vaccine candidates for attenuation, as had been done initially with measles vaccine. Only yellow fever (Theiler and Smith 1937), polio (Sabin et al. 1954), measles (Katz et al. 1958), mumps (Hilleman et al. 1968), and rubella (Plotkin et al. 1969), a remarkably small number of viruses, had been attenuated to produce a successful live virus vaccine. To attenuate VZV, Takahashi subjected a varicella virus obtained from an infected child named Oka to the following maneuvers: 11 serial passages in human embryonic lung fibroblasts at 34°C; 12 serial passages in guinea pig fibroblasts at 37°C; and then roughly ten additional passages in human embryonic lung fibroblasts at 37°C. Although this strategy succeeded in Takahashi's hands, it was not uniformly productive. After years of attempts, Merck scientists were unable to attenuate VZV in this fashion; their virus was either too reactogenic or not sufficiently immunogenic (Neff et al. 1981).

Initial small vaccine trials consistently showed safety and efficacy of Takahashi's vaccine in healthy Japanese adults and children. The vaccine terminated outbreaks of varicella (Takahashi et al. 1974) and interrupted transmission in families when administered as prophylaxis to children recently exposed to their siblings with chickenpox (Asano et al. 1977). Remarkably, the vaccine safely protected children who were somewhat immunocompromised from developing severe chickenpox (Takahashi et al. 1990). Especially compelling were studies indicating that children receiving chemotherapy for leukemia in remission could be safely immunized and achieve detectable immune responses to VZV, although the protective efficacy of the vaccine in these children was not extensively tested (Izawa et al. 1977).

The newly evolving techniques of molecular biology in the 1980s played a particularly important role in the development of varicella vaccine. Manifestation of some form of rash is not uncommon after VZV vaccination, and it was therefore critical to have a rapid means of diagnosing VZV and determining whether an eruption was wild-type in etiology or related to the Oka vaccine strain, particularly during the initial evaluations of vaccine safety and efficacy. Before the advent of molecular

methods, it was necessary to propagate the virus and then analyze its DNA; later, it became possible to diagnose and type VZV without having to isolate the virus, by employing the polymerase chain reaction (PCR) to amplify the viral DNA directly, followed by restriction enzyme analysis of the amplification products.

These studies were initially led by Philip LaRussa in the Department of Pediatrics and Saul Silverstein in the Department of Microbiology at the Columbia University College of Physicians and Surgeons. The presence of a unique splicing site for Pst 1 in gene 54 of wild-type VZV was exploited by these investigators and has been critical in interpreting specimens from clinical events such as breakthrough chickenpox and possible zoster in vaccinees (LaRussa et al. 1992). More recently, single nucleotide polymorphisms (SNPs) in gene 62 have been noted that are specific to vaccine-type VZV (Loparev et al. 2007). However, differentiating between vaccine and wild-type viruses in this fashion must be interpreted with caution (Lopez et al. 2008), as rarely VZV may have a single SNP that is characteristic of the Oka strain but have all the other characteristics of a wild-type strain, and the latter is indeed clear based on history (Lopez et al. 2008).

In the late 1970s, the mortality rate for the prevalent childhood disease chickenpox in young, varicella-susceptible children thought to be cured of leukemia was 7% in studies in the U.S. (Feldman et al. 1975). Despite the fears of chickenpox in such settings, development and use of the vaccine was extremely controversial. Pediatrician thought leaders, Henry Kempe from Colorado University School of Medicine (Kempe and Gershon 1977) and Saul Krugman from New York University (NYU) Medical Center were among the first to advocate testing of varicella vaccine in the U.S. Krugman, then Chairman of Pediatrics at NYU, had widely respected vaccinology credentials from his roles in the development of measles, rubella, and hepatitis B vaccines. Well aware of the potential complications of chickenpox in children and adults (Krugman 1960; Krugman et al. 1957), Krugman was extremely impressed with the successful development of what appeared to be a highly protective vaccine against varicella by Japanese investigators. As more Japanese studies appeared in the medical literature (Hattori et al. 1976; Izawa et al. 1977; Sakurai et al. 1982; Kamiya et al. 1984), it gradually became apparent that protection of immunocompromised children from varicella by vaccination was not only possible but also of potential clinical significance (Kempe and Gershon 1977).

Consequently, with the support and guidance of William S. Jordan and Harry Meyer at the National Institute of Allergy and Infectious Diseases (NIAID), a workshop meeting of American, European, and Japanese representatives of academia and commercial vaccine producers was convened in Bethesda, Maryland in February 1979 to discuss issues surrounding varicella vaccine, including whether clinical trials should be carried out in the U.S., as well as the possible development of other herpesvirus vaccines. The meeting's participant roster reads like a contemporary "Who's Who" of medical virology and included, among many others, Philip Brunell, Anne Gershon, Harry Ginsberg, Maurice Hilleman, Kenneth McIntosh, Myron Levin, Joseph Melnick, Thomas Merigan, June Osborne, Joseph Pagano, Philip Pizzo, Stanley Plotkin, Gerald Quinnan, and of course Michiaki Takahashi.

The recommendation emanating from the 3-day conference was that research on varicella vaccine be encouraged in the U.S. Jordan (1917–2008) served as the Director of the Microbiology and Infectious Diseases Program at NIAID, whose mission was the advancement of research initiatives concerning vaccines within the National Institutes of Health (NIH), a position in which he could advocate for varicella vaccine investigation. He was highly respected in the medical community, having served in several senior academic positions, including Dean at Western Reserve, the University of Virginia, and the University of Kentucky Medical Schools prior to his NIH appointment. Jordan was subsequently awarded the Gold Medal of the Sabin Vaccine Foundation in 2004 for his lifetime work on numerous vaccines. He established the well-known scientific review known as the Jordan Report, still issued by NIH several times a year and considered to be the most complete reference available on vaccine research and development.

Following the pivotal 1979 meeting, varicella vaccine began to be intensively studied in American adults and in leukemic children in remission. Over the next decade, it was consistently shown in a number of clinical trials that varicella vaccine was safe and effective, not only in varicella-susceptible adults but also in these high-risk children. The availability of the antiviral drug acyclovir to treat those who developed rash and fever related to the vaccine made studies in immunocompromised children possible, as it provided an additional safety “net” yet did not interfere with immune responses to the vaccine (Brunell et al. 1982; Gershon et al. 1984; Arbeter et al. 1990). These studies that were led by well-established investigators, many such as Stanley Plotkin at the Children’s Hospital of Philadelphia, recognized as distinguished vaccinologists, at an early stage of vaccine development in the U.S. were crucial to varicella vaccine’s eventual global success. The studies identified and quantitated adverse effects such as vaccine-associated rashes, breakthrough varicella infections, and other types of vaccine failures. Additional work has shown varicella vaccine to be one of the safest vaccines available.

The largest of the early U.S. clinical trials, a direct result of the 1979 workshop, was a collaborative vaccine study sponsored by NIAID and involving over 500 children with leukemia in remission, led by the author, then in the Department of Pediatrics at New York University. Although approximately 25% of vaccinated children developed a vaccine-associated rash requiring treatment with oral or intravenous acyclovir, none developed life-threatening varicella from either the vaccine or subsequently from the wild-type virus after exposure to a person with chickenpox or zoster. The vaccine was 85% effective in preventing chickenpox entirely; most children were given two doses at 3 months’ interval (Gershon et al. 1984; Gershon et al. 1996a). Breakthrough disease in these children, who were at risk of severe or even fatal chickenpox before vaccination, was uniformly mild and did not require specific antiviral treatment.

The favorable results of these studies in high-risk children rapidly led to clinical trials of vaccine safety and efficacy in healthy children. Almost immediately, interest in immunizing healthy children eclipsed that in immunocompromised children. Despite the absence of observed mortality related to the Oka vaccine strain, concern persisted that it was not entirely non-pathogenic and that vaccination of large numbers

of immunocompromised children might yield hitherto unforeseen serious adverse events. It seemed more reasonable to try to build a background level of immunity to varicella by universally vaccinating healthy children. Many of the original theoretical safety concerns, such as oncogenic potential and enhanced zoster incidence following varicella vaccination had been examined and essentially dispelled by this time. Additionally, since varicella caused approximately 150 annual deaths in the U.S. in the modern, pre-vaccine era, mainly in previously healthy adults and children, and was associated with in excess of 10,000 annual hospitalizations, the availability of a safe varicella vaccine was clinically useful and less controversial than had been initially thought (Gershon et al. 2008).

An early randomized, double blind, placebo controlled study in healthy children given a single dose of vaccine by Merck investigators showed 100% efficacy in the first year after vaccination (Weibel et al. 1984). Unfortunately, the dose of vaccine virus in this study, 17,000 plaque forming units (pfu), was more than tenfold higher than that used in the varicella vaccine which was eventually licensed in the U.S. (Varivax™, 1,350 pfu). Although the dose of vaccine was not considered to be of importance in the ultimate immune response, it is now clear that dosage is not only important but critical for the development of immunity; there is a direct relationship between the dose of virus and vaccine immune response. For this reason, the Merck study failed to accurately predict the effectiveness of the subsequently licensed vaccine, although it provided proof-of-concept regarding vaccine efficacy. A subsequent randomized, double blind study of Varilrix™ varicella vaccine, manufactured by Glaxo SmithKline, was carried out in Finland by the highly respected Finnish vaccinologist, Professor Timo Vesakari, and it also demonstrated over 90% efficacy at a dose of 10,000 pfu (Varis and Vesikari 1996), which is 0.7-logs higher than the minimum titer of the GSK vaccine currently licensed in Canada, Australia, and some European countries.

Additional, uncontrolled, open label clinical trials eventually leading to licensure of Varivax™ in the U.S. examined safety and immunogenicity in over 9,000 children. These studies also predicted high degrees of vaccine efficacy based on antibody testing (White 1996). However, these predictions may have been overestimated because the antibody test used to determine immunity to VZV in these trials was overly sensitive and a suboptimal surrogate for immunity. Post-licensure case-control studies have indicated vaccine effectiveness of about 85% after a single dose in children aged 1–12 years (Vazquez et al. 2001, 2004). This degree of protection contrasts with the protection rates in excess of 95% seen with many other childhood vaccines such as those against rubella and measles.

Fortunately, in the pivotal NIAID study of vaccine efficacy in leukemic children, one of the primary outcomes used to determine vaccine efficacy was whether or not the child was protected following an intimate exposure to a person with varicella (Gershon et al. 1984). This was based on the original recommendation of Wolf Szmuness of the New York Blood Center. Szmuness, a refugee from Poland after World War II, was a close friend and colleague of Saul Krugman; they collaborated in a study on the efficacy of hepatitis B vaccine in New York City's gay community. Serendipitously, Szmuness participated in many of the planning discussions between

the author and Krugman in late 1979, resulting in expansion of the outcome measurements to include protection after a household exposure as well as immunologic surrogates.

As a result of the successful clinical trials in healthy children, Merck's varicella vaccine was licensed in 1995, with recommendations by both the Advisory Committee for Immunization Practices (ACIP) and the Committee on Infectious Disease of the American Academy of Pediatrics (AAP) for single dose, universal vaccination of all healthy children aged 1–13 years in the U.S. (Committee on Infectious Diseases 1995; CDC 1996). Following licensure, the concept, leadership, administration, and monitoring of universal vaccination against varicella were largely due to the outstanding efforts of Jane Seward, Deputy Director of the Division of Viral Diseases in the National Center for Immunizations and Respiratory Diseases at the CDC (Seward et al. 2002, 2004; Seward and Orenstein 2006).

Varicella vaccine (and more recently, zoster vaccine) remains the first and only live, attenuated herpesvirus vaccine to be licensed anywhere in the world. Because all known herpesviruses cause lifelong, latent infection, one of the original safety concerns about a VZV vaccine was that it might lead to a higher prevalence of zoster in vaccinated populations. However, it was subsequently shown that zoster was actually less common after vaccination than after natural infection (Hardy et al. 1991). This phenomenon was demonstrated in several additional studies in immunocompromised hosts (Brunell et al. 1986; Takahashi et al. 1985; Broyer et al. 1997), and extrapolated data from this population has led to the expectation of less zoster in healthy, vaccinated individuals. It will take many years to prove the latter because of the typically prolonged latency period of the virus after natural varicella and possibly varicella vaccine.

Regarding other safety issues, there are a handful of case reports in which immunocompromised children have had rash and/or pneumonia after vaccination, and were subsequently successfully treated with antivirals (Sharrar et al. 2000; Galea et al. 2008), although it was realized in retrospect that most of these children did not meet the criteria for vaccination in the first place. There have also been several cases of zoster in which Oka strain viral DNA has been transiently demonstrated in the cerebrospinal fluid of vaccinees (Levin et al. 2003; Civen et al. 2008; Galea et al. 2008), and six reported instances of person-to-person transmission of the Oka vaccine strain through contact resulting in mild disease (Gershon et al. 2008). Overall, based on the enormous volume of use of this vaccine in the U.S., with over 50 million doses distributed as of this writing, the associated risks appear to be incredibly small especially when compared to the virulence of the wild-type virus.

In order to evaluate the safety and efficacy of varicella vaccine following licensure, Seward and her CDC colleagues devised a creative investigative plan. Owing to the persistent annual case burden of natural varicella, it is not practical for it to be a reportable disease in the U.S. Seward, therefore, established three sentinel surveillance sites to monitor vaccine use and varicella and zoster incidence in counties in California, Texas, and Pennsylvania in 1995. It was assumed that the effects of varicella vaccination there would be reflective of the larger U.S. population. Since then, the incidence of varicella has fallen by 80% in these locations in both

vaccinees and also in unvaccinated individuals, the latter finding indicative of herd immunity (Seward et al. 2002). Varicella-associated hospitalization rates have decreased by 88% (Zhou et al. 2005), and age-adjusted mortality has fallen by 66% (Nguyen et al. 2005).

Optimal strategies for the use of varicella vaccine continue to evolve. Numerous outbreaks in school and daycare settings among groups of children with a high immunization penetrance must be addressed. Most, but not all of these breakthrough infections, resulted in non-serious illnesses, and the majority of infected children were previously unvaccinated. However, these outbreaks suggest a potential degree of either primary (i.e., failure to seroconvert) or secondary (i.e., waning of immunity) vaccine failure.

A re-evaluation of the seroconversion rate after one dose of vaccine was, therefore, undertaken using a highly sensitive and specific test for immunity, the fluorescent antibody to membrane antigen (FAMA) assay (Williams et al. 1974). Since its development in 1974, the assay has been performed by one person, Sharon Steinberg, a Research Associate currently at Columbia University. Although it is a rather difficult assay to perform, requiring live cell cultures infected with VZV presumably to preserve the conformation of viral antigens, it has proven to be highly predictive of immunity to varicella (Michalik et al. 2008). In a study of 148 healthy children in New York, California, and Tennessee vaccinated with a single dose and analyzed by FAMA, seroconversion rates were surprisingly low, 76% (Michalik et al. 2008). This degree of primary vaccine failure could account numerically for most of the breakthrough varicella observed in outbreaks.

It has been hypothesized that the seroconversion rate after a single vaccine dose in healthy children in early studies was overestimated by the original antibody test. It is possible that one dose of vaccine fails to provide enough of an immune stimulus to assure a durable, memory T cell response. Some vaccinees fail to seroconvert after a single dose and subsequently develop unmodified varicella, which may be mild, as varicella often is; others may rapidly lose detectable antibodies. Children in the latter group may be those who develop a modified form of breakthrough varicella upon exposure to wild-type VZV. These observations prompted the recommendation for two doses of vaccine for all children, not just older ones (Arvin and Gershon 2006). Among many others, Ann Arvin, a herpes virologist and viral immunologist at Stanford University Medical Center, played an important role in the ultimate recommendation for a routine two-dose schedule of varicella vaccine by the CDC in June 2006 (Arvin and Gershon 2006).

One study from the CDC has also suggested that there is some degree of secondary vaccine failure over time (Chaves et al. 2007). Because this study did not consider primary vaccine failures, the interpretation of the epidemiological data may be biased (Michalik et al. 2008). Other investigations have not indicated significant secondary vaccine failures (Vazquez et al. 2004). The question of whether there is significant waning of immunity after varicella vaccination, therefore, remains controversial.

The potential consequences of vaccine failure are significant as they may deleteriously impact both public health, such as the transmission of wild-type VZV and the accumulation of young adults who are susceptible to chickenpox, and individual

health concerns, such as an increased risk of development of latent infections. Investigations into the mechanisms by which VZV establishes latency from cell free enveloped virions in skin vesicles and is transmitted as aerosolized cell free virions from vesicles suggest that with fewer cases of breakthrough disease there will be lower rates of latent VZV infection and subsequent zoster due to fewer VZV eruptions (Chen et al. 2003, 2004).

It is hoped, but remains unproven, that administration of more than one dose of varicella vaccine to all children will offer a solution to vaccine failure. After a second dose of varicella vaccine, considerable boosting of immunity occurs. With measles–mumps–rubella–varicella (MMRV) combined vaccination, the seroconversion rates for varicella increased from 89 to 99%, and the geometric mean titer (GMT) increased from 12 to 469 units/mL after the second dose, as measured by a glycoprotein ELISA test (Shinefield et al. 2005). Similar immune boosts were not seen for the measles, mumps, or rubella components, suggesting that for varicella in contrast to the other viral antigens, the immune response is incomplete after a single dose. Another study, conducted over a 10-year period following immunization with either one or two doses of monovalent varicella vaccine, projected that the incidence of breakthrough varicella was more than three times lower in children who were given multiple doses (Kuter et al. 2004). Additional data on the effectiveness of two doses in the prevention of breakthrough varicella are warranted now that two doses have become the standard in the U.S.

Recent developments on the Oka vaccine virus, spearheaded by Michael Oxman of the University of California at San Diego Medical Center and Myron Levin at the University of Colorado Medical Center, demonstrate its use as a therapeutic, rather than a preventive vaccine to prevent reactivation zoster in the elderly who are latently infected with VZV from a past episode of chickenpox. The vaccine formulation contains approximately tenfold the dose (i.e., 17,000 pfu) of the Merck varicella vaccine; this high dose was required to boost cell-mediated immunity in the elderly population. The vaccine has shown efficacy in the prevention of both zoster and post-herpetic neuralgia, the latter a potentially severe morbidity of zoster, especially in the elderly (Oxman et al. 2005). It was demonstrated to be 64% effective in preventing zoster in the 60–69 age group and 41% effective in those 70–79 years of age; vaccination was 55% effective in preventing post-herpetic neuralgia in the latter age group (Oxman et al. 2005). The vaccine appears to be well tolerated in these elderly individuals and associated with few, if any attributable, serious adverse events.

Consideration ought to be given to change the nomenclature of Oka vaccines to VZV vaccines at this point, with subheadings of varicella and zoster, although the mechanisms of prevention of VZV disease are different for the two vaccines. When vaccinating prophylactically against varicella, both humoral and cellular immune responses to VZV are engendered in susceptible hosts. In the absence of rash or breakthrough varicella, latent infection may not occur, thus precluding the future development of zoster. In zoster vaccination, the recipient already harbors latent VZV related to previous varicella, thus the vaccine is considered therapeutic rather than preventive. The mechanism for the prevention of zoster reactivation involves boosting the cell-mediated immune response to VZV.

A theoretical, long-term consequence of universal varicella vaccination of children is that the incidence of zoster in unvaccinated individuals will increase due to less community exposure to circulating wild-type virus. This is much less of a concern for vaccinees as zoster is less frequent after immunization than after natural infection (Hardy et al. 1991). Boosting of immunity can prevent zoster, as demonstrated in the Shingles Prevention Study (Oxman et al. 2005) and in other studies as well (Gershon et al. 1996b). Whether reduced circulation of wild-type VZV actually increases the burden of zoster is difficult to ascertain. The results of investigations in the vaccine era have been inconsistent as the prevalence of zoster is increasing in the U.S. (Insinga et al. 2005; Jumaan et al. 2005; Leung et al. 2005; Mullooly et al. 2005; Yih et al. 2005). Prior to the development of varicella vaccine, increases in zoster prevalence were observed several times in the twentieth century (Ragozzino et al. 1982; Brisson et al. 2001; Leung et al. 2005). Additional data are necessary to answer such concerns regarding zoster. The use of the Oka vaccine strain to prevent zoster could be useful in managing this theoretical circumstance, should it develop, and the recommended age of vaccination could be lowered.

At present, VZV vaccines have worldwide utility. An exception might be in countries where the rate of infection with human immunodeficiency virus-type I (HIV) is high and both varicella and zoster are common. Zoster causes tremendous morbidity in HIV-infected individuals. Varicella vaccine is useful in children with HIV infection as long as the child is relatively immunologically intact at the time of immunization (Levin et al. 2006). It is also highly successful in preventing both varicella and zoster in HIV-infected children whose immune systems remain relatively well preserved (Son et al. 2008; Wood et al. 2008). Interestingly in one study, the incidence of zoster in unvaccinated HIV-infected children did not increase, despite a well documented decrease of 64% of the circulating wild-type virus after 1999 (Son et al. 2008). It is rewarding to realize that a vaccine once considered highly controversial and of limited benefit has become widely used worldwide and is improving the quality of life in both healthy and immunocompromised individuals.

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# Chapter 16

## Polysaccharide Vaccines

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By the first half of the twentieth century, many of the most clinically important infectious diseases of humans, including smallpox, yellow fever, and influenza, had been controlled through the use of either live-attenuated or killed-whole cell vaccines. Certain bacterial infections, such as cholera and typhoid had been successfully addressed using killed organisms (refer to [Chap. 6](#)); others, such as tetanus and diphtheria, had been controlled using vaccines comprising immunogenic bacterial component proteins (refer to [Chap. 7](#)). Vaccinology's subsequent "golden age," made possible by advances in the laboratory in the late 1940s that allowed the growth of viruses *ex vivo* in cell culture systems (refer to [Chap. 9](#)), led directly to successful vaccines against the scourges of childhood, such as polio, measles, mumps, and rubella. Despite these apparent successes, other clinically important pathogens continued to elude a vaccine solution. It was through the study of these organisms that landmark discoveries in microbiology and immunology paved the way for a novel approach to vaccines.

### 16.1 Polysaccharides as Immunogens

It was recognized as early as Pasteur's time that certain bacteria produced polysaccharides. The concept of using bacterial polysaccharides as immunogens derived from the seminal discovery by roommates and scientific colleagues Oswald Avery and Alphonse Dochez at the Rockefeller Institute in New York of a soluble, type-specific substance produced in broth cultures of pneumococci and in the blood and urine of patients with pneumococcal pneumonia that precipitated with homologous anti-pneumococcal serum (Dochez and Avery [1917](#)). Unlike proteins, the substance

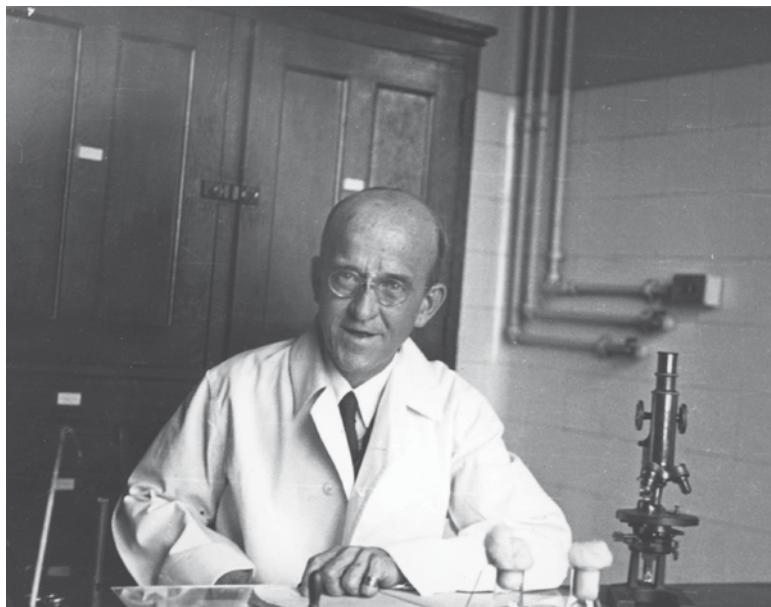
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resisted heating and trypsin digestion (Dochez and Avery 1917). That this material, later found to be the polysaccharide component of the bacterial capsule, could engender antigenic specificity went against the prevailing dogma in the field that recognized the supremacy of proteins as immunogens. Dochez moved on to other communicable disease challenges and met with continued success in his research career, discovering the streptococcal etiology of scarlet fever and the viral etiology of the common cold, and serving on Rockefeller's Board of Scientific Directors for nearly two decades (Corner 1964). However, Avery devoted much of his subsequent career accumulating evidence of the immunochemical importance of bacterial polysaccharides and, later, of deoxyribonucleic acids (DNA).

Oswald Avery (1877–1955), Canadian by birth, spent his entire professional life in New York City. After completing medical school at Columbia and an additional 6 years performing bacteriologic research at the Hoagland Laboratory in Brooklyn, he accepted an offer to join the research staff of the pneumonia service at the fledgling Rockefeller Institute Hospital in 1913 (MacLeod 1957). From that point, Avery's (Fig. 16.1) research efforts became focused largely on the pneumococcus and the relationship between its chemical composition and its biological properties and clinical manifestations (Dubos 1976). Towards the end of his active career, this research thread eventually led to the revolutionary discovery, by Avery and two colleagues, Colin MacLeod and Maclyn McCarty, that DNA, not protein, was responsible for the transformation of pneumococcal types (Avery et al. 1979) and was, therefore, the carrier of genetic information.



**Fig. 16.1** Oswald Avery (Rockefeller Archive Center)

Armed with the knowledge of a “soluble specific substance” that in some fashion afforded pneumococci antigenic specificity, Avery formed a collaboration in 1922 with Michael Heidelberger, an organic chemist working in kidney diseases at the Rockefeller Institute Hospital, in order to identify and characterize the material and, in so doing, possibly establish the “molecular basis of immunological specificity” (Dubos 1976). Together, the two not only identified the “soluble substance” as largely comprising complex polysaccharides (Heidelberger and Avery 1923, 1924), an unexpected finding as immunologic specificity was attributed largely to proteins at that time, but they also elucidated a founding principle of immunochemistry (Heidelberger 1927). Moreover, their observations laid the foundation for a quantitative understanding of the antigen-antibody interaction (Heidelberger and Kendall 1929) and new approaches, based on polysaccharides, to preventive vaccines.

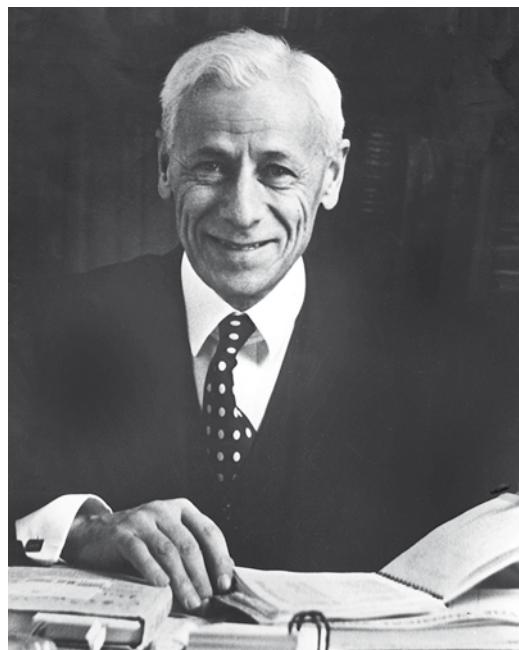
The initial findings by Avery and Heidelberger regarding the polysaccharide identity of the “specific soluble substance” and that the polysaccharides of different pneumococcal serotypes were biochemically distinct were so unexpected to even the pair of careful scientists that they felt compelled to subject the material to a wide variety of purification procedures and chemical analyses before definitively concluding that the immunologic specificity was not due to contaminating proteins (Heidelberger 1977; Dubos 1976). Nonetheless, as with other new paradigms that move scientific knowledge forward, they do not occur without significant upheaval (Kuhn 1996). The attribution of the immunological specificity of pneumococci to their polysaccharide capsule met with initial controversy but was eventually accepted due to the sheer volume of corroborating scientific data from a broad array of microbial species (Dubos 1976). Based on this early work, Wolfgang Casper, a young physician working in Oscar Schiemann’s Berlin laboratory, demonstrated in 1927 that purified preparations of pneumococcal capsular polysaccharides were immunogenic and protected mice against the strains of virulent organisms from which they were derived (Schiemann and Casper 1927).

Shortly thereafter, Heidelberger (1888–1991) (Fig. 16.2) moved to the Columbia University College of Physicians and Surgeons where he spent the next nearly 30 years unraveling the immunochemistry and biochemistry of antibodies and continuing his quantitative studies on pneumococcal capsules and other microorganisms (Heidelberger 1979). He blazed new trails with his scientific work and also with his international humanitarian efforts on behalf of the United Nations Association (Heidelberger 1979). His work and that of Avery would lead directly to vaccination using capsular polysaccharides and, ultimately, to the successful use of polysaccharide–protein conjugate vaccines.

## 16.2 Pneumococcal Vaccine

Sir William Osler’s “captain of the men of death” (Osler 1901), the pneumococcus, was isolated in 1881 by both Pasteur in the course of his rabies studies and by George Sternberg, the latter considered to be the founding father of American bacteriology.

**Fig. 16.2** Michael Heidelberger  
(Albert and Mary Lasker  
Foundation; Columbia  
University)



Sternberg would later become the Surgeon General of the United States and in that capacity, he would leave a significant imprint on vaccine history through the establishment of the Army Medical School (which would become the Walter Reed Army Institute of Research) and the Yellow Fever Commission, headed by Major Walter Reed (Malkin 1993). By the late 1880s, the pneumococcus had been shown to be the primary causative agent of lobar pneumonia (Sternberg 1897), the most common and most lethal acute infectious disease of Osler's time.

The existence of multiple pneumococcal serotypes, demonstrated by Neufeld, using the Quellung reaction in the early part of the twentieth century (Neufeld 1902), coupled with the immunochemical revelations of Avery and Heidelberger in the 1920s, led directly to the introduction of serum therapy for pneumococcal pneumonia. In 1913, Neufeld, working at the Koch Institute in Berlin, as well as Rufus Cole and Dochez from the Rockefeller Institute Hospital in New York reported success with the use of type I pneumococcal antisera in patients with pneumonia (Corner 1964). Although the results of its use in humans proved favorable in some studies, serum therapy never fulfilled its theoretical potential or the initial promise seen in animals (Parish 1965). This approach was permanently discarded with the advent of clinically effective antimicrobial chemotherapy in the late 1930s, an advance that limited vaccine research as well for years to come (Heidelberger 1979). However, as shown early during the clinical use of antibiotics, vaccine prophylaxis remained the most effective means of improving mortality in bacteremic pneumococcal disease (Austrian and Gold 1964).

Almroth Wright, the British bacteriologist responsible for the first large-scale vaccination using killed typhoid vaccine at the end of the nineteenth century (refer to Chap. 6), was the first to attempt prophylactic vaccination against pneumococcus, using whole-cell heat-killed organisms in approximately 50,000 South African gold miners in 1911, (Wright et al. 1914a, b). This approach, adopted prior to an understanding of serotype-specific immunity, provided only limited and transient protection. Following the demonstration by Avery and Heidelberger of the immunologic specificity of the bacterial capsular polysaccharide, a series of studies performed in the 1930s characterized the type specificity of pneumococcal polysaccharides in humans (Francis and Tillett 1930) and demonstrated the presence of circulating antibodies to homologous polysaccharides in the convalescent sera of patients with lobar pneumonia (Finland and Sutliff 1931). Max Finland and colleagues from the Thorndike Laboratory at Boston City Hospital subsequently showed that pneumococcal polysaccharides were immunogenic when given parenterally, resulting in the appearance of antibodies against homologous types that were protective in mice (Finland and Sutliff 1932; Finland and Dowling 1935; Finland and Ruegsegger 1935). They observed this with a number of pneumococcal types of varying preparations, dosage ranges, and modes of administration (Finland and Brown 1938), thus paving the way for clinical trials of polysaccharide vaccines.

During the 1930s, a series of five pneumococcal polysaccharide vaccine field trials was conducted in the Depression-era Civilian Conservation Corps camps on both coasts under the auspices of the U.S. Army and the U.S. Public Health Service (Ekwurzel and Simmons 1938). A total of approximately 61,000 individuals were vaccinated with a product containing 1 mg each of type I and type II polysaccharides; each of the trials included a group of uninoculated, unmatched controls. Although the initial studies suggested vaccine efficacy, the results were inconclusive. The final two trials, performed in the winter of 1936–1937, enrolled 70,000 mostly young adult men, of whom nearly 30,000 were vaccinated and the remainder served as controls. These studies were designed to definitively assess efficacy; however, multiple factors, including the relative young age of the vaccinees, the differing risk environments in the camps, incomplete pneumococcal typing, and the lack of subject randomization might have impacted their validity (Ekwurzel and Simmons 1938). Nonetheless, at both trial locations, the incidence of pneumonia due to homologous types in vaccinated subjects was reduced as compared with that in controls, suggesting a potential beneficial effect and confirming the trend noted in their earlier studies; no effect on survival could be ascertained, as the total number of deaths was too low for an assessment. Additionally, the vaccine had no impact on the occurrence of other respiratory diseases.

A prolonged epidemic of pneumococcal pneumonia among recruits at an Army Air Force Technical School in Sioux Falls, South Dakota, between 1942 and 1944 provided the milieu for what would become the first definitive trial of a polyvalent pneumococcal polysaccharide vaccine in humans. The study, designed to mitigate sources of bias and address some of the confounding variables noted in other trials, was performed under the aegis of the Commission on Pneumonia of the Armed

Forces Epidemiological Board by Colin MacLeod (who was then the Director of the Commission on Pneumonia), Heidelberger, and colleagues from the Army Medical Corps (Woodward 1994).

The researchers immunized 8,586 troops with a quadrivalent vaccine comprising capsular polysaccharides from types I, II, V, and VII pneumococci; 8,449 troops, randomly assigned to an unimmunized control group that was shown to be matched for age and length of service, received injections of isotonic saline with phenol (MacLeod et al. 1945). Epidemiologic data compiled by Lieutenant Richard Hodges at the School had previously demonstrated that nearly two-thirds of the cases of pneumococcal pneumonia were due to the types contained in the vaccine. In addition to assessing the impact of vaccination on the incidence of disease in vaccinees, MacLeod and colleagues also chose to evaluate its effect on transmission and pneumococcal carriage rates.

After the second week following inoculation, none of the vaccinees and 23 of the control subjects developed pneumonia due to pneumococcal types contained in the vaccine, a statistically significant difference (MacLeod et al. 1945). This time frame for protection correlated with the kinetics of serum antibodies directed against the inoculated polysaccharides. Interestingly, the incidence of vaccine type-specific pneumonia in the unimmunized population, although higher than that in the vaccinated group, was still dramatically lower than predicted based on the epidemiology of the epidemic during preceding seasons (MacLeod et al. 1945). Both vaccinated and unvaccinated groups appeared to have reduced carriage rates of pneumococcal types contained in the vaccine, which was postulated to be the mechanism of the overall disease reduction among the squadron. There were no differences in the incidence of pneumonia due to pneumococcal types not contained in the vaccine between vaccinated and control subjects, confirming the type specificity of the vaccine (Table 16.1).

MacLeod's trial clearly demonstrated that a large outbreak of pneumococcal pneumonia in a confined population could be rapidly terminated by the use of a multivalent polysaccharide vaccine. Shortly thereafter, Heidelberger reported the successful development of an immunogenic hexavalent polysaccharide vaccine, comprising the serotypes most commonly responsible for human pneumococcal disease at the time (Heidelberger et al. 1948). Based on the work of MacLeod and others, two hexavalent pneumococcal polysaccharide vaccines were licensed and commercially produced in the late 1940s; both were subsequently voluntarily withdrawn from the market owing to their lack of use in the setting of newly discovered antibiotics and the promise of the latter to relegate pneumococcal disease to the dustbin of history (Austrian 1975).

The sense of complacency within the medical and scientific communities regarding the lack of perceived need for pneumococcal vaccines in the fledgling antibiotic era would only be transitory. In 1964, Robert Austrian and Jerome Gold, from the University of Pennsylvania School of Medicine, published the results of a 10-year study that revealed the pneumococcus to be a persistent cause of morbidity and mortality despite the impression within the medical profession that it "no longer constitutes a serious medical problem" due to the availability of antibiotics

**Table 16.1** Prevention of pneumococcal pneumonia by immunization with specific capsular polysaccharides

Interval	Number of cases of pneumonia			
	Types I, II, V, VII in immunized subjects	Types I, II, V, VII in non-immunized subjects	All other types in immunized subjects	All other types in non-immunized subjects
Weeks				
1	2	0	1	1
2	2	3	5	3
3	0	3	7	5
4	0	2	8	12
6	0	2	6	7
8	0	2	3	4
10	0	1	4	4
12	0	0	2	4
14	0	2	2	1
16	0	3	2	4
16+	0	8	16	14
Total	4	26	56	59

From MacLeod et al. 1945

(Austrian and Gold 1964). They demonstrated that pneumococcal infections were not, in fact, on the decline and that antimicrobial therapy did not significantly alter the outcome of bacteremic pneumococcal pneumonia among those individuals who were at risk to die within the first 5 days of infection (Austrian and Gold 1964). Prophylaxis using a multivalent polysaccharide vaccine was still considered to provide the best opportunity for protection.

Austrian (1917–2007) devoted his career to pneumococcal research, spending the last 45 years of his life based at the University of Pennsylvania. He was a persistent advocate of a vaccine approach to preventing infection, providing data and consultative guidance in support of pneumococcal vaccine development at Merck. With his staunch support, pneumococcal vaccine became yet another such agent produced under the direction of Maurice Hilleman. Austrian (Fig. 16.3), who was awarded the Lasker award in 1978 for his work on pneumococcal infections, directed the pivotal clinical trials of the pneumococcal polysaccharide vaccine, sponsored by the National Institute of Allergy and Infectious Diseases (Klein and Plotkin 2007) and performed in South African gold miners.

The most fertile gold fields in the world, in the Transvaal area of northern South Africa, opened the same year, 1886, that the pneumococcus was proven to be the most common cause of lobar pneumonia; since then, gold mines and pneumococcal disease have been inextricably linked (Austrian 1978). The mines employed hundreds of thousands of young, native African men for 6–9-month stints, a rotational system that ensured a continuous supply of immunologically naïve hosts into the environment. Pneumococcal disease, causing sinusitis, meningitis, and pneumonia, was endemic in the mines and periodically erupted in epidemic form (Austrian 1978). This likely reflected the overcrowded living conditions and the intimate intermingling of numerous

**Fig. 16.3** Robert Austrian  
(University of  
Pennsylvania)



susceptible individuals with those harboring divergent pneumococcal types, although the effects of working 2 miles underground in extreme conditions of temperature and humidity likely contributed to disease patterns.

As with Sir Almroth Wright's studies 60 years earlier, South African gold miners became the test subjects in Austrian's pivotal vaccine trials involving a hexavalent vaccine and subsequently one comprising 13 pneumococcal polysaccharide capsular types. The first trials began in 1972 at the East Rand Preparatory Mine, just east of Johannesburg, and involved 12,000 miners (Austrian 1978). Overall, the polyvalent vaccines were found to be associated with a statistically significant 80% reduction in the incidence of pneumococcal pneumonia and bacteremia caused by relevant capsular types (Austrian et al. 1976).

On the basis of these and other trials (Smit et al. 1977), a tetradecavalent vaccine was licensed in 1977, followed in 1983 by a vaccine containing 23 of the most clinically relevant capsular types (Austrian 1996). Subsequent analyses in various at-risk populations have shown aggregate protective efficacy of polyvalent pneumococcal vaccines to range between 56 and 67% for infection caused by relevant serotypes (Shapiro et al. 1991; Sims et al. 1988; Bolan et al. 1986). Additionally, the vaccine has shown important "herd" effects on the unvaccinated population (Austrian 1978; Kyaw et al. 2006). More recently, the use of pneumococcal polysaccharides conjugated to protein carriers, discussed below, has demonstrated promise in improving the utility of these vaccines in pediatric and other high-risk

populations (Eskola and Anttila 1999; Black et al. 2000; CDC 2000), in reducing the carriage rates of pneumococci (CDC 2005), and the prevalence of antimicrobial-resistant organisms (Kyaw et al. 2006).

### 16.3 Meningococcal Vaccine

The clinical entity of meningococcal meningitis was first reported in 1805 in Switzerland (Vieusseux 1805) and in 1806 in the United States in Medfield, Massachusetts (Danielson and Mann 1806). In the latter report, the severe consequences of this “singular and very mortal disease” were described in great clinical detail, although it would be more than 80 years before the causative agent, *Neisseria meningitidis* – the meningococcus, was isolated from cerebrospinal fluid (CSF) of infected individuals (Weichselbaum 1887). As with the pneumococcus, multiple serotypes of meningococci were recognized in the early twentieth century, and shortly thereafter passive therapy using immune sera was applied to the infection with some success (Flexner 1913). However, the use of this approach was supplanted by the advent of antimicrobial chemotherapy in the 1930s.

An epidemic of meningitis was initially reported from Algiers in the mid-nineteenth century; multiple outbreaks of meningococcal disease occurred in Africa during the last decade of the nineteenth century and the first decade of the twentieth century (Greenwood 1999). Epidemic disease erupted during World War I among troop recruits, and based on concerns regarding potential outbreaks associated with American entry into World War II, the Commission on Meningitis became part of the original charter of the Armed Forces Epidemiological Board (Woodward 1994). In excess of 5,000 cases of meningococcal disease were documented among non-deployed U.S. troops between 1941 and 1943; most of these occurred within a recruit’s first 3 months of military service (Woodward 1994).

With the concerns regarding potential communicable disease transmission among new military recruits and in the context of the recently completed pneumococcal polysaccharide field trials and preparation for MacLeod’s trial underway, a vaccine approach to meningitis became a consideration. Elvin Kabat (1914–2000), a “young whirlwind” of a student who had initially joined Heidelberger’s laboratory in 1933 simply as a “helper”, in short order adapted Heidelberger’s quantitative method of immunochemistry to bacterial agglutination (Heidelberger 1979). Kabat (Fig. 16.4) obtained his PhD in Heidelberger’s laboratory at Columbia in 1937, the year that the first antibiotic, sulfa, was introduced as a treatment for meningococcal meningitis (Schwentker et al. 1937).

Shortly after the attack on Pearl Harbor, Kabat proposed using the recently purified group A meningococcal polysaccharide as an immunogen, based on demonstrating the presence of anticapsular antibodies in convalescent sera of experimentally infected animals and analogous to the contemporaneous work with pneumococcal vaccines (Kabat 1983). After antibody responses in preliminary subjects, measured by precipitin assay, were determined to be poor as compared with those observed

**Fig. 16.4** Elvin Kabat  
(Courtesy of  
Dr. Geoffrey Kabat)



using pneumococcal polysaccharide immunogens, the effort was abandoned (Kabat et al. 1945). In retrospect, Kabat believed that this represented a failure of technology; their precipitin assay was too insensitive to detect the antibodies, an issue that would be resolved by the time of the next vaccine effort two decades later. Additionally, the poor immunogenicity of Kabat's polysaccharide preparation was also probably related to its low molecular weight.

By the early 1960s, less than 25 years after the introduction of sulfa drugs, the majority of clinical meningococcal isolates were resistant to these agents (Artenstein and Gold 1970). An outbreak of group B meningococcal disease at Fort Ord, California, in 1964 (Gauld et al. 1965; Cataldo et al. 1968) and of group A disease in Morocco shortly thereafter (Alexander et al. 1968) confirmed the clinical consequences of drug resistance. As had occurred with pneumococcal disease, such events led experts in the field to believe that a meningococcal vaccine represented the best hope for prevention of infection in susceptible individuals.

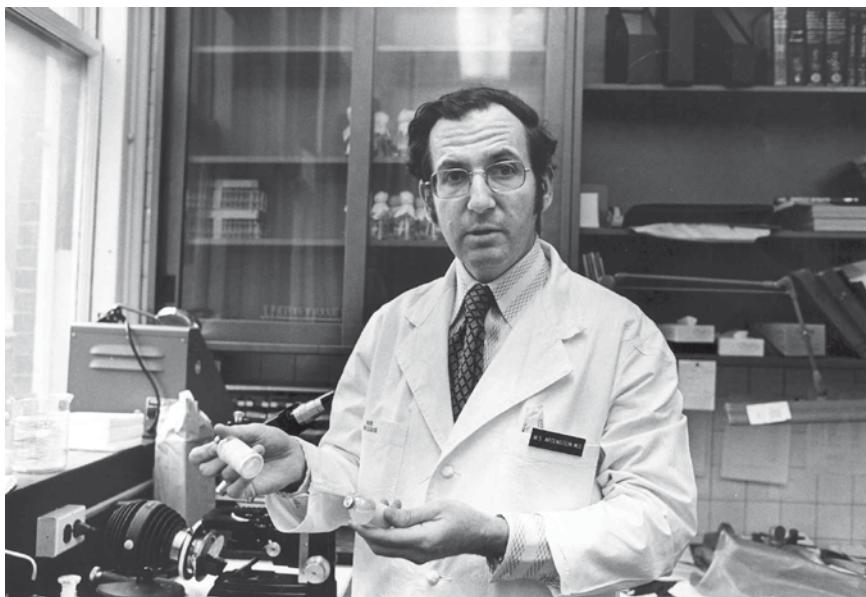
Because of the relevance of epidemic meningitis in military populations and the contemporaneous example of the rapid emergence of antimicrobial-resistant pneumococci, by the mid-1960s military scientists were firmly engaged in laboratory investigations into the pathogenesis and immunology of meningococcal infections. The studies, performed by three physician scientists working at the Walter Reed Army Institute of Research (WRAIR) were reported in 1969 in a series of papers that detailed the human immunologic response to the meningococcus. The team included Malcolm Artenstein (1930–1976), the civilian Chief of the Department of Bacterial Diseases, who had previously been part of the group there that first isolated

the rubella virus (refer to [Chap. 13](#)); and Emil Gotschlich and Irving Goldschneider, both young military officers who would go on to productive civilian research careers at the conclusion of their mandatory service. Gotschlich left WRAIR in 1968 and returned to the Rockefeller University where he would continue a distinguished career in research that would be recognized by the Lasker Award for Clinical Research in 1978; he eventually rose to the position of Vice President for Medical Sciences there. Goldschneider continued his successful immunologic research at the University of Connecticut Health Center.

Using prospectively collected sera from military recruits at Ft. Dix, New Jersey, one of the major basic training embarkation sites for the U.S. Army at the time, the WRAIR group demonstrated the protective role of circulating capsular antibody against clinical disease. Pre-existing antibodies were detected in 89% of recruits, none of whom acquired meningococcal disease; 38% of those lacking serum bactericidal antibodies developed systemic disease upon exposure to pathogenic strains of the organism (Goldschneider et al. [1969a](#)). They also determined that immunity to the meningococcus was serogroup specific, based on the capsular polysaccharide (Goldschneider et al. [1969b](#)), and they further described novel methods for the preparation of high molecular weight group A and C polysaccharides (Gotschlich et al. [1969](#)). In contrast to Kabat's experience with meningococcal polysaccharide immunogens two decades earlier, the WRAIR investigators demonstrated their polysaccharides to be highly immunogenic in human volunteers after a single intradermal injection (Gotschlich et al. [1969a](#)) and to lead to a significant reduction of the carriage rates of group C meningococci, the major reservoir of transmission, in the nasopharynx of recipients (Gotschlich et al. [1969b](#)).

Armed with these data, which included an elucidation of the correlate of protective immunity, Artenstein ([Fig. 16.5](#)) and colleagues at the WRAIR designed and executed a series of large field trials of the meningococcal group C polysaccharide vaccine. A number of epidemiologic factors complicated the design of a group-specific efficacy study: the relatively low incidence of meningococcal disease, even during epidemics, necessitated a large sample; the unpredictability of outbreaks in different training camps necessitated the involvement of multiple, large military recruit centers; and the rapid acquisition of the meningococcal carrier state among new recruits required that informed consent and vaccination had to be accomplished within days of arrival (Artenstein et al. [1970](#)). Additionally, the potential introduction of bias based on the herd effect of vaccination on the nasopharyngeal carriage rate in the control group was accounted for by designing the studies to immunize only 20% of the population (Gotschlich et al. [1969b](#)).

The first efficacy trial involved 13,763 men randomly assigned to receive group C meningococcal polysaccharide vaccine at five large Army basic training facilities; in the second, 14,482 were vaccinated at three sites (Artenstein et al. [1970](#); Gold and Artenstein [1971](#)). In aggregate, more than 114,000 men served as unimmunized controls in the two studies. Over an observation period of 8 weeks, which was the duration of basic training, no systemic reactions and only minimal local reactions to the vaccine were noted. Seventy-three cases of group C meningococcal disease occurred in the controls as compared with two in the vaccinated groups; the overall reduction in attack rates from all studies was consistent with an 89.5% group-specific, protective

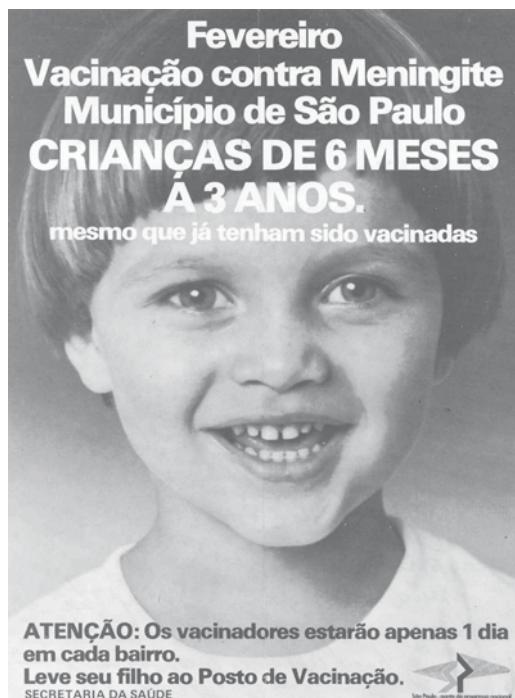


**Fig. 16.5** Malcolm Artenstein (Dr. Andrew W. Artenstein)

effect of the vaccine. Although the acquisition of group C meningococcal carriage was reduced in vaccinees, carriage rates for all other serogroups were similar.

Within 18 months after completion of the efficacy studies, routine vaccination using group C meningococcal polysaccharide was instituted at U.S. military training centers and resulted in the virtual elimination of group C disease as a military health problem (Artenstein 1975). Shortly thereafter, a group A polysaccharide vaccine proved highly effective in preventing group A meningococcal disease in Egypt (Wahdan et al. 1973). A quadrivalent, purified polysaccharide vaccine comprising group A, C, Y, and W-135 components was licensed in 1981 and has been successfully applied to prevent meningococcal disease in those at high risk.

However, meningococcal polysaccharide vaccines were suboptimally immunogenic and demonstrated relative poor efficacy in younger children, findings observed in a field trial conducted in the midst of a group C epidemic in São Paulo, Brazil, in 1974 (Morais et al. 1974) (Fig. 16.6). As with pneumococcal polysaccharides and as discussed below, conjugation to antigenic proteins represented a means to enhance immunogenicity. The introduction of a monovalent, group C meningococcal polysaccharide–protein conjugate vaccine in England in late 1999 led to an initial dramatic decrease in the incidence and mortality of meningococcal disease in targeted pediatric populations (Ramsay et al. 2001). Similar results were demonstrated elsewhere in Europe over the last decade (Larrauri et al. 2005; de Greeff et al. 2006). A quadrivalent polysaccharide product conjugated to diphtheria toxoid protein, licensed in 2005, may improve the immunogenicity and protective efficacy of the multivalent product in the pediatric population.



**Fig. 16.6** Poster advocating meningococcal vaccination for children between the ages of 6 months and 3 years around São Paulo, Brazil, 1974 (Dr. Andrew W. Artenstein)

The development of effective vaccines against group B meningococci, still a major cause of epidemic and sporadic disease throughout the world, is complicated by the poor immunogenicity of the organism's polysaccharide and its cross-reactivity with sialylated structures in human tissues (Stephens et al. 2007). Therefore, alternative approaches based on outer membrane proteins from genetically modified group B strains (Jódar et al. 2002) or on novel candidate antigens identified via genomic screening (Pizza et al. 2000; Giuliani et al. 2006) may offer opportunities for advances in this effort.

## 16.4 *Hemophilus influenzae* Vaccine

*Hemophilus influenzae* type b (Hib) was long recognized as a cause of meningitis in infants and young children (Rivers and Kohn 1921), although the organism was initially a cause of great controversy because of its hypothesized, and later disproved, role in the etiology of influenza in the late nineteenth and early twentieth centuries (refer to Chap. 11). In 1931, while still at the Rockefeller Institute, Margaret Pittman (refer to Chap. 7) demonstrated the existence of both encapsulated and nonencapsulated forms of *H. influenzae* and determined that there were six types of the former

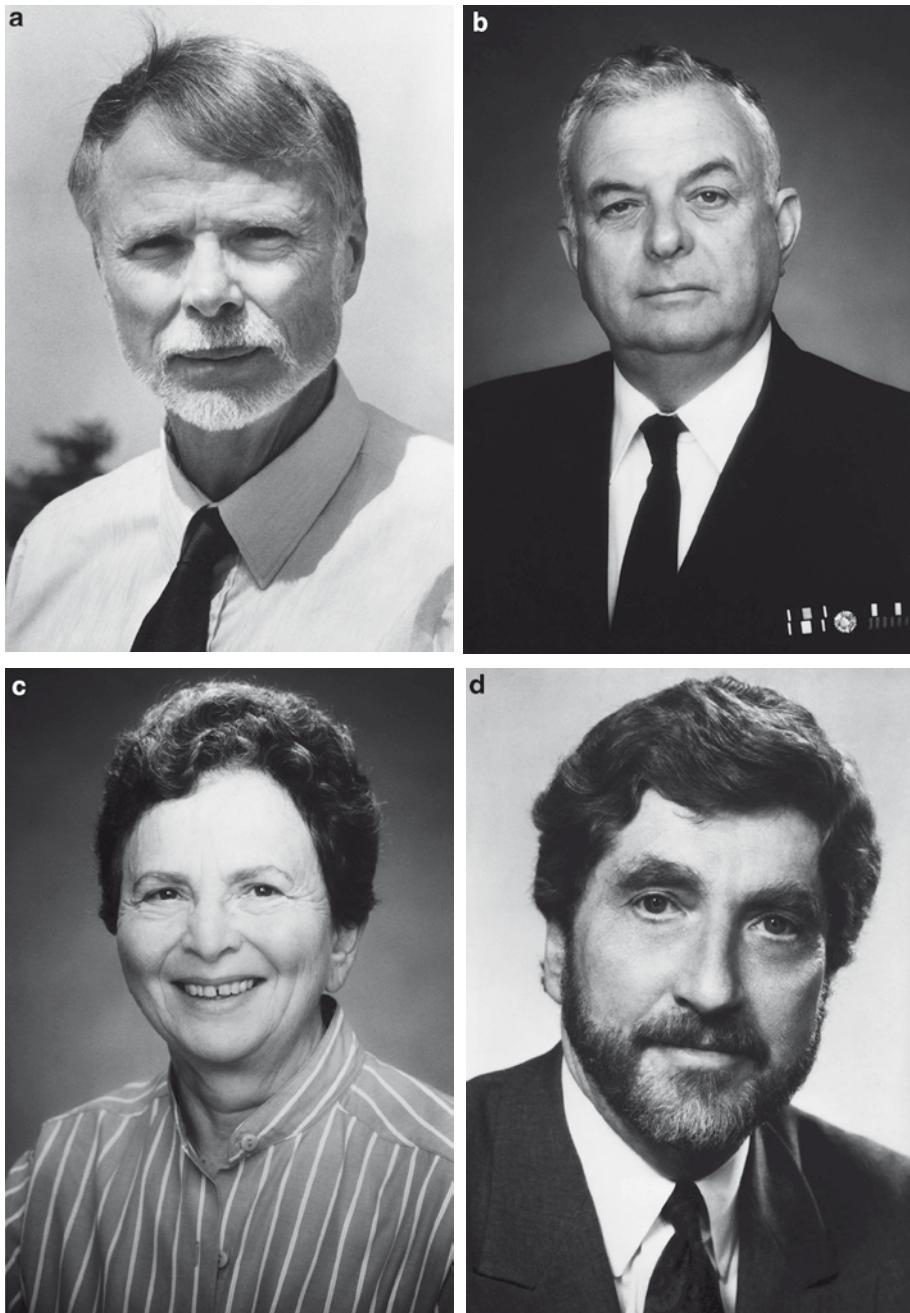
based upon the structure of their capsular polysaccharides; clinical strains were predominantly of type b (Pittman 1931a). Additionally and as contemporaneously proven with both pneumococcal and meningococcal capsular serotypes, she showed that immunologic responses in animals were type-specific (Pittman 1931b).

The virulence of Hib appeared to derive from the ability of its polysaccharide capsule to protect the organism from the lytic action of serum complement (Sutton et al. 1982). The protective effect of rabbit hyperimmune serum was noted to be mediated by the content of Hib anticapsular antibodies (Alexander et al. 1944), an observation that explained an earlier finding of an inverse relationship between the age distribution of Hib meningitis and the presence of serum bactericidal antibodies (Fothergill and Wright 1933). Vertically acquired maternal antibodies directed against the Hib polysaccharide capsule protected newborns during their first approximately 6 months of life; subsequently, as maternal-derived protection waned, protective levels of anticapsular antibodies were maintained by continuous exposure to cross-reacting respiratory and intestinal bacteria (Schneerson and Robbins 1975). The vast majority of Hib meningitis affected children between 6 months and 5 years of age.

Epidemiologic studies reported in the 1970s and 1980s described the burden of morbidity and mortality related to Hib disease and underscored the need for a preventive vaccine approach to the problem. The attack rate among young children was approximately 0.4% (Parke et al. 1972), although significantly higher rates were noted in groups at particularly high risk, such as Alaskan Eskimo children, Australian aboriginal children, those with congenital humoral immunodeficiencies, as well as those in nursery, day-care, and other crowded environments (Robbins et al. 1996a). Mortality rates ranged between 5 and 10%; severe neurologic sequelae occurred in 30% of survivors (Sell et al. 1972). Additionally, resistance to ampicillin and other antimicrobials used in the treatment of Hib meningitis was steadily increasing throughout this time (Robbins et al. 1996b).

It was against the aforementioned epidemiologic backdrop and in the context of successful meningococcal polysaccharide vaccine trials and contemporaneous pneumococcal vaccine trials that first-generation Hib polysaccharide vaccines were studied. In pediatric clinical trials in the United States and Finland, the latter involving nearly 98,000 children, both the immunogenicity and the durability of anticapsular antibody responses were found to be age-related (Smith et al. 1973; Peltola et al. 1977). In the Finnish trial, which was designed to assess the efficacy of a group A meningococcal polysaccharide vaccine in children and in which Hib vaccine was actually used in the control group, a protective efficacy of the latter product of 90% was demonstrated in those vaccinated at 18 months of age or older; no difference in the incidence of invasive Hib disease was noted between control and vaccinated children younger than 18 months of age (Peltola et al. 1984). On this basis, Hib capsular polysaccharide vaccine was licensed in 1985 and recommended for children at 24 months of age.

However, the lack of immunogenicity and protective efficacy in young children, the group at highest risk for invasive Hib disease, was predicted to be a use-limiting gap of the purified capsular polysaccharide vaccine even before its licensure. Medical researchers Porter Anderson and David Smith (Fig. 16.7), initially at the Children's Hospital in Boston and later at the University of Rochester, and Rachel



**Fig. 16.7** (a) Porter Anderson, (b) John Robbins, (c) Rachel Schneerson, (d) David Smith (Albert and Mary Lasker Foundation)

Schneerson and John Robbins (Fig. 16.7) from the National Institute of Child Health and Human Development at the National Institutes of Health in Bethesda independently and collaboratively began working on ways to improve upon the level and durability of immunologic responses to Hib polysaccharide in the late 1960s.

The concept that the immunogenicity of certain ligands or haptens could be enhanced through their covalent attachment to proteins was initially suggested by pathological anatomist and immunologist Karl Landsteiner in a series of papers in the 1920s. Landsteiner, a Nobel Laureate for his 1901 discovery of blood groups and their immunologic properties, had earlier provided the initial description of non-human primates as a permissible model for poliomyelitis (refer to Chap. 9) in 1908 (Corner 1964). Oswald Avery and Walther Goebel, Landsteiner's colleagues at the Rockefeller Institute during the 1920s and 1930s, confirmed and extended the latter's observations by demonstrating that the antigenicity of simple carbohydrate components of the pneumococcus could be improved by covalently binding them to proteins (Avery and Goebel 1929).

Smith, Anderson, Schneerson, and Robbins integrated and extended the previous observations to develop conjugate vaccines of the Hib capsular polysaccharide covalently bound to diphtheria toxoid carrier proteins. In this fashion, the scientists essentially formulated the polysaccharide as a hapten, thus converting it from a T-cell-independent to a T-cell-dependent antigen and inducing effective and durable humoral immune responses in infants, associated with an anamnestic response (Eskola et al. 1990). The first Hib conjugate vaccine was licensed in 1987 for children; in late 1990, after trials of multiple conjugate products had demonstrated efficacy in infants as young as 2 months of age, routine vaccination in this age group was initiated (Peter 1998).

Prospective efficacy trials of three different Hib vaccines, in which the capsular polysaccharides are conjugated to either diphtheria toxoid, the outer membrane protein complex of group B meningococcus, or tetanus toxoid, were performed in the 1990s and demonstrated the efficacy of each of these approaches (Chandran et al. 2008). The incidence of invasive Hib disease showed a significant decline related to the use of the polysaccharide product in children in the late 1980s; disease burden in infants also declined, even prior to the advent of their routine immunization, probably as a result of a "herd" effect on the Hib carriage rate in vaccinees and the subsequent transmission to others (Adams et al. 1993). Since the introduction of routine immunization of infants and children using Hib conjugate vaccines, the burden of invasive Hib disease in developed countries has declined by 98% as compared to the pre-vaccine era (Bisgard et al. 1998; CDC 2002); promising success has also been observed in areas of the developing world in which Hib vaccines have been deployed, thus raising the possibility that this disease might eventually be eliminated in young children.

## 16.5 Other Polysaccharide–Protein Conjugates

As described above, the practical concept of protein conjugation to enhance the immunogenicity of capsular polysaccharides resulted from "the confluence of two great streams of research in the 1920s and 1930s": the elucidation of the pathogenic

and chemical nature of bacterial capsules and the description of the immunological consequences of the modification of proteins (Gotschlich 2009). These areas integrated the innovative work of Landsteiner, Avery, Heidelberger, Goebel, and others, creating a framework that would be successfully exploited first by Smith, Anderson, Schneerson, and Robbins, five decades later, in the arena of Hib vaccine conjugates (Robbins et al. 1996b). Their work, of course, was also facilitated by the maturation of modern immunology, with its augmented understanding of the role of T cells in the humoral response.

Other polysaccharide–protein conjugate vaccines followed on the success of Hib products. Meningococcal monovalent and quadrivalent conjugate vaccines have been licensed and are in routine use; the former have been shown to be effective (Ramsay et al. 2001; Larrauri et al. 2005; de Greeff et al. 2006), and the latter are under evaluation. A pneumococcal conjugate vaccine containing seven capsular types that account for 80% of invasive disease in children conjugated to a diphtheria toxin mutant was licensed in 2000. Since its routine use in childhood vaccination was introduced, it has demonstrated in excess of 90% efficacy against vaccine-associated serotypes, has reduced the burden of invasive pneumococcal disease by about 80% in children, and has resulted in a reduction in the incidence of otitis media (Poehling et al. 2006; Center 2007; Grijalva and Griffin 2008). A typhoid vaccine containing purified Vi polysaccharide (refer to Chap. 6) conjugated to recombinant *Pseudomonas aeruginosa* exotoxin A protein has shown greater than 90% efficacy in Vietnamese children and is under evaluation in infants (Lin et al. 2001). It is expected that novel conjugate vaccines against additional pathogens will continue to be developed.

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# Chapter 17

## Hepatitis B

Baruch S. Blumberg

### 17.1 Introduction

The discovery of hepatitis B virus (HBV) did not arise from a goal-directed search for this pathogen but from studies in basic science that were initially set on a different path. Much of the early research that resulted in the discovery of the virus, the invention of diagnostics, and the development of a vaccine was conducted at the Division of Clinical Research of Fox Chase Cancer Center (Blumberg et al. 1967). This chapter will focus on this early period and will also include a discussion of the worldwide use of the initial and subsequent vaccines that were associated with dramatic decreases in the incidence and prevalence of acute and chronic HBV infection, the HBV carrier state, and hepatocellular carcinoma (HCC), the latter marking HBV vaccine as the first one to prevent cancer. The nonpathological effects of chronic HBV infection, including a discussion of the relationship of genetic polymorphisms to susceptibility to chronic infection, and the relation of HBV infection to gender selection will conclude the chapter.

### 17.2 Disease History

HBV has probably been an infectious pathogen of humans since the origin of hominids. Homologous viruses, “hepadnaviruses,” are found in many other species, indicating a long prehuman interaction with these agents (Sherker and Marion 1991). HBV infection is one of the most common viral causes of disease and a major cause of jaundice throughout the world. Illness associated with jaundice was recognized in ancient folk pharmacopeias. Epidemic jaundice was described in the writings of Hippocrates in the fourth century B.C. (Zuckerman 1976); descriptions of what appears to be hepatitis have been noted in other ancient texts including the

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Babylonian Talmud from the fifth century A.D. The diseased liver also has a prominent role in classical mythology. Prometheus was punished by the gods for bringing fire and, in some accounts, the arts of civilization to humanity by having the lobes of his liver eaten by an eagle as he was left bound and defenseless on Mt. Caucasus; regeneration took place during the night readying the sufferer for further assaults during the next cycle.

Evidence that the virus is transmitted by injection or other means of transfer of human blood and/or blood products was recorded as early as the late nineteenth century when the use of HBV-contaminated smallpox vaccine caused jaundice in over 15% of vaccinated shipyard workers in the north German port of Bremen (Lürman 1885). A particularly impressive epidemic occurred during World War II. In preparation for the U.S. military assault on German-dominated North Africa in 1942, Allied invading forces were inoculated with yellow fever vaccine, some of which had been diluted with human plasma that was contaminated with hepatitis virus (refer to Chap. 10). Over 28,000 of the soldiers developed jaundice, and 62 died. In a retrospective serological analysis performed more than 40 years after the outbreak and with the causative agent of hepatitis B known, it was demonstrated that 97% of the recipients of the serum-contaminated vaccine had evidence of HBV infection (Seefe et al. 1987). Since human blood products were included in many inoculated treatments used in the premodern era of medicine, reports of jaundice in recipients were well described (Findley and MacCallum 1937; Beeson 1943; Cameron 1943; Havens 1947).

Before the availability of HBV diagnostics, the impact of donor blood from infected carriers could not be measured. A comparison of the incidence of HBV infection related to family member and volunteer blood donations to that of “professional” donors showed that the latter statistic was significantly higher than the former and associated with both clinically apparent and subclinical hepatitis (Senior et al. 1974). Additionally, prior to the wide use of disposable needles and the advent of other safeguards in healthcare environments to prevent exposures to human blood, medical equipment was routinely re-used, often following inadequate disinfection, representing another potential source of HBV transmission.

### 17.3 Clinical Epidemiology

HBV is a partially double-stranded DNA virus belonging to the *Hepadnaviridae* family, a category that was created when it was recognized that the newly discovered HBV did not fit into any previous classification. There is significant diversity in host response following infection; only a minority of those infected becomes acutely ill. Acute hepatitis usually starts with flu-like symptoms, anorexia, and vomiting that may be accompanied by fever, urticaria, and arthritis. The urine may darken in days to weeks often followed by jaundice. The symptoms usually wane with the onset of jaundice.

The existence of two distinct clinical and epidemiologic types of viral hepatitis was postulated by Saul Krugman and his colleagues based on their landmark yet controversial studies at the Willowbrook State School on Staten Island in New York during the late 1950s and 1960s (Krugman et al. 1967). The studies at Willowbrook, an overcrowded state institution designated for the care of severely mentally retarded children, disclosed extraordinarily high rates of clinical and subclinical hepatitis among the residents and staff. Although their experiments were subsequently the subject of considerable ethical debate, important findings emerged from them, including an understanding of the routes of transmission of hepatitis and the concept of using HBV-immune globulin to prevent hepatitis (Krugman 1986).

The majority of individuals with HBV infection will develop protective antibodies (anti-HBs) directed against the viral surface antigen (HBsAg), as well as other antibodies directed against other viral components. Multiple protein products of the virus may be found in the blood or cells of individuals during the course of disease and subsequent recovery: HBsAg (surface antigen), HBcAg (core antigen), HBeAg, HBxAg, and the polymerase and reverse transcriptase enzymes produced by the P reading frame, among others. Antibodies against several of these proteins, including those directed against core antigen (anti-Hbc) and the protective anti-HBs are variably produced in infected individuals.

Most individuals experiencing acute HBV infection will recover spontaneously within weeks or months, and most retain anti-HBs that confer lifelong protection. A small percentage of individuals with acute HBV infection develop fulminant hepatitis that rapidly progresses to death with high frequency. However, approximately 10% of acute cases of HBV infection progress to a chronic viral carrier state, signified by the persistence of HBsAg in their blood. These patients generally remain asymptomatic for decades yet are at risk to develop chronic liver disease and HCC. In many parts of the world, chronic carriage begins a few weeks after birth or at a young age. There are large geographic differences in the prevalence of the HBV carrier state, varying from in excess of 15% in East Asia and Oceania, 5–10% in Africa and Eastern Europe, to less than 1% in Europe and North America.

Approximately two billion people – one-third of the world's current population – have been infected with HBV, and about 350–400 million are chronically infected, the latter associated with nearly one million deaths a year due to HBV infection (Dienstag 2008). To comprehend the relative mortality burden of HBV worldwide in terms of another prevalent chronic viral infection, one need only examine the estimated mortality expected from the number of HBV carriers as compared to those infected with the human immunodeficiency virus (HIV-1) in the 1990s. Based on a more than tenfold relative prevalence of HBV at the time, the mortality burden was more than twice that of HIV-1, despite the higher mortality rate associated with the latter. Since HBV has been common in humans for tens of thousands of years, and HIV-1 has only been associated with human disease for less than 40 years, the cumulative mortality due to HBV is historically significantly greater than that due to HIV-1.

## 17.4 Discovery of Hepatitis B Virus

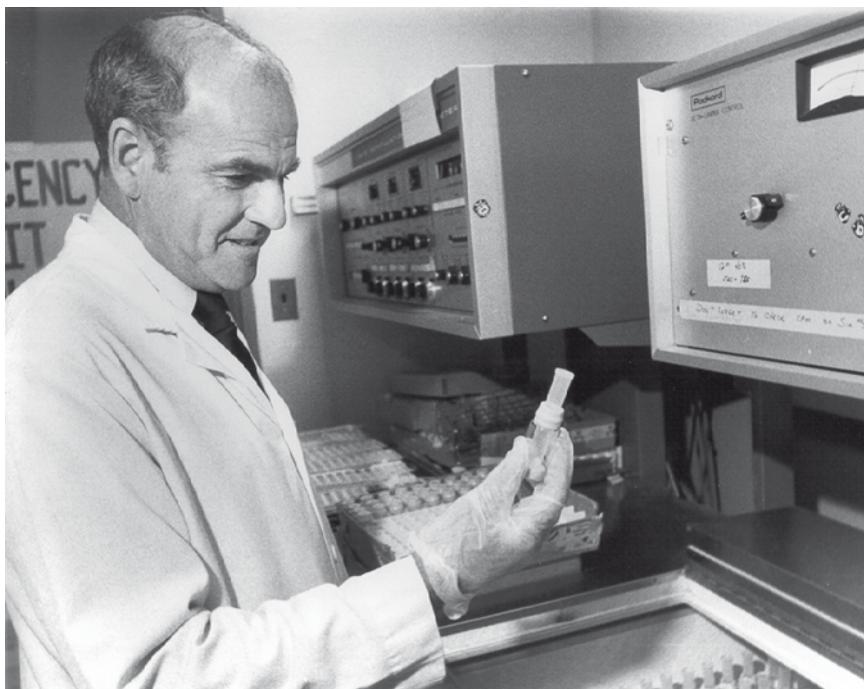
The discovery of HBV did not originate as a directed search for the virus but as an investigation in basic medical research. It was a circuitous and convoluted process whose outcome would have been difficult to predict at its onset (Blumberg 2002). A striking feature of medicine is the great variation in host response to disease-causing agents. Starting in 1957, we set out to discover the relationship between inherited and acquired polymorphic variation in circulating proteins to disease susceptibility (Blumberg 1961). This is analogous, at the phenotype or proteome level, to current work examining the possible relationship between genomic polymorphic variation and disease using single nucleotide polymorphisms (SNPs) and other databases as probes.

I had become interested in the genetic basis of disease during medical school and my subsequent clinical training at Bellevue and Presbyterian Hospitals in New York City; doctoral work at Oxford University and a 7-year stint at the National Institutes of Health solidified my engagement in this line of research investigation (Blumberg 2002). Such work was interesting and complex, as it required worldwide collection of specimens in varying disease environments to compare the distribution of the polymorphic alleles and to try to understand their relation to disease (Fig. 17.1). In the late 1950s, protein variation was assessed in the laboratory using the recently introduced method of gel electrophoresis.

Because many serum proteins are polymorphic, patients who had received multiple blood transfusions were likely to have been exposed to a variety of exogenous noninherited proteins, which would represent foreign antigens, provoking a host antibody response directed against them. Thus, their blood could be used as “reagents” to discover and study antigenic protein polymorphisms. Using the double-diffusion in agar gel technique, described by Ouchterlony in 1961, we identified a complex, inherited antigenic system of the serum low-density lipoproteins – the “Ag System” (Allison and Blumberg 1961). Using these and other antilipoprotein antibodies identified by others and us, associations with cardiovascular disease, Alzheimer’s disease, and diabetes were found.

We continued to test the sera of transfused patients against a panel of sera with the expectation that we would find additional antigen-antibody systems of interest. In 1964, a precipitin band in the gel, indicating an antigen-antibody interaction, was detected between the sera of a transfused hemophiliac patient from New York and, among others, the sera of Australian aborigines (Blumberg et al. 1965). Much of our subsequent research utilized these sera.

The reactant protein was initially named “Australia antigen” (Au) after its geographic origin; the problem was to learn what it represented. Au was rarely found in healthy Americans but was common in those with leukemia, which generated the hypothesis that individuals at high risk of leukemia might also have high frequencies of the antigen. Since patients with Down’s syndrome, a chromosomal defect associated with mental retardation, are at high risk for an unusual form of leukemia, we predicted that they would have a high frequency of Au. This was confirmed in a series of studies in large institutions for the mentally retarded (Blumberg et al. 1967).



**Fig. 17.1** Baruch Blumberg (Dr. Baruch Blumberg)

Subsequently, a series of observations raised the possibility that Au was associated with hepatitis. The epidemiology was suggestive: it was found in transfusion recipients, in institutionalized patients, and sporadically in patients with hepatitis: the former two representing settings known to facilitate the transmission of infectious diseases. But the most convincing observation was in a patient with Down's syndrome, who did not have Au when first seen but developed it on subsequent testing. In this case, the appearance of Au coincided with the onset of hepatitis. We then formally tested that hypothesis by comparing the prevalence of Au in patients with and without clinical hepatitis; Au was found to be significantly more common in those with the disease.

Next a series of investigations was designed to determine whether Au was a hepatitis virus or a viral component. Its identity to the component of the HBV, which subsequently became known as HBsAg, was confirmed by clinical observations, electron microscopic (EM) findings, transmission studies in animals, and other investigations. The hepatitis hypothesis was published in 1967 (Blumberg et al. 1967), and the initial observations were soon confirmed by other investigators in Japan, Italy, and the United States (Okochi and Murakami 1968; Prince 1968; Vierucci et al. 1968). Investigators at the New York Blood Bank associated Au with

the elusive etiology of post-transfusion hepatitis or hepatitis B that had been postulated by Krugman and other pioneers in the field before the actual discovery of HBV (Prince 1968). The identification of one hepatitis virus facilitated the identification of others, such as hepatitis A, C, D, and E viruses, which greatly increased the probabilities of control and treatment of these disease entities.

There were immediate applications of the discovery of HBV. The “Au test” became widely used for the diagnosis of acute and chronic hepatitis, representing a major step forward; a virus could be diagnosed within a few hours by direct detection. Previously, viral diagnosis often depended on comparing the titers of specific antibodies early in infection to titers during convalescence, a process that generally took weeks (Merigan 1997).

In 1969, blood donor testing to detect asymptomatic carriers of HBV was adopted by the Philadelphia General Hospital (Blumberg et al. 1969; Senior et al. 1974); within 4 years and after some controversy, testing became mandated in blood banks. Although post-transfusion hepatitis due to HBV was brought under control through testing, it was not until the late 1980s that the viral etiology of another major form of post-transfusion hepatitis, hepatitis C (HCV), was elucidated. Subsequently, the development of reliable diagnostic tests for HCV in the early 1990s further reduced the frequency of post-transfusion viral hepatitis to a low level.

## 17.5 Inventing a Vaccine

One of the major problems in the invention of vaccines is the identification of the specific antibody or cellular immune response that provides protection. The failure to do this has slowed the development of vaccines against HIV, tuberculosis, malaria, and other pathogens. The identification of a correlate of protection against HBV was possible soon after the research began.

By 1968, we had recognized that antibodies directed against the surface antigen of HBV were protective. We had rarely seen individuals who had both HBsAg, indicating infection, and anti-HBs in their blood, as the presence of the latter is consistent with protection. Further, it was demonstrated that individuals who had anti-HBs before they were transfused with donor blood containing HBV were much less likely to develop hepatitis than those lacking anti-HBs (Okochi et al.). Anti-HBs were subsequently shown to be protective in a multiyear study in a renal dialysis unit where HBV infection was endemic (Lustbader et al. 1976).

A peculiar feature of HBV, which was recognized after EM visualization of viral particles, was that in addition to the whole virus particles there were very large numbers of spherical and rod-shaped particles containing only HBsAg that were noted in the peripheral blood of carriers and other infected individuals (Bayer et al. 1968; Dane et al. 1970). In some carriers, these particles amounted to more than 1% of their total serum proteins. They subsequently formed the basis of the initial HBV vaccine.

In 1969, the Institute for Cancer Research filed U.S. and foreign patents for the process of creating a viral hepatitis vaccine “from blood containing Australia antigen, having particles resembling viruses which are substantially free of nucleic acid, of a size range of 180–210 Å, substantially free of infectious particles. The vaccine, where required, is attenuated or altered. The preferred procedure of removing impurities including infectious components involves centrifugation, enzyme digestion, column gel filtration, differential density centrifugation in a solution of sucrose, dialysis, differential centrifugation in a solution of cesium chloride, and dialysis” (Blumberg and Millman 1972; Blumberg 1972). No vaccine had previously been produced from the blood of viral carriers, and none has since. However, the medical and scientific world was not quite ready for a vaccine for HBV.

Studies by Krugman and colleagues increased the interest in the Blumberg/Millman vaccine. They inoculated children with a preparation of HBV-positive serum which had been boiled for 1 min, and subsequently challenged these children with inoculation of untreated serum containing the virus (Krugman et al. 1971). The heated serum provided substantial, but not complete, protection against HBV infection and provided additional proof of concept to potential manufacturers that a vaccine was possible. Shortly thereafter, Merck Pharmaceuticals provided an assurance of interest to Fox Chase Cancer Center (FCCC) (Hilleman 1971); by 1975, a sufficient amount of preclinical work had been done in laboratories in the United States and elsewhere to recommend moving forward with the vaccine. Following the execution of a licensing agreement between FCCC and Merck, the noted vaccine expert Maurice Hilleman (Chaps. 13 and 14) was given responsibility for the project leading to the production of an experimental HBV vaccine for clinical trials (Buynak et al. 1976).

## 17.6 Field Trials

By the early 1980s, a series of vaccine field trials were completed, primarily through the efforts of Wolf Szmuness (1919–1982) and colleagues (Szmuness et al. 1980; Szmuness et al. 1981). Szmuness was a Polish physician who had survived a Siberian labor camp and latter day pogroms against the Jews in his home country to become a laboratory technician at the New York Blood Center in the 1970s. Although prevented from functioning as a physician because of his foreign background, he rose in the ranks there through sheer intellectual prowess to become an independent researcher and the lead investigator of the HBV vaccine trials. These trials have been described as among “the best organized and executed trials of any human vaccine” (London and Blumberg 1985) and as “a milestone in preventive medicine” (Kellner 1985). It was primarily on their basis that the vaccine received Food and Drug Administration (FDA) approval; the pivotal clinical trial will therefore be described in some detail (summarized from London and Blumberg 1985).

The first challenge was to choose a study population with a sufficiently high risk of infection to make a vaccine trial feasible. Szmuness believed that the trial should

be carried out in a population which stood to benefit from a potentially effective vaccine (Szmuness et al. 1980). Among the populations at high risk considered for the trial were residents of institutions for the mentally retarded, such as those in whom we had earlier reported a high HBsAg frequency, patients undergoing maintenance hemodialysis, members of the medical staff of hemodialysis centers, American Indians, and homosexual men. By the late 1970s, very few new residents were being admitted to state institutions for the retarded, and the rate of new infections in long-term residents was quite low. Quarantine procedures instituted in most hemodialysis units had greatly reduced the incidence of HBV infections.

Szmuness ascertained that the risk of infection among homosexual men in New York City was high and that the potential study subjects were cooperative, intelligent, and well educated (Szmuness et al. 1975). The prevalence of serological markers of HBV infection was 68% among more than 10,000 men surveyed, and the annual incidence of infection was projected to be 19.2% (Szmuness et al. 1978) and estimated at 30% in subsequent studies (Szmuness 1980). The study included 1,083 male subjects enrolled between November 1978 and October 1979; 549 received vaccine and 534 placebo. The vaccine was administered in three doses: the first two were given 1 month apart, and the third was given 6 months after the first. Ninety-three percent of the subjects received all three inoculations. Most participants were followed for endpoints for a year after the last dose of vaccine.

The results were convincing. First, there were no differences in the frequency or severity of adverse events between the vaccine and placebo groups. Second, the antibody response in the vaccinated groups was excellent. Seventy-seven percent of the vaccinees had significant levels of anti-HBs within 2 months of the first inoculation, increasing to 96% after the third dose; only 2–5% of placebo recipients without evidence of active HBV infection developed anti-HBs. Third, there was a clear difference in the number of HBV infections between the vaccine and placebo recipients. Of 122 infections, including subclinical ones, 93 (76.2%) occurred in the placebo recipients and 29 (23.8%) in the vaccinees, a highly statistically significant result ( $p < 0.0001$ ). Fifty-two of the subjects had a clinical event classified as hepatitis B, based on alanine aminotransferase levels  $\geq 90$  IU plus the appearance of HBsAg in their serum, but only seven of these occurred in vaccinated men, only one of whom had completed the course of vaccine but had not developed anti-HBs. The efficacy ratio (i.e., the incidence in placebo recipients over those in vaccinees) reached 14.0 for the period between 5 and 18 months after vaccination. Finally, an unforeseen but clinically and biologically important finding was that those vaccinated subjects who failed to produce anti-HBs were no more likely to develop persistent infection than placebo recipients who became infected.

Thus, the pivotal randomized, double-blinded, clinical trial proved HBV vaccine efficacy (Szmuness 1980), a conclusion supported by multiple subsequent trials in different risk groups and geographic settings (Maupas et al. 1981; Francis et al. 1982; Desmyter et al. 1983; Benahamou et al. 1984; Chan et al. 2004). Within 2 years of the publication of the successful pivotal trial, the U.S. FDA approved the plasma-derived HBV vaccine. The Szmuness trial was not only effective but also efficient; just over a thousand people were involved, as compared to the more than

one million children involved in the testing of polio vaccine and the thousands that have been involved in the so-far unsuccessful trials of an HIV vaccine.

Millions of doses of the plasma-derived vaccine were used worldwide. While reports of adverse events on occasion led to suspension of the vaccine programs, they were subsequently reinstated (Marshall 1998). The effectiveness of using HBsAg derived from plasma as a means of inducing a protective antibody response validated its manufacture by recombinant methods; such technology is now the major source of HBV vaccine (McAlear et al. 1984). It was the first vaccine produced by the recombinant method and for many years the only one. Recombinant technology has helped to provide worldwide vaccine access, as the cost of its manufacture and distribution has decreased.

## 17.7 Global Vaccination Programs

National vaccination programs began soon after the original vaccine formulation became available (Ginsberg and Shouval 1992; Bonnanni 1995; De la Torre and Esteban 1995; Gatcheva et al. 1995). The International Conference on Prospects for Eradication of Hepatitis B Virus held in February 1990 explored the possibilities for control and eradication of the disease (Blumberg 1990). In 1992, the World Health Organization (WHO) placed HBV vaccine on the Extended Program on Vaccination, setting a target date of 1997 for its integration into national programs; by 2004, there were 129 countries with such programs (Van Damme and Vorsters 2002). HBV is now one of the most widely used vaccines in the world.

Systematic vaccination results have been impressive. Newborn and childhood vaccination started in Taiwan in 1984 with robust national participation and data collection and demonstrated a highly significant reduction in the prevalence of carriage, decreasing from 9.8 to 0.7% over a 15-year period (Chan et al. 2004). This study and others have also noted a significant, but of diminished magnitude, reduction in carrier prevalence among unvaccinated children (Da Villa et al. 1992, 1995), indicating a possible “herd immunity” explanation that could hasten the overall effect of such programs and accelerate control and possibly eradication of HBV. A striking decline in lethal fulminant hepatitis associated with vaccine use has also been observed in young children. From 1975 to 1984, the average infant mortality from fulminant hepatitis was 5.36/100,000; from 1985 to 1998, after the vaccination program had started, it was 1.71/100,000 (Kao et al. 2001).

Several HBV carriage surveys before and after vaccination programs have been summarized and confirm striking decreases in the prevalence of HBV carriers and in the incidence of clinical hepatitis in various global settings: in the Peoples Republic of China, the prevalence of carriers decreased from 16.0 to 1.4% after the introduction of HBV vaccination; in the Gambia, rates fell from 10.0 to 0.6%; in Japan, from 2.7 to 0.9%; in Saudi Arabia, 6.7 to 0.3%, and in Catalonia, 9.3 to 0.9% (Blumberg 2004). In the United States, the rate of new HBV infections has declined significantly from a peak of more than 70,000 cases in 1984 to less than 20,000 in

2006. The decline has been greatest among children born since 1991, when routine pediatric vaccination was recommended by the U.S. Public Health Service (CDC 2008). In Alaska, following an intensive vaccination campaign among Native Americans, the incidence declined from a baseline of 215 cases per 100,000 to 7–14 cases per 100,000 in 1993 after the program was implemented; no cases were reported in 1995 (McMahon et al. 1996).

## 17.8 The First Cancer Vaccine

Perhaps the most conceptually important outcome of these global vaccination programs has been the favorable impact on the incidence of HCC, primary cancer of the liver, throughout the world. HCC is the third most common cause of death from cancer in men and the seventh in women worldwide. Most HCC is caused by infection with HBV or HCV; HBV purportedly accounts for more than two-thirds of the cases. In Taiwan, the annual incidence in the vaccine-impacted population (6–14 years of age) declined from 0.7 cases per 100,000 from 1981 to 1986, before vaccination programs were fully implemented, to 0.36 per 100,000 from 1990 to 1994 after the vaccination program was in place (Chan et al. 2004).

HBV vaccine was the first vaccine to demonstrate a favorable impact on the prevention of cancer throughout the world. It was not until 2007 that the second vaccine for the prevention of cancers, those caused by human papilloma virus (HPV), was introduced (Chen and Berek 2007). There are now early studies assessing the possibility of vaccine protection against Epstein-Barr virus-induced nasopharyngeal cancer (Hepeng 2008). The American Cancer Society estimates that worldwide, as many as 20% of cancers may be causally associated with infectious agents (American Cancer Society 2008); therefore current successes should provide impetus for the development of novel vaccines that may further lessen the global burden of cancer. The apparent success of HBV vaccine and the promise of HPV vaccination programs serve as models for future cancer prevention.

## 17.9 The Biology of HBV: Genetics and Gender

Throughout history, physicians and scientists have generally encountered viruses and other microorganisms primarily in their pathological phase. But, microorganisms have a rich interaction with humans beyond their roles in disease causation. HBV has infected about one-third of the current global population and has probably interacted with hominids since the origin of the species. It is important to understand the possible effects that may result from its control and eradication.

Initial research on hepatitis B began as a study on the inheritance of susceptibility to HBV chronic infection (Blumberg et al. 1966); more recent population and genomic studies have added rich detail to this theme (reviewed in Blumberg 2006a, b).

There are multiple genetic loci at which one allele will increase susceptibility to chronic infection while an alternate allele will increase the probability of developing protective antibody. An added interesting aspect of these observations is that these same alleles may affect susceptibility to other infectious agents. Two such examples include the DRB1\*1302 allele at the MHC Class II locus on chromosome 6, which is related to susceptibility to HBV chronic infection, and the responses to falciparum malaria and papilloma virus; and the VDR locus on chromosome 12 that is related to responses to HBV, *Mycobacterium tuberculosis*, and *M. leprae*. We have categorized the organisms with affinities to the same genetic locus as Microorganism Gene Affinity Clusters (MIGAC).

In some cases, an allele that confers an advantage to the host for one member of the cluster may be disadvantageous for another. These allelic variations constitute genetic polymorphisms and, as such, may be associated with both favorable and unfavorable effects for the host population. For example, HBV carriers bind larger quantities of iron than uninfected individuals, a possible selective advantage in regions with low dietary iron intake (Sutnick et al. 1974; Felton et al. 1979). It is interesting to conjecture how members of a specific gene affinity cluster will be affected if one of the infectious agents in the cluster is greatly decreased as the result of a successful vaccination program.

HBV interacts differently in male and female hosts. Infected men are more likely to become carriers, while women more likely to develop protective antibody (anti-HBs). As carriers are more likely to develop chronic liver disease and HCC, women appear to have a selective advantage over men in this regard. Also females who become carriers are less likely to develop HCC than male carriers, a further survival advantage. Extensive studies on genetic susceptibilities using SNP data have also found that androgens and estrogen differentially affect susceptibility to chronic disease (Chen 2008).

Even prior to the use of sensitive methods for the detection of HBsAg, HBV infection was noted to be common in individuals with renal failure undergoing hemodialysis. Within the renal transplant subpopulation, investigators observed that patients who developed anti-HBs rejected transplanted kidneys more rapidly than those who were carriers of HBV and that this effect, although independent of the gender of the recipient, was seen only when the kidney donor was a male (London et al. 1969, 1977). There appeared to be a specific relation between anti-HBs and an antigen present in male but not female donor kidneys.

The most perplexing observation relative to the association between HBV and gender concerns the effect of the parental HBV infection on the gender of their offspring. A family study in Greece showed that if either parent was a carrier of HBV infection, the gender ratio of their offspring (i.e., number of male offspring divided by the number of female offspring) was greater than in families in which one of the parents, particularly the mother, harbored anti-HBs (Drew et al. 1978). Similar observations have been noted in other studies in Greece, France, New Guinea, the Philippines, and in east Greenland (Blumberg 2006a). In at least one of these studies, the differences in ratio were due to a decreased number of female births. There was also diminished fertility noted in the carrier families. These family

studies were confirmed by population studies; the prevalence of HBV appears to be strongly correlated with the ratio of males to females in the population. After intensive HBV vaccination programs, the male to female gender ratio was decreased in two populations (Oster 2005). This research suggests that the high gender ratios found in Asia and elsewhere could be accounted for in part by the high prevalence of HBV in these populations, and that HBV vaccination programs could alter the ratio. These conclusions have been contested, making it apparent that an understanding of HBV and its gender implications requires further study (Lin and Luoh 2006; Gupta 2008).

## 17.10 Conclusions

HBV was discovered in a non-goal-directed project, as is often the case in the solution of medical and biological problems. The original vaccine was derived from a portion of the virus – the surface antigen particles that exist in large quantities in the blood of carriers of HBV, representing a novel method of making a vaccine without the requirement for cell or tissue culture. HBV vaccine was subsequently the first vaccine to utilize recombinant methods of production and is now one of the most commonly used vaccines in the world. The global reductions in the prevalence of HBV carriage and the incidence of acute and chronic liver disease associated with vaccination have been profound. A similar favorable impact on the worldwide incidence of HCC has made HBV vaccine the first vaccine shown to prevent cancer. It is hoped that future vaccines will further reduce the tragic toll of this disease. The susceptibility to persistent HBV infection and its attendant complications appears to be affected by polymorphic susceptibility genes that also interact with other infectious agents and may have nonpathologic, even beneficial effects. As successful HBV vaccination programs proceed, it will be important to understand these complex phenomena.

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# Chapter 18

## Japanese Encephalitis

Scott B. Halstead

### 18.1 Brief History of the Disease

Summer-fall encephalitis outbreaks, consistent with Japanese encephalitis (JE), were recorded in Japan as early as 1871; the largest, in 1924, involved more than 6,000 cases, 60% of them fatal (Hiroyama 1962). A filterable agent from human brain tissue was isolated in rabbits that year and in 1934, Hayashi transmitted the disease experimentally to monkeys by the intracerebral inoculation of human brain suspension (Hayashi 1934; Inada 1937). The virus was subsequently adapted to mice, and soon after, a serological diagnostic test was developed based on the presence of specific neutralizing antibody in recovered patients (Hayashi 1934; Kawamura et al. 1936; Taniguchi et al. 1936). Inoculation of mouse brain with JE and related St. Louis encephalitis (SLE) flaviviruses provided antigens that enabled workers to confirm encephalitis cases serologically, including a cluster of cases that occurred in 1934 and 1935 in Beijing (Kuttner and Tsun 1936).

The virus was initially called Japanese B encephalitis (the modifying “B” has since fallen into disuse) to distinguish the disease from Von Economo’s type A encephalitis, which had different clinical and epidemiologic characteristics. A mosquito-borne transmission for JE was suggested when the virus was isolated from *Culex tritaeniorhynchus* mosquitoes in 1938. Two decades later, field studies established the role of aquatic birds and pigs in the viral enzootic cycle (Buescher and Scherer 1959; Scherer et al. 1959). Viruses isolated from human cases in Japan in 1935 and in Beijing in 1949 provided prototype Nakayama, Beijing, and P3 strains that were widely used in vaccine production for many years.

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## 18.2 Why the Disease is Important

During the first half of the twentieth century, summer outbreaks of JE were reported principally in temperate areas of Asia, including Japan, China, and Korea (Igarashi 1992). In the summer of 1945, an outbreak of JE occurred on Okinawa among residents and U.S. military personnel; in 1948, another extensive outbreak was reported in which about 8,000 Japanese civilians and 35 American soldiers were affected (Sabin 1947; Tigert et al. 1956). Annual outbreaks of several thousand cases occurred in Japan until as recently as 1966. In Korea, after 5,616 cases and 2,729 deaths were reported in 1949, epidemics continued every 2 or 3 years, culminating in an unprecedented 6,897 cases in 1958 (Halstead and Grosz 1962; Sohn 2000). China has accounted for the majority of cases in the region; between 1965 and 1975, more than 1 million cases were reported (Yu 1995). In the decades that followed, Chinese public health efforts placed a great emphasis on vaccination, resulting in a dramatic decline in cases. Recently, China integrated JE into the routine immunization system in all rural endemic provinces in an effort to improve coverage.

In Japan, Korea, and Taiwan, the introduction of routine immunization programs after 1965 led to the near elimination of the disease. However, enzootic transmission of the virus continues in these locations and small outbreaks occur periodically, as in Taiwan or Korea in 1982 (Wu et al. 1999; Sohn 2000). Viral surveillance using sentinel pigs and horses in Japan documents high annual levels of zoonotic transmission of JE virus, signifying continued risk of human disease should vaccination programs be discontinued (Igarashi 2002; Konishi et al. 2006; Kurane 2006).

Although sporadic cases were observed in northern Thailand from the early twentieth century, JE was not recognized as a major public health problem in Southeast Asia until 1969, when 685 cases were reported from the Chiang Mai Valley (Grossman et al. 1973). Since then yearly outbreaks have produced thousands of cases and hundreds of associated deaths, making JE a leading cause of childhood mortality and disability (Chunsuttiwat and Warachit 1995). In 1974, the first of several epidemics was recorded in an area of Myanmar (Burma) adjacent to the Chiang Mai Valley (Thein et al. 1988). In Vietnam, since reinstatement of notification in 1979, several thousand JE cases have been reported annually.

The disease is now recognized as a public health threat in the densely populated deltas of the Mekong and Red Rivers and nationwide, with incidence rates exceeding 20 per 100,000 in areas of the northern delta near Hanoi (Nguyen and Nguyen 1995). The disease probably occurs at similar rates in ecologically similar areas of Cambodia and Laos, and appears to be the etiology of 18–31% of hospitalized encephalitis cases in Cambodia. JE is enzootic elsewhere in Southeast Asia (Dirk Van Peenen et al. 1975; Macdonald et al. 1989; Cardosa et al. 1995; Buhl et al. 1996; Ostlund et al. 2004; Kari et al. 2006) and has recently become a reportable disease in the region, which should enhance the database for decision-making in the future. Recognition of its magnitude led Thailand to adopt universal childhood immunization and Vietnam to augment their programs (Nguyen and Nguyen 1995; Vasakarava 1995).

JE transmission was first recognized in Southwest Asia after outbreaks in Sri Lanka and India in 1948 and 1952, respectively. Reports were limited to southern India until the mid-1970s when large outbreaks were subsequently reported from various states. JE is currently considered hyperendemic in India and southern Nepal; more than 80,000 cases were reported over the past two decades with a 35% mortality rate, both likely underestimates due to incomplete disease surveillance (Jacobson and Sivalenka 2004). The disease has spread as far west as the Indus valley in Pakistan (Igarashi et al. 1994).

The expanded geographical range and amplification of JE across Asia correlate with the agricultural development and intensive rice cultivation, supported by irrigation schemes that increase the vector populations (Service 1991). A large outbreak occurred in Sri Lanka in 1985 after rerouting a river to a previously arid district. Subsequent attempts at vector control failed to prevent an outbreak the following year, prompting the ministry of health to introduce vaccine in these districts, which has reduced morbidity. Similarly, in southern Nepal, hyperendemic transmission of malaria and JE were documented to follow deforestation and development in the Terai (Peiris et al. 1992; Joshi 1995). Migratory viremic birds or windblown mosquitoes are other possible mechanisms of JE viral importation over significant distances. Such factors are hypothesized to have led to outbreaks on Saipan and the Torres Strait islands between New Guinea and northern Australia (Paul et al. 1993; Hanna et al. 1996).

While JE incidence may fluctuate yearly or regionally, widespread childhood immunization is desirable because viral eradication from nature is not possible. As shown by experience in economically advanced Asian countries, clinical JE can be eliminated through universal vaccine coverage. As the scope of JE has been increasingly recognized throughout Asia, vaccination is gaining wide support from public health authorities.

## 18.3 Inactivated Virus Vaccines

### 18.3.1 *Mouse Brain Vaccines*

Inactivated mouse brain-derived JE vaccines were produced in Russia and Japan in the 1930s; the former was shown to be efficacious against Russian autumnal encephalitis, i.e., JE (Smordintsev et al. 1940). With the specter of deployed forces in JE-endemic areas during World War II, eminent virologists in the U.S., such as Albert Sabin (refer to Fig 12.3) at Cincinnati Children's Hospital, turned their attention to this pathogen. Sabin, who later achieved international recognition for the successful development of an oral polio vaccine (refer to Chap. 12), led an effort to prepare a simple, uncentrifuged, 10% suspension of infected mouse brain inactivated with formalin. The vaccine was variably immunogenic, but efficacy field trials were not completed (Sabin 1943, 1947; Sabin and Duffy 1947a, b; Sabin et al. 1947). From 1948 to 1951, all U.S. soldiers assigned to Asia were vaccinated with a more

stable, inactivated chick embryo-derived vaccine that had shown 80% efficacy in Japanese children in combination with a mouse brain-derived product (Smadel et al. 1947; Tigertt et al. 1950; Ando and Satterwhite 1956; Sabin et al. 1956); its use was discontinued in 1952 after data failed to produce convincing evidence of immunogenicity and efficacy (Sabin and Schlesinger 1947; Pond and Smadel 1954).

The Nakayama strain of JE virus isolated from the CSF of a patient in 1935 and maintained by continuous mouse brain passage, was the principal strain used in mouse brain-derived vaccines first produced in Japan and subsequently in many Asian countries using the method pioneered by Russian workers and Sabin of antigen concentration and partial purification (Oya 1988). The strain was originally chosen because it had good propagation characteristics and provided cross-protection against other JE strains in mice; subsequently, those of the JaGAr01/Beijing type (e.g., Beijing-1, known as P1 in China, and the equivalent P3 strain; see later discussion), from diverse areas of Asia, were noted to confer a broader neutralizing antibody response against various JE viral isolates than the Nakayama strain (Kobayashi et al. 1984; Kitano et al. 1986; Hashimoto et al. 1988). The Beijing-1 strain grows to higher titer, produces higher heterologous antibody titers in immunized mice than the Nakayama strain, and is formulated in half the volume. Biken, the principal Japanese manufacturer of JE vaccine, used the Beijing-1 strain since 1989 in vaccine produced for domestic consumption, whereas the Nakayama strain was used in vaccines distributed internationally. Successive refinements of the inactivated, mouse brain vaccine were undertaken in Japan to remove mouse neuroproteins (Kanamitsu et al. 1970; Yamashita et al. 1970; Oya 1988), but less purified vaccines are produced by several commercial manufacturers in South Korea and by government pharmaceutical organizations elsewhere in Asia.

The efficacy of Biken's Nakayama vaccine was evaluated in two prospective field trials. In the Taiwan trial in 1965, two doses yielded 80% efficacy in the first year after immunization (Hsu et al. 1971, 1972; Okuno et al. 1975). A subsequent blinded, randomized, placebo-controlled trial comparing two doses of the monovalent Nakayama vaccine with a specially formulated bivalent vaccine containing Nakayama plus Beijing-1 antigens and a tetanus toxoid placebo in more than 65,000 children in northern Thailand demonstrated identical, 91% efficacies of both JE vaccines after 2 years of follow-up (Hoke et al. 1988). Reduced attack rates of dengue and dengue hemorrhagic fever, related flaviral infections, were also observed in JE-vaccinated groups, although the differences were not significant. Experimental studies in monkeys and neutralizing antibody data in humans suggest that JE vaccines might also provide cross-protection against West Nile virus (Goverdhan et al. 1992); however, breakthrough West Nile infections have been documented following JE immunization (Johnson et al. 2005).

### 18.3.2 Cell Culture Vaccines

Attempts to produce a cell culture-derived JE vaccine were motivated by concerns about potential contaminating neural antigens and allergic reactions associated with

crude mouse brain vaccines and the desire to improve immunogenicity and ease of production. Among the numerous primary and continuous cell culture systems that were examined, primary hamster kidney (PHK) cells were discovered to produce the highest infectious yield (Lee, Grayston and Kenny 1965). The P3 strain of JE virus was recovered in 1949 from a patient's brain during the P1 (i.e. Beijing-1) strain epidemic. After 70 passages in mouse brain, it was demonstrated to be more immunogenic and induce broader responses in mice than did mouse brain-derived Nakayama strain vaccine; it was synergistic in mouse protection tests when combined with Nakayama strains in a bivalent, inactivated, PHK cell culture vaccine, although this formulation was not evaluated in humans (Gu and Ding 1987). A formalin-inactivated vaccine prepared from the P3 strain in PHK cells was produced exclusively in the People's Republic of China (PRC) and comprised the country's principal JE vaccine from 1968 to 2000 (Gu and Ding 1987).

Two doses of inactivated PHK cell-derived vaccine, given 1 week apart, were relatively poorly immunogenic in naïve children, with low neutralizing antibody responses of brief duration (Wang et al. 1979; Gu and Ding 1987). However, a booster dose resulted in anamnestic responses in more than 93% of recipients, with preservation of seropositivity for years in a majority of vaccinees and uniform seroconversion following subsequent booster dose (Gu and Ding 1987; Luo et al. 1994). Extensive, randomized field trials among 480,000 children have demonstrated efficacies in the range of 76–95% with this vaccine (Oya 1988).

In accordance with recent technical guidelines developed by the WHO (Hombach et al. 2005a, b; Ferguson et al. 2007), several groups developed experimental, formalin inactivated, whole-virion vaccines from infected Vero, or African green monkey kidney cell cultures that met mouse protection potency standards established for the inactivated mouse brain vaccine (Sugawara et al. 2002). The Walter Reed Army Institute of Research (WRAIR), in collaboration with international scientists, has been a leader in this regard (Srivastava et al. 2001).

The WRAIR has a long history of producing vaccines against diseases of military relevance; many, such as adenovirus, rubella, meningococcus, hepatitis A, and Japanese encephalitis have become licensed products (Artenstein et al. 2005). The list of contributors includes a number of renowned vaccine researchers: Sabin, Smadel, Hilleman, Buescher, Artenstein, Parkman, Meyer, Russell, and Innis, among others (refer to other chapters in the work). Charles Hoke, as Chief of the Virology Department at the Armed Forces Research Institute of the Medical Sciences (AFRIMS), WRAIR's Bangkok laboratory, planned and executed the pivotal efficacy study of inactivated mouse brain JE vaccine in 1984 (Hoke et al. 1988) that led to its eventual licensure by the Food and Drug Administration (FDA).

In the 1980s and 1990s, as reports accumulated on the frequent and sometimes serious adverse events associated with the licensed, killed, mouse brain vaccine, the need for a safer product became urgent. Ken Eckels, a WRAIR vaccine researcher who subsequently played an important role in the development of the first inactivated hepatitis A vaccine and directed the development of a variety of other vaccines for the military, discovered that dog kidney-adapted vaccine strain SA 14–14–2, brought to WRAIR in 1987 by Dr. Yu Yong Xin of China's National Institute for the Control of Pharmaceutical and Biological Products (NICPBP),

grew vigorously in Vero cells and was highly immunogenic after formalin inactivation. Yu had been working to adapt and grow test lots of the vaccine strain in validated primary dog kidney cells (PDK), such as those used in licensed measles and rubella vaccines in the U.S.

Phase II studies using inactivated SA 14–14–2 grown in Vero cells and formulated with alum, IC51 vaccine, showed dose-related, high seroconversion rates associated with homologous strain neutralizing antibody that persisted for 2 years in 85% of subjects (Lyons et al. 2007). In a multi-center, non-inferiority, phase III, randomized controlled trial involving 867 adults using WHO guidelines (Hombach et al. 2005b), seroconversion rates and neutralizing antibody titers were found to be higher in those given two doses of study vaccine as compared with three doses of licensed mouse brain vaccine (Tauber et al. 2007). Short-term adverse events were minimal and their incidence did not differ from those in the licensed vaccine group (Tauber et al. 2007). This vaccine marketed by Novartis as Ixiaro, has been licensed for use in individuals 17 years and older in the United States, the European Union and Australia.

The Research Foundation for Microbiological Disease of Osaka University produced a Vero-based vaccine formulated in alum, BK-VJE, using the Beijing-1 JE strain; it has been shown to be well tolerated, with only mild elevations of liver function tests, and immunogenic, with neutralizing antibody seroconversion observed in all seronegative subjects. Additionally, high levels of neutralizing antibodies have been noted after a single dose in children, previously immunized with mouse brain vaccine (Abe et al. 2006). High seroconversion rates have been observed with multiple dose regimens of other Vero cell-derived, inactivated JE vaccine formulations in children, although mild adverse events have been noted in as many as 45% of subjects following administration of the first dose. The Kitasato Institute in Japan has prepared stable and immunogenic, inactivated JE vaccines in Vero cells harvested without serum as a stabilizer (Toriniwa and Komiya 2007, 2008).

## 18.4 Live Attenuated Vaccines

### 18.4.1 SA 14–14–2

Many workers attempted attenuation of JE in a variety of cell cultures, including chick embryo and mouse embryo skin cells (Hammon et al. 1963, 1966; Inoue 1964; Kodama et al. 1968; Yoshida et al. 1981). Biologically, attenuation may be correlated with decreased binding to mouse brain cell receptors (Ni and Barrett 1998). Initial findings of loss of neurovirulence in animals suggested the possibility of safe use in humans, but these early tissue culture vaccines proved to be over-attenuated (Hammon et al. 1966).

The development of a successful, live, attenuated JE vaccine based on viral strain SA 14–14–2 was achieved by Dr. Yu Yong Xin. The parent virus, SA 14, a low, peripheral, pathogenic strain of JE isolated in 1954 from *C. pipiens* larvae collected in Xian, had demonstrated a remarkable drop of neurovirulence after 11 serial

passages in weanling mice and 20 passages in PHK cells. After 100 further PHK cell passages at 36–37°C, three of nine plaques tested showed low neurovirulence in monkeys; by further plaqueing and selection, a stable avirulent strain, SA 14–5–3, was selected for human trials (Yu et al. 1973, 1975, 1981; Ao et al. 1983; Eckels et al. 1988; Xin et al. 1988; Yu et al. 1989; Sil et al. 1992; Wills et al. 1992; Hase et al. 1993). SA 14–5–3 demonstrated safety in animals: it did not kill 3-week-old mice by either subcutaneous or direct intracerebral inoculation; intrathalamic and intraspinal inoculation in monkeys resulted in no mortality or morbidity and only minimal inflammatory response, limited to areas around the injection sites.

SA 14–5–3 vaccine was shown to be safe in humans and although associated with seroconversion rates in excess of 85% in endemic areas, rates in non-endemic areas were only 61% (Yu et al. 1973). Expanded field trials in southern China involving more than 200,000 immunized children confirmed the vaccine's safety and durable efficacy, 88–96% over 5 years (Yu 1981). Nonetheless, the vaccine's poor immunogenicity in flavivirus-naïve subjects from non-endemic areas suggested that SA 14–5–3 virus, like previous live, cell culture-passaged, vaccine candidates had been over-attenuated and did not replicate uniformly in humans. Additional serial passage via subcutaneous inoculation of suckling mice using non-neural tissues (Yu et al. 1975), followed by plaque selection and cloning in PHK cells resulted in the SA 14–14–2 strain that maintained reduced neurovirulence and was equally attenuated, but was more immunogenic in animals and humans, associated with seroconversion rates greater than 90% in non-immune subjects (Yu et al. 1981; Ao et al. 1983).

The underlying molecular basis of the vaccine's neuro-attenuation is still under investigation. Genomic sequence analyses of the virulent parent SA 14 virus and its derived vaccine viruses demonstrate a small number of stable, amino acid substitutions that are associated with attenuation (Aihara et al. 1991; Ni et al. 1994, 1995). Attenuation of JE viruses involves the selection of neutralization escape variants, through which attenuation appears to be associated with single base mutations, resulting in single envelope protein amino acid changes linked with altered early virus-cell interactions but not with replication (Cecilia and Gould 1991; Hasegawa et al. 1992; Ni and Barrett 1996).

The reduced neurovirulence of the SA 14–14–2 strain, as compared with the parent virus, was confirmed in both weanling mice and rhesus macaques by the absence of mortality and only minor morbidity with intracerebral inoculation of the former, and by the absence of clinical illness and only minor inflammatory reactions in the central nervous system of the latter animals with combined intrathalamic and intraspinal inoculation (Yu et al. 1962). Ultrastructural studies of 5-week-old mice inoculated intracerebrally showed that the parent virus produced cytopathologic changes in the majority of neurons, particularly in the rough endoplasmic reticulum and Golgi apparatus of the neuronal secretory system; the neurons of mice given the vaccine strain appeared normal, and viral replication could not be confirmed (Hase et al. 1993).

After clinical efficacy was demonstrated in field trials, the SA 14–14–2 vaccine was licensed in the PRC in 1988 and currently, 50 million doses are distributed annually in southwestern and western China. Protection rates of SA 14–14–2 vaccine among 1–10 year-old children in five major Chinese trials conducted between

1988 and 1999 were consistently above 98% (Wang et al. 1993; Halstead and Jacobson 2008); it was found to be “non-inferior” to the widely used inactivated vaccine using tissue culture neutralizing antibody immunogenicity endpoints (unpublished). Subsequently, the annual distribution of inactivated vaccine has decreased from its peak of approximately 70 million doses in the late 1990s to about 13 million in 2004, concurrent with increased use of the live, attenuated vaccine. As of 2005, a variety of JE vaccine strategies remain in use: 16 provinces use live, attenuated SA 14–14–2 vaccine; eight inactivated P3 vaccine; three a combination of both; and three provinces in higher altitude, non-endemic areas of north and far western China do not employ JE vaccination (Yu 2004).

### ***18.4.2 Internationalization of SA 14–14–2***

In 1982, when the author, then Deputy Director of the Health Sciences Division of the Rockefeller Foundation, visited the NICPBP and met Dr. Yu, a new chapter in the history of JE vaccine was initiated: an attempt to work with Chinese vaccine manufacturers to internationalize SA 14–14–2 so it might meet the growing needs for an effective and affordable JE vaccine in Southeast and Southwest Asia. Recognizing the regulatory problem posed by the growth of virus on PHK cells, a vaccine substrate that had not been used to produce any licensed vaccine in the U.S. or Europe, it was decided to re-adapt SA 14–14–2 to a licensed substrate, PDK cells. This led to the collaboration between Dr. Yu and Eckels at the WRAIR and the adaptation of SA 14–14–2 virus to PDK cells. Because of the limited relations between the U.S. and China at the time, it required 3 years’ petition to U.S. authorities to re-export the new version of the organism to China. When a small field trial was eventually completed in susceptible Chinese adults, the PDK-adapted vaccine demonstrated unacceptably low immunogenicity, and further efforts along this line were abandoned.

Meanwhile, with the licensure of Dr. Yu’s original formulation of PHK cell-derived SA 14–14–2 vaccine in China in 1988, the full-scale manufacture of the product proceeded at the Chengdu Institute of Biological Products (CDIBP), the one Chinese facility at the time to have large-scale lyophilization equipment. The Rockefeller Foundation, in collaboration with this Institute, endeavored to improve the production of SA 14–14–2 by using baby hamster kidney cells (BHK) that were free of adventitious agents, in a specially designed and newly built manufacturing facility that would be recognized by the WHO as qualifying for the export of vaccine from China to other Asian countries enzootic for JE. Two internationally recognized expert consultant vaccinologists, Drs. Alex Shelokov and C.J. Lee, were sent to Chengdu to assess the production and safety of BHK cells produced in China and to develop a plan to bring the production of Chinese vaccine up to international standards. A U.S. engineering and architectural firm was engaged to design a Good Manufacturing Practices (GMP)-compliant facility up to U.S. FDA standards that was capable of producing 30 million doses per year. Costs to design, construct, and equip this facility were estimated at \$26 million; however,

construction was impeded by a \$5 million CDIBP shortfall - money needed to purchase GMP-compliant lyophilization and filling equipment.

At this time, a case-control study, designed by Liu Zenghle and Sean Hennessy of the University of Pennsylvania and undertaken through Rockefeller contacts at the West China Medical University in Chengdu, documented excellent protection of children by the SA 14–14–2 vaccine administered in rural areas surrounding Chengdu (Hennessy et al. 1996). Once published, this study attracted a Korean businessman, Mr. Hyunsoo Kim, chief executive officer of Boran Pharmaceuticals, who proposed to import SA 14–14–2 vaccine and sell it for use in Korea and, ultimately, throughout the JE endemic areas of Asia, fortuitous because this author's retirement from the Rockefeller Foundation in 1995 effectively ended their support for the internationalization effort. Efforts to obtain a license for SA 14–14–2 in Korea were stalled when the eight manufacturers of JE mouse brain vaccine brought political pressure on the Korean FDA, the latter subsequently citing the unknown dangers of using PHK cells as a reason to withhold consideration of SA 14–14–2. With the expiration date for the 200,000 vaccine doses specially prepared for export to Korea rapidly approaching, this author suggested that it be given to a population at risk for JE.

Following the suggestion by Theodore Tsai of the American CDC, a team comprising Hyunsoo Kim, Sun Heang Shin, and the author visited Kathmandu, Nepal in February, 1999 where it was learned that the northwestern provinces of the Nepalese Terai had suffered recurrent epidemics of JE over the past decade. Working with epidemiologist M.K. Banerjee and with the support of M. B. Bista, Director of the Epidemiology and Disease Control Division of the Nepal Ministry of Health, a plan to deliver all 200,000 doses was developed. The vaccine was to be administered as a candidate requiring written consent of vaccinees, parents, or guardians. Applications for an investigational new drug study and a case-control study to measure vaccine efficacy were approved by the Nepal Research Council by July 1999.

A group including the author, pediatrician Dr. Young Mo Sohn, epidemiologist Dr. Heechoul Oh, public health specialist Dr. Myung Ho Kim, epidemiologist J.B. Tandan, and project administrator Ms. Sun Heang Shin visited both the Bheri Zonal Hospital to recruit the hospital director and staff into the study and the Vector Borne Research and Training Center, Hetauda, Nepal to meet with technicians who performed the IgM-capture ELISA test for JE antibodies, required for case identification. Refrigerated vaccine was shipped to Nepal and during late July, vaccine was administered to children and adult volunteers in the Bardia, Banke, and Kailali Districts.

At 79 health posts more than 160,000 subjects, 1–15 years of age were given vaccine containing  $10^{5.8}$  PFU/0.5 mL of live, attenuated virus that had been packaged for export in individual syringes. Once clinical records were reviewed and families interviewed in hospital, it became apparent that none of the 227 clinically diagnosed JE cases nor any of the 20 serologically proven cases had received vaccine. The efficacy of a single dose of SA 14–14–2 vaccine in preventing JE cases when given just a few days prior to the onset of a JE epidemic was 99.3% (Bista et al. 2001); follow-up 1 year later showed the efficacy remained greater than 98% (Ohrr et al. 2005).

The selection and interviewing of controls was almost entirely the responsibility of Dr. J.B. Tandan. Parents or guardians of JE cases and controls were interviewed in 2004 for vaccination history by a hospital nurse or field worker, respectively; a child was listed as vaccinated if the parents reported vaccination during 1999 and there was additional proof, either a record of the child's name in the vaccine registry, inspection of an individual vaccination card (in wide use in Nepal), exact recall of vaccination post, or name of vaccinator. Five years after receiving vaccine, its protective efficacy continued to be in excess of 95% (Tandan et al. 2007). This longitudinal experience confirmed the benefits of SA 14–14–2 in epidemic JE based on its rapid induction and durability of protection, probably life-long, following a single dose. SA 14–14–2 vaccine is comparable to yellow fever 17D, long regarded as the prototype live, attenuated vaccine because of its low rate of adverse events and long duration of immunity (refer to Chap. 10).

Elsewhere, Dr. Julie Jacobson, working at PATH, an international, non-profit, health promotion organization, established an epidemiological study site for JE in Hyderabad, Andhra Pradesh, India in 2003. A close collaboration with state and national health officials allowed Jacobson, director of the JE prevention program, to approach the Government of India following the disastrous 2005 epidemic in Uttar Pradesh with an offer to make available millions of doses of live, attenuated SA 14–14–2 vaccine. The offer was possible as PATH had negotiated a landmark agreement with Chinese JE vaccine manufacturer CDIBP to honor public-sector pricing for the live, attenuated SA 14–14–2 product for lower income, endemic countries in Asia and had provided support to the CDIBP to select a qualified engineering and design company to oversee the construction of a new vaccine production facility that will meet WHO standards and make them eligible to bid to sell vaccines to agencies supported by international development funding.

As a result more than 11 million children in India were vaccinated against JE in 2006, a mass campaign that reached 88% of the target population in 11 high-risk districts. Additionally, PATH staff planned and executed a successful trial showing the effectiveness of live, attenuated measles and JE vaccines when co-administered to 15 month-old children, an important first step to integrate JE vaccine into Expanded Program on Immunization schedules (Hombach et al. 2005b; Gatchalian et al. 2008). With PATH technical assistance, SA 14–14–2 vaccine was licensed for use in India, a significant achievement as India's National Regulatory Authority is WHO-approved.

## 18.5 New Vaccine Approaches

A novel, promising genetic approach to JE vaccines was developed by Tom Monath, a tropical medicine physician who had spent the first phase of his virology research career with the CDC and the U.S. Army Medical Research Institute for Infectious Diseases before entering the civilian vaccine development industry, and Thomas Chambers, at the St. Louis University School of Medicine. They pursued the construction of flavivirus chimeras in which the yellow fever 17D genome and SA 14–14–2 contribute non-structural and structural genes, respectively (ChimeriVax-JE;

Acambis, Cambridge, MA, USA) (Chambers et al. 1999); the DNA is transcribed to RNA and subsequently electroporated into Vero cells. The resultant infectious clone has the neurovirulence properties of SA 14–14–2 and favorable Vero cell growth characteristics of 17D; attenuation is dependent on clusters of amino acid mutations in the JE envelope protein (Monath et al. 1999, 2002a; Arroyo et al. 2001), on the attenuated phenotype of 17D, and on the chimerization process which further attenuates the virus over that of either gene donors.

The YF/JE chimera has proved to be highly immunogenic in rhesus monkeys, protects against intracerebral and intranasal challenge using a wild-type JE strain (Raengsakulrach et al. 1999; Monath et al. 2000), and is non-transmissible by mosquito vectors of JE or YF viruses. Its clinical development to date has included a total of 3,045 healthy adult and pediatric subjects who have participated in numerous clinical trials (Monath 2008). In an initial phase I trial in 36 healthy adults, half of whom had previous immunity to yellow fever, adverse events following vaccination were mild and similar to those observed with 17D vaccine: injection site reactions, headache, fatigue, and fever (Monath et al. 2002b). A low-level, transient viremia was present in the majority of both yellow fever naïve and immune subjects, and was similar in magnitude and duration to that induced by yellow fever 17D vaccine. All recipients of the chimeric vaccine, including yellow fever immune subjects, seroconverted against JE within 30 days following vaccination; previously immune subjects responded with higher JE neutralizing antibody titers than naïve subjects (Monath et al. 2002b). Similarly, prior yellow fever immunity did not appear to reduce the response to the chimeric vaccine (Guirakhoo et al. 1999).

A double-blind, phase II trial of the chimeric vaccine confirmed a transient viremia in most subjects, with virus titers inversely correlated with inoculum dose, and a seroconversion rate of nearly 100% against the parental virus, irrespective of dose, in flavivirus naïve volunteers, although the rate was lower against three wild-type JE strains (Monath et al. 2003). Despite waning of neutralizing antibody titers by 6 months, immunological memory was uniformly elicited with inactivated JE vaccine (Monath et al. 2003). Pivotal, phase III studies on safety and efficacy in adults have been completed and showed that the chimeric vaccine was associated with higher immunogenicity, statistically superior efficacy as compared with licensed, inactivated JE vaccine, and was safe and well tolerated. Antibody responses are durable, making the single dose, chimeric product suitable for protection of both travellers to and residents of JE-endemic areas. Pediatric clinical trials in India assessed the safety and tolerability of ChimeriVax-JE in children and its co-administration with measles vaccine.

## 18.6 Conclusion

The history of JE vaccines continues to evolve. Live, attenuated SA 14–14–2 vaccine is available in Korea and parts of South Asia but is not yet WHO prequalified. Inactivated vaccine based on the SA 14–14–2 strain IC51 is now licensed in the U.S., the European Union and Australia; chimeric vaccine is not yet licensed for

use anywhere in the world. Multiple, inactivated vaccines derived from Vero cells are under study in Japan. Based on the absence of person-to-person transmission, with its potential for herd immunity, JE control ultimately depends on individual protection; the world appears to be on the verge of having several safe and potent JE vaccines available. Time will tell whether Southeast Asia, India, Pakistan, and Bangladesh will join Japan, Korea, Taiwan, and China to successfully relegate JE to the dustbin of history.

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# Chapter 19

## Hepatitis A

Leonard N. Binn and Stanley M. Lemon

### 19.1 The Setting for Vaccine Development

Although hepatitis A is arguably among the most ancient of human diseases and among the first to have been recorded by physicians, the responsible infectious agent, a unique picornavirus, hepatitis A virus (HAV), was identified less than 40 years ago (Feinstone et al. 1973). The discovery of the virus built on decades of previous research led by investigators such as Paul Havens and Saul Krugman, who had defined many of the clinical features of the disease, clearly distinguished it from “homologous serum jaundice” (known today as hepatitis B) and documented the predominantly fecal-oral nature of HAV transmission (Havens 1945; Krugman and Ward 1958). However, the discovery of the virus, accomplished by immune electron microscopy of suspensions of fecal samples collected from infected human volunteers (Feinstone et al. 1973), marked a turning point in the control of hepatitis A that is matched only by general improvements in public health and sanitation that have resulted in decreasing infection prevalence in many regions of the world. The discovery of HAV led relatively rapidly to the general availability of sensitive and specific diagnostic tests and, not long afterwards, to the successful commercial manufacture of a safe and potent vaccine. These are the events that we attempt to describe here.

As important to vaccine development as discovery of the virus was the development of tractable animal models of hepatitis A. Early efforts in the 1970s at Merck, Sharp, and Dohme by Phil Provost and Maurice Hilleman, the latter already credited with the development of a number of successful vaccines against human

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viral pathogens (refer to Chapters 13 and 14), demonstrated the utility of marmosets and tamarins as animal models (Mascoli et al. 1973). Experimental infections of marmosets provided potent sources of virus, enabling its characterization, and served as a source of immune serum, facilitating the development of a serologic assay and providing the materials and methods needed for vaccine development. Jules Dienstag, Stephen Feinstone, and Robert Purcell at the U.S. National Institutes of Health (NIH) developed a chimpanzee model that closely paralleled human disease (Dienstag et al. 1975). Studies in the chimpanzee confirmed the hepatotropic nature of HAV infection and demonstrated copious viral shedding from the liver into the gut via the biliary system (Schulman et al. 1976).

Stanley Lemon, James LeDuc, and Leonard Binn at the Walter Reed Army Institute of Research (WRAIR) subsequently described infection of the New World owl monkey, *Aotus trivirgatus* (LeDuc et al. 1983). LeDuc, a U.S. Army scientist stationed at the Gorgas Memorial Institute in Panama, demonstrated that owl monkeys captured in the wild frequently became infected with HAV and showed this animal species to be highly susceptible to HAV infection (Lemon et al. 1982). This was confirmed in subsequent experimental challenges of owl monkeys carried out collaboratively between the United States Army Medical Research Institute of Infectious Diseases (USAMRIID), in Frederick, Maryland, and the WRAIR in Washington, D.C. (LeDuc et al. 1983).

These and other studies firmly established the owl monkey as an excellent model of human hepatitis A infection and provided compelling evidence for infection of the gastrointestinal epithelium following oral challenge with virus (Lemon et al. 1988; Asher et al. 1995). While virus could be detected within the liver 5 days after oral inoculation, HAV antigen was found in epithelial cells of the intestinal crypts and cells of the lamina propria of the small intestine as early as 3 days after infection (Asher et al. 1995). The much lower costs and ready availability of colony-bred *Aotus* provided a significant advantage over the use of the endangered chimpanzee for such studies; the larger size of the owl monkey as compared to marmosets or tamarins made the former species generally easier to work with.

## 19.2 Scientific Underpinnings

Like many recent vaccines, the hepatitis A vaccine did not emerge as the result of the work of a single investigator or group of investigators, or indeed even a single institution or commercial entity. The story is one to which the U.S. Army and the WRAIR contributed substantially. Long interested in hepatitis A because of the well-known viral ability to play a decisive role in military campaigns, the Army had years previously funded the human volunteer studies directed by Joseph Boggs that produced the biologic materials in which the virus was subsequently identified by Feinstone, Kapikian, and Purcell at the NIH (Boggs et al. 1970; Feinstone et al. 1973).

Astute vaccinologists at Merck, Sharpe & Dohme recognized the commercial potential of a vaccine against hepatitis A (something that was not well appreciated

by the marketing division at Merck for a number of years) and laid the scientific foundations for its development. In the late 1970s, 6 years after the discovery of the virus, the Merck group led by Hilleman, himself a WRAIR scientist earlier in his career, published the first credible reports of the cultivation of HAV in mammalian cells (Provost and Hilleman 1979). The virus used in these studies, the CR326 strain, had been recovered in Costa Rica by Victor Villarejos in the course of collaborative studies of hepatitis epidemiology in Central America. Passage of the virus in marmosets facilitated its isolation in cell culture; HAV was found not to be cytopathic and specific immunoassays were required to detect its growth in cells (Provost and Hilleman 1979). Not long afterward, the HM175 strain of HAV was isolated directly in African green monkey kidney (AGMK) cells in Purcell's laboratory at the NIH using fecal material brought there by Ian Gust, a visiting scientist from Australia (Daemer et al. 1981). These two virus strains, CR326 and HM175, would play central roles in future vaccine development efforts.

Several years after the isolation of virus in cell culture, the RNA genome of HAV was reverse-transcribed, and the first molecular clones of cDNA were prepared by John Ticehurst working in the Purcell laboratory at NIH (Ticehurst et al. 1983). A complete cDNA copy of the genome was subsequently cloned and fully sequenced by a group headed by Dino Dina at Chiron, then a small California biotech company (Najarian et al. 1985). Data from these efforts firmly placed the virus within the family *Picornaviridae*, something that had been under debate within the field. Shortly thereafter, in work also from Purcell's laboratory, RNA transcripts derived from a genome-length cDNA clone were shown to be infectious when transfected into cultured cells (Cohen et al. 1987), thereby ushering HAV into the era of reverse molecular genetics.

### 19.3 Strategies for HAV Vaccine Development

From the outset, the goal was to develop a vaccine capable of producing neutralizing antibodies, since the value of passive immunization with pooled human immune globulin in providing a high, albeit transient level of protection had been known for almost 40 years (Gellis et al. 1945). Early on, three general strategies were envisioned for vaccine development; two would rely on tried and proven techniques based on decades of experience with other successful virus vaccines, and one that would attempt to use the new molecular biology to express the capsid proteins of HAV in yeast or bacteria in order to produce a recombinant hepatitis A vaccine. A major consideration at the time was that, although HAV could be adapted to growth in cell types suitable for vaccine manufacture, replication was slow and virus yields were relatively low.

The concept of a recombinant approach to HAV vaccine generated considerable enthusiasm from many quarters in the 1980s. Emerging data at the time suggested that a yeast-derived recombinant hepatitis B vaccine would likely replace the earlier, plasma-derived vaccine (refer to Chapter 17). Additionally, a recombinant

vaccine would obviate difficulties for vaccine manufacture engendered by the inefficient growth of HAV in cell culture. However, hopes for a quick solution to the vaccine problem via the wonders of recombinant DNA were soon abandoned when it was recognized that immunization with any of the three major HAV capsid proteins failed to stimulate neutralizing antibodies. Although weak neutralizing activity could be obtained with various HAV peptide sequences (Emini et al. 1985), this was clearly insufficient as the basis for an effective vaccine. The first crystal structures of picornaviruses revealed the strongly conformational nature of their critical neutralization epitopes and explained the aforementioned findings (Hogle et al. 1985; Rossmann et al. 1985). No comparable crystallographic or cryo-electron microscope structure yet exists for the HAV capsid, but the conformational nature of HAV neutralization epitopes has been documented in studies of cell culture-derived mutants that escape neutralization by monoclonal antibodies (Ping and Lemon 1992). Although it was eventually shown that a workable immunogen could be produced by promoting the self assembly of recombinant HAV capsid proteins expressed in a baculovirus system (McLinden et al. 1991), such a vaccine strategy was never commercialized.

## 19.4 Efforts toward Development of an Attenuated Vaccine

Recognition that HAV was a picornavirus led vaccinologists to explore vaccine strategies that had succeeded previously with other members of this virus family, particularly poliovirus. Given the relatively low yields of HAV being achieved in cell culture, the notion of producing an oral, live attenuated vaccine akin to the Sabin polio vaccine was attractive at several levels. First, the general impression among those in the field was that such a vaccine would require a lesser amount of virus and thus be easier to produce than an inactivated, Salk-like vaccine. Also, since the natural route of infection was known to be oral, it was considered likely that an oral vaccine might produce better immunity, perhaps protecting against transmission of the virus as well as hepatocellular disease, much as the Sabin vaccine produced systemic as well as mucosal immunity. It was recognized from the outset that wild-type HAV grew poorly in cell culture, and that with adaptation to cell culture the magnitude of the virus yield would be increased and the time required to reach such yields would be reduced. The virus could also be adapted to maximal growth at temperatures lower than the human core temperature. Both approaches appeared to result in genetic changes in the virus that reasonably could be expected to produce an attenuated phenotype.

The Merck group, led by Provost and Hilleman, invested significantly in the attenuated virus approach using the CR326 virus as starting material, while Purcell and his colleagues initiated a comparable set of studies with the HM175 virus at NIH. Work done several years later by Suzanne Emerson at the NIH revealed that the adaptation of the HM175 virus to growth in cell culture led to genetic changes in the nonstructural proteins of HAV, particularly in 2B, the basis of which remains

unexplained today (Emerson et al. 1992; Funkhouser et al. 1994). Other changes in the 5' nontranslated RNA of the virus identified in the Lemon laboratory at the University of North Carolina were responsible for fine tuning replication in particular cell types, most likely by altering interactions of the viral internal ribosome entry site with host cell-specific proteins (Schultz et al. 1996; Funkhouser et al. 1999). Unfortunately, these changes, which were poorly understood from a genetic perspective at the time, did not result in an attenuation phenotype that was acceptable for a vaccine.

Data generated by both the Merck and NIH groups demonstrated that cell culture-adapted viruses were clearly limited in their ability to replicate in nonhuman primates, particularly upon oral challenge, resulting in a generally poor and variable immune response (Provost et al. 1982, 1983). Both the NIH HM175 and Merck CR326 candidate vaccines made it to phase I studies in humans, carried out at the WRAIR by Maria Sjogren (Sjogren et al. 1992) and at Johns Hopkins by Karen Midtun (Midtun et al. 1991), respectively. The results were disappointing. Although the vaccines were found to be safe, relatively high doses were required to achieve what was considered to be a useful immune response. Oral administration of the NIH candidate failed to elicit antibodies; intramuscular administration of  $10^6$ - $10^7$  TCID<sub>50</sub> was needed for their detection (Sjogren et al. 1992). Contemporaneously, the results of a human trial of a prototype inactivated vaccine developed at WRAIR became known, providing a critical proof of concept for this approach to vaccine development, sharply tilting the momentum within the field toward a killed virus approach, and ending the progress of the Merck attenuated candidate.

An interesting footnote to the attenuated vaccine story is the subsequent development of an attenuated HAV vaccine in China. Relatively few details have been published concerning the Chinese vaccine, developed by J.S. Mao, a previous visiting scientist in Purcell's laboratory at the NIH. The vaccine, based on cell culture propagation of the H2 strain of HAV, has apparently been used widely in China and was reported in 2000 to have provided 95% protection against clinically-evident hepatitis during outbreaks of disease in young school children (Zhao et al. 2000). A report of a more recent study conducted in India suggests that an H2-based Chinese vaccine elicited a protective antibody level in a comparable number of school children there (Bhave et al. 2006). Little is known about the H2 strain, although its nucleotide sequence, as deposited in GenBank, is remarkably close to the HM175 virus studied in the Purcell laboratory.

## 19.5 Inactivated Hepatitis A Vaccines

The first experimental HAV vaccine was made by Merck from purified suspensions of inactivated HAV-infected marmoset liver (Provost and Hilleman 1978). The inactivated HAV liver suspensions were highly antigenic in marmosets, protecting them against subsequent challenge with the CR326 virus. The development of methods for propagating HAV in marmoset liver and, later, in fetal rhesus monkey

kidney cell cultures provided better sources for viral antigen and enabled Hilleman's group to continue their efforts to develop an inactivated vaccine concurrently with their efforts towards an attenuated vaccine (Hilleman 1993). The Purcell group collaborated with workers at SmithKline Beecham in Rixensart, Belgium, in an effort to produce a commercial vaccine based on cell culture production of the HM175 virus. However, the first vaccine based on inactivated HAV produced in cell culture to be evaluated in humans was developed by a team at WRAIR.

Efforts had been initiated at WRAIR in 1980 to propagate the virus in cell culture and to develop quantitative virus- and antibody-mediated neutralization assays. These efforts occurred in parallel with the aforementioned work on the development of the owl monkey as a suitable animal model for vaccine evaluation. Initial studies in several types of primate cell cultures confirmed earlier reports from Merck and NIH, as field isolates from military-associated outbreaks and marmoset-passaged HM175 virus obtained from Purcell were serially passed and detected by radioimmunoassay and fluorescence microscopy (Binn et al. 1984). Primary AGMK cells suitable for vaccine production were infected with several strains, and after serial passage, increasing viral yields were obtained. Harvests of HM175 virus from serum-free cultures were shown to be immunogenic in rabbits and guinea pigs, indicating their potential for use in vaccine production.

To provide a quantitative viral assay, a radioimmunofocus assay (RIFA), similar to a plaque assay was developed. Foci of infected BSC-1 cells grown under an agarose overlay were detected with [<sup>125</sup>I]-labeled IgG prepared from a plasma unit collected from a colleague at WRAIR who had experienced acute hepatitis A while on duty in Africa (Lemon et al. 1983). As in early studies of the poliovirus plaque assay, the number of radioimmunofoci was found to be directly related to sample dilution, indicating that each focus resulted from a single infectious HAV particle. Moreover, the development of foci was prevented by specific antibodies, thus enabling the development of the radioimmunofocus inhibition test (RIFIT) for neutralizing antibody to HAV (Lemon and Binn 1983). HAV produced in cell culture was found to hemagglutinate several species of red blood cells, and the agglutination was specifically inhibited by immune chimpanzee serum and human and animal convalescent sera (Eckels et al. 1989). The RIFA and RIFIT assays became standards in the field for quantitation of infectious virus and virus-neutralizing antibodies. A modification of the RIFA, incorporating murine monoclonal anti-HAV labeled with an infra-red responsive fluorescent tag, (Counihan et al. 2006) remains in use today for quantifying cell culture-produced virus.

To demonstrate the feasibility of producing an inactivated vaccine from cell culture-produced virus, the HM175 virus was adapted to BSC-1 cells after ten passages in AGMK cells, thereby generating high virus yields. Three experimental lots of formalin-inactivated vaccine were produced by Binn and his coworkers in BSC-1 cells at WRAIR (Binn et al. 1986). Formalin treatment rapidly inactivated virus infectivity; no viable virus was detected by the fourth day. Each of the three vaccine lots was found to be antigenic in guinea pigs. Safety and immunogenicity tests in six naïve owl monkeys ensued, with each receiving three doses of vaccine by the intramuscular route. Although none of the monkeys excreted virus in their feces,

each animal developed neutralizing antibodies after the second dose. Subsequent oral or intravenous challenges revealed that the vaccinated monkeys were protected. Unlike control monkeys, neither virus shedding nor biochemical evidence of hepatic inflammation were detected in the vaccinated animals (Binn et al. 1986). The results confirmed that an effective HAV inactivated vaccine could indeed be produced from cell culture.

However, to develop a similar inactivated HAV vaccine for use in humans it was necessary to find a different cell substrate for viral production. MRC-5 human diploid cells had been used previously for vaccine manufacture, and the AGMK cell-passaged HM175 isolated at WRAIR was subsequently adapted to MRC-5 cells. After six passages in these cells, high yields of virus were once again obtained, a seed virus lot was generated, and a formalin inactivated HAV vaccine was prepared (Binn et al. 1988). The candidate was safety tested in cell cultures and owl monkeys and was found to produce neutralizing antibody in 87% of guinea pigs after two doses and in all five owl monkeys. Challenge of the monkeys after a third dose indicated that the vaccine was protective, as none of the animals excreted virus in their stools or experienced elevated liver enzymes (Binn et al. 1988). Additional safety tests required by the FDA did not detect any adventitious agents. Thus, pre-clinical findings indicated that this cell culture-produced inactivated vaccine was both safe and protective.

## 19.6 The First Human Trial of an Inactivated HAV Vaccine

In March of 1986, after investigational new drug (IND) approval, the first trial of an inactivated HAV vaccine prepared in cell culture was initiated in eight volunteer members of the WRAIR staff. Each received three intramuscular doses of vaccine on days 0, 28, and 56, and a booster dose 6–8 months later. None of the volunteers had any signs or symptoms of hepatitis, detectable viral antigen in their stools, or elevations in serum liver enzymes (Sjogren et al. 1988). After the first three doses, four of the volunteers had neutralizing antibody; after the fourth dose, all responded with neutralizing antibody, although detected by a less sensitive radioimmunoassay in six volunteers. As the radioimmunoassay was not sufficiently sensitive to detect antibody in recipients of immune globulin, who were known to be protected from disease, these early serologic results of immunization with the inactivated vaccine were highly encouraging. Moreover, the vaccine-related antibodies were subsequently found to persist for several years.

Further testing of the WRAIR vaccine in 21 additional volunteers provided additional evidence for its immunogenicity and safety. Volunteers received either three doses of vaccine at 0, 1 and 6 months, or four doses at 0, 1, 2, and 6 months. Two months after the last dose of vaccine, 86% of each group had neutralization titers of 1:40 or greater by RIFIT. Five asymptomatic volunteers experienced minimal and transient elevations of alanine aminotransferase (ALT) that were not associated with the appearance of IgM anti-HAV antibodies, the latter the best

indicator of acute HAV infection. The results indicated the three- and four-dose regimens were equivalent (Sjogren et al. 1991a). Although a more potent vaccine would be required for commercialization, the WRAIR vaccine had provided critical proof of concept and pointed the way for future efforts in the field.

### ***19.6.1 Commercial Development and Proof of Vaccine Efficacy***

Subsequent vaccine development efforts occurred primarily within the commercial sector, with two manufacturers scrambling for the lead in gaining FDA approval and marketing an inactivated HAV vaccine. The long-standing effort at Merck was increasingly focused on developing an inactivated rather than an attenuated vaccine based on the CR326 strain of HAV, while SmithKline Beecham began efforts toward commercial development of a vaccine based on the HM175 virus isolated at NIH. These commercially produced vaccine candidates differed from the prototype WRAIR vaccine in that they contained greater amounts of partially purified antigen with aluminum hydroxide added as an adjuvant. Clinical investigators at WRAIR remained involved, conducting early safety and immunogenicity studies of a vaccine candidate produced by SmithKline Beecham. In these studies, none of the study subjects developed overt signs of hepatitis or elevated ALT levels, yet all developed neutralizing antibody after two doses, with antibody responses markedly increased after a third, booster dose (Sjogren et al. 1991a). Delaying the booster dose to 12 months did not improve the response (Sjogren et al. 1991b).

Efforts toward developing an inactivated vaccine also moved quickly at Merck, using the attenuated CR326 virus as the basis for antigen production (Hilleman 1993). Doubts over the size of the market and thus the commercial potential of such a vaccine seemed to plague their development program, but by 1991 both the Merck and SmithKline Beecham vaccines were ready to enter large scale efficacy testing.

The Merck product, subsequently licensed as VAQTA, was a highly purified preparation of inactivated virus particles; the SmithKline Beecham candidate, eventually marketed as Havrix, contained substantial amounts of contaminating cellular proteins associated with the inactivated virus (<5 µg nonviral proteins per dose, compared with <0.1 µg for the Merck vaccine) (Lemon and Thomas 1997). Despite this, the two vaccines appeared to have comparable immunogenicity and reactogenicity. Some debated the need for clinical trials to demonstrate efficacy, given that the vaccines were producing levels of neutralizing antibody substantially in excess of those associated with the accepted protection afforded by passive immunization with pooled human globulin. However, there was never much doubt that the FDA would require clinical demonstration of efficacy for vaccine licensure.

The first clinical proof of efficacy was obtained with Merck's formalin-inactivated HAV vaccine (Werzberger et al. 1992). The vaccine was evaluated in Hasidic Jewish children residing in Kiryas Joel, a community in southern New York State which had historically experienced a high rate of hepatitis A infections each summer.

The study, led by Alan Werzberger, a community pediatrician, involved a double-blind comparison of vaccine versus placebo. Enrollment was initiated almost simultaneously with the onset of an outbreak of hepatitis A that swept through the children in the community, as had occurred in prior years. A single vaccine dose demonstrated 100% efficacy in preventing hepatitis A. No cases occurred in children who had been immunized more than 21 days previously (Werzberger et al. 1992). Since the incubation period of hepatitis A is about 28 days, this suggested the possibility of some protection even if vaccine were given following exposure.

Continuing progress in evaluating the SmithKline Beecham vaccine, including studies carried out in collaboration with the NIH and WRAIR groups (Karron et al. 1988; Purcell et al. 1992) and studies by the Ministry of Public Health of Thailand provided, in part, the basis for approval of a large-scale, double-blind efficacy trial of the inactivated HM175 virus vaccine. Directed by Bruce Innis, a WRAIR investigator, the trial involved more than 40,000 children in 148 primary schools in the Kamphaeng Phet Province of rural, northern Thailand. Participants were given either the formalin-inactivated, candidate hepatitis A vaccine or recombinant hepatitis B vaccine as a control, both at 0, 1, and 12 months (Innis et al. 1994). No serious adverse events were noted. Prior to the 1 year booster dose, 94% of the initially seronegative children had seroconverted; 99% were seropositive after the booster. During the trial, there were 40 cases of hepatitis A, of which 38 occurred in the control group, indicating that the vaccine had 95%, cumulative, protective efficacy through the 18 month observation period (Innis et al. 1994). Despite the fact that data supporting the efficacy of the Merck vaccine had been published 2 years earlier, the SmithKline Beecham vaccine, Havrix, became the first HAV vaccine to be licensed in the U.S. in 1995. The Merck vaccine, VAQTA, was licensed the following year. Contemporary efforts in several other countries, most notably Japan and Switzerland, also eventually led to the successful registration of inactivated vaccines based on different virus strains.

## 19.7 The Impact of Hepatitis A Vaccines

Following the licensure of HAV vaccines in 1995, the Advisory Committee on Immunization Practices in the U.S. promulgated recommendations for targeted vaccination of selected high-risk groups and routine vaccination of certain pediatric populations living in states with high incidence rates (CDC 1996, 1999). As a result, national HAV infection rates declined by 76% and the epidemiology shifted from hepatitis A being primarily a pediatric infection to one most commonly affecting young adult men (Wasley et al. 2005). HAV vaccines have had a dramatic impact on the incidence of disease in other settings as well. In Israel, where hepatitis A vaccination at 18 and 24 months of age became mandatory in 1999, in excess of 90% overall reductions in disease have been observed as compared with prevaccination rates (Dagan et al. 2005). Broader application of HAV vaccines throughout the world will depend on epidemiologic and economic considerations.

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# **Chapter 20**

## **Rotavirus**

**Penelope H. Dennehy**

### **20.1 Introduction**

#### **20.1.1 *Rotavirus Disease Burden and Epidemiology***

Rotavirus is the leading cause of severe diarrhea in infants and young children worldwide (de Zoysa and Feachem 1985). Each year rotavirus causes approximately 114 million episodes of gastroenteritis in children less than 5 years of age (Parashar et al. 2006a); by age five nearly every child will have an episode of rotavirus gastroenteritis. About 600,000 children die every year from rotavirus, representing approximately 5% of all deaths in children younger than 5 years worldwide; more than 80% of all rotavirus-related deaths occur in resource-poor countries in south Asia and sub-Saharan Africa (Parashar et al. 2006a).

In epidemiologic studies, the incidence of rotavirus disease was observed to be similar in both industrialized and developing countries, suggesting that adequate disease control would not simply be achieved by improvements in water supply, hygiene, and sanitation, and that *vaccination* was the only control measure likely to have a significant impact on the incidence of severe, dehydrating rotavirus disease. In view of the high, global disease-burden, safe and effective rotavirus vaccines for children are a high priority, particularly in resource-poor countries.

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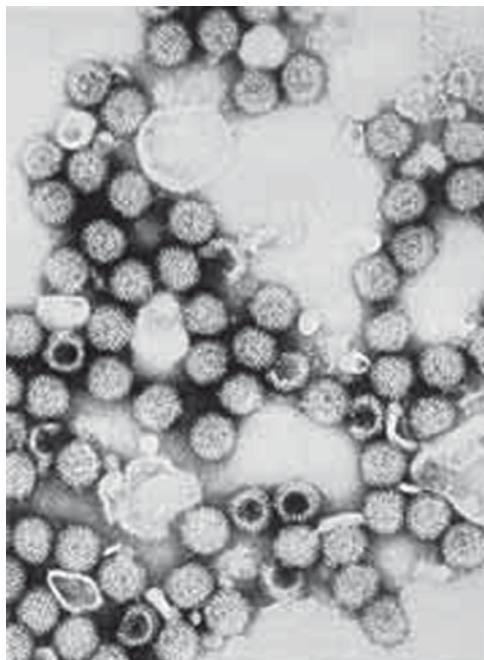
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### 20.1.2 Brief History of Rotavirus Discovery

Human rotaviruses are part of a large family of viruses causing neonatal diarrhea in a variety of domestic animals and birds. Rotavirus particles have a distinctive wheel shape on electron microscopy giving rise to their name, derived from the Latin “rota,” meaning wheel (Fig. 20.1) (Flewett et al. 1974a; Wyatt and Zapikian 1977). The first description of rotavirus in humans was made by Dr. Ruth Bishop and a team of investigators at The Royal Children’s Hospital (RCH) in Melbourne, Australia in 1973 (Bishop et al. 1973). Bishop, who began her career as a bacteriologist studying intestinal flora in children with malabsorptive diseases, began collaborating with Rudge Townley, a gastroenterologist at RCH who had perfected a technique for taking intestinal biopsy samples from babies, in the early 1970s. She then joined forces with virologist Ian Holmes and electron microscopist Brian Ruck, both of whom were working in Melbourne University’s microbiology department.

Using electron microscopy, her team examined biopsy specimens of duodenal mucosa taken by Geoff Davidson, another gastroenterologist at RCH, from children with gastroenteritis. Bishop’s team observed viruses, which they called duovirus because they were seen in the duodenum and had a double capsid (Fig. 20.1).



**Fig. 20.1** Rotavirus electron micrograph  
(Dr. Penelope Dennehy)

Shortly thereafter, Bishop and other investigators confirmed the association between the presence of duovirus in feces and acute gastroenteritis. The name ‘rotavirus’ was later suggested by an Irish investigator, Dr. Thomas Henry Flewett (1974b). Further work over the years by Bishop and her team led to an improved understanding of rotavirus infection in humans and to the development of vaccines. In 1998, Bishop was rewarded for her work on rotavirus by being named as co-recipient of the WHO Children’s Vaccine Initiative Pasteur award.

### **20.1.3 Virology**

To understand how vaccines have been developed to prevent rotavirus disease, one must understand the structure of the virion. Rotaviruses are segmented, double-stranded RNA viruses containing two structural proteins: VP7, a glycoprotein (G protein), and VP4, a protease-cleaved protein (P protein). These two proteins define the serotype of the virus and as targets of the potentially protective neutralizing antibody response, are considered critical to vaccine development (Hoshino and Kapikian 1994).

A typing system has been developed for each protein. Fifteen G types and 26 P types have been described, but only 5 G serotypes (G1, G2, G3, G4, and G9) and 3 P genotypes (P[4], P[6], and P[8]) are commonly associated with human illness. Until recently, four rotavirus strains (G1, G3 and G4 combined with P[8] and G2 combined with P[4]) comprised 96% of the globally identified strains (Santos and Hoshino 2005). Previously rare G serotypes, such as G5, G6, G8, G10, and in particular, G9 have recently emerged, although clinically predominant serotypes vary from year to year and region to region. The development of a successful rotavirus vaccine requires the vaccine to be efficacious against all the major P or G serotypes causing disease in a specific region.

### **20.1.4 Natural Protection**

Naturally acquired rotavirus infection provides the greatest protection against reinfection causing severe disease. After the first infection, infants and young children are protected against subsequent symptomatic diseases regardless of whether their first infection was symptomatic or asymptomatic (Velázquez et al. 1996). Following a single infection, 40% of children are protected against any subsequent infection with rotavirus; 75% are protected against diarrhea caused by a subsequent rotavirus infection; and 88% are protected against severe rotavirus (Velázquez et al. 1996). Second, third, and fourth infections confer progressively greater protection. No child with two previous infections in this study developed severe rotavirus diarrhea.

## 20.2 Approaches to and Development of Rotavirus Vaccines

Studies of natural protection suggested that a realistic goal for a rotavirus vaccine was to duplicate the degree of protection against disease engendered by natural infection. Therefore, vaccine program objectives have included the prevention of moderate-to-severe disease but not necessarily that of mild disease associated with rotavirus. Additionally, as no reliable immune correlate of protection has emerged from studies of natural protection in humans, each new vaccine candidate has required testing in large field trials of clinical efficacy.

Vaccine efforts have been primarily directed at the development of live, attenuated, oral rotavirus vaccines that aim to mimic the protection provided by naturally occurring rotavirus infection. Attenuation of rotavirus for vaccine use may be achieved in several ways. The most extensively evaluated approach is based on the “Jennerian” concept involving immunization of infants with animal rotaviruses that are considered naturally attenuated for humans (Hoshino and Kapikian 1994). More recently, human rotaviruses attenuated by passage in cell culture have been developed and tested, as have viruses recovered from asymptomatic human neonates that may be naturally less virulent.

### 20.2.1 *The Classical “Jennerian” Approach: Monovalent, Animal Rotavirus-Based Vaccines*

Research to develop a safe, effective rotavirus vaccine began in the mid-1970s when investigators demonstrated that previous infection with animal rotavirus strains protected laboratory animals from experimental infection with human rotaviruses (Zissis et al. 1983). It was felt that live animal strains that were naturally attenuated for humans *might* induce immune protection against disease in children. The first human rotavirus vaccine programs began in the early 1980s when animal rotaviruses, shown to be well-tolerated and immunogenic in small studies in adults, were tested in larger, placebo-controlled trials in children. Three non-human rotavirus vaccines, bovine rotavirus strains RIT 4237 (G6P[1]) and WC3 (G6P[5]) and a simian (rhesus) RRV strain (G3P[3]), provided the bulk of information on this vaccine strategy.

The first vaccine trials involved candidate RIT 4237 (Delem et al. 1984), developed by Smith Kline-RIT, Belgium, and studied in clinical trials in Finland, Yugoslavia, Italy, Switzerland, Austria and the UK. RIT 4237 was shown to be safe and effective in Finland and provided in excess of 80% protective efficacy against severe rotavirus disease due to heterotypic human rotaviruses (Vesikari et al. 1985). However, later studies in developing countries were disappointing, showing little or no efficacy and leading to the abandonment of this vaccine strategy (Hanlon et al. 1987; Lanata et al. 1989).

Initial studies with WC3, developed by H. Fred Clark, Paul Offit, and Stanley Plotkin, research scientists associated with The Children’s Hospital of Philadelphia

(CHOP) and The Wistar Institute, also appeared promising (Clark et al. 1988). Plotkin had a long and distinguished resume in vaccinology, having invented the rubella vaccine now used throughout the world (refer to Chap. 13) and worked extensively on vaccines for polio, rabies and cytomegalovirus. He and Clark began laboratory studies of rotavirus at the Wistar Institute in 1980; a year later Clark, a veterinarian, isolated a strain of rotavirus, the Wistar Calf-3 or WC3 strain, from a calf with diarrhea. Offit, a young pediatric infectious diseases physician at CHOP, joined Plotkin and Clark during 1981 in the efforts to develop a vaccine against rotavirus using the WC3 strain. After initial development at CHOP and the Wistar Institute, the WC3 vaccine candidate was licensed to Pasteur-Merieux serums et vaccins of Lyon, France, the predecessor of Sanofi-Pasteur vaccines, who sponsored several clinical trials to determine its safety and efficacy. These studies indicated that while WC3 was safe for use as a vaccine, it failed to provide sufficient protection against rotavirus infection (Bernstein et al. 1990; Georges-Courbot et al. 1991). Further studies on WC3 as a monovalent vaccine were abandoned.

The RRV strain was developed by Dr. Albert Kapikian and his colleagues at the National Institute of Health (NIH) by adapting to cell culture a strain of rotavirus, MMU 18006, from a rhesus monkey suffering from diarrhea (Hoshino et al. 1984). Kapikian began his career at NIH in 1957 studying the epidemiology and etiology of various viral diseases and in 1972, identified the Norwalk virus, the first virus to be associated with acute epidemic gastroenteritis. In 1973, at the National Institute of Allergy and Infectious Diseases (NIAID) Laboratory of Infectious Diseases, he became the first in the U.S. to detect and visualize human rotavirus, discovered earlier that year by Ruth Bishop in Australia. RRV, rhesus rotavirus, was a G3-like virus that replicated well in the human intestine without causing excessive diarrhea, making it a logical vaccine candidate. Studies done with RRV vaccines in the early 1980s demonstrated inconsistent protection against rotavirus gastroenteritis (Flores et al. 1987; Vesikari et al. 1990; Santosham et al. 1991; Madore et al. 1992; and Padilla-Noriega et al. 1992). For this reason, it was not advanced as a monovalent vaccine.

### ***20.2.2 The Modified “Jennerian” Approach: Reassortant Rotavirus Vaccines***

The ability of rotaviruses to re-assort their genomic segments during in vitro infections with heterogeneous serotypes suggested a potential alternative vaccine approach and facilitated the production of reassortant viruses that contain genes derived from both animal and human rotavirus parents. The VP7 gene was thought to be important in protection; therefore, early human-animal reassortant rotaviruses for use as vaccines included human VP7 genes with their other 10 genes derived from an animal rotavirus strain (Kapikian et al. 1996). Kapikian, later named as co-recipient with Ruth Bishop of the Pasteur award in 1998 and the Albert B. Sabin Gold Medal winner from the Sabin Vaccine Institute in 2005 for his career's work on rotavirus vaccines, developed and patented this “modified Jennerian” rotavirus vaccine

strategy with his NIAID colleagues (Flores and Kapikian 1990). In view of the inconsistency of protection observed with monovalent animal rotavirus-based vaccines, subsequent vaccine efforts focused on taking advantage of such reassortant rotavirus strains.

The next generation of vaccines was formulated to include more than one rotavirus G-serotype to provide heterotypic as well as homotypic immunity. Using these techniques, three live, reassortant, animal–human rotavirus vaccines for oral delivery were developed: two at the NIH and one at CHOP. At NIAID, Kapikian developed a reassortant, animal–human rotavirus vaccine based on the RRV strain. Reassortant rhesus rotaviruses carrying the human G1 VP7, G2 VP7, and G4 VP7 were created by mixed infection and selection; G3 reassortants were not specifically targeted, as rhesus virus is a G3-like strain. This multivalent, live, oral, reassortant vaccine, rhesus rotavirus-tetravalent, became generally known as RRV-TV and was licensed to Wyeth Lederle Vaccines (Radnor, Pennsylvania) for commercialization as RotaShield. RRV-TV was extensively evaluated in field trials in the U.S., Finland, and Venezuela and proved 80–100% effective in preventing severe diarrhea due to rotavirus in each of these settings (Rennels et al. 1996; Joensuu et al. 1997; Pérez-Schael et al. 1997; and Santosham et al. 1997). On this basis, the vaccine was licensed in August 1998 for routine use in children in the U.S. at 2, 4 and 6 months of age (CDC 1999a).

Within 9 months of inclusion of this vaccine in the U.S. immunization schedule and the immunization of over 600,000 infants, several cases of vaccine-associated intestinal intussusception were reported (CDC 1999b). The period of the greatest risk was shown to be 3–10 days after the first of the three oral doses (CDC 1999b; Kramarz et al. 2001; Murphy et al. 2001, 2002, 2003). Although the true overall incidence of this adverse event proved difficult to assess, a group of international experts suggested a consensus rate of 1 per 10,000 vaccinated infants (Peter and Myers 2002). The pathogenic mechanisms involved in intussusception following vaccination remain currently unknown.

As a consequence of this rare but potentially dangerous adverse effect, Wyeth withdrew RotaShield from the U.S. market 14 months after its introduction. Unfortunately, the vaccine was never evaluated in terms of its risk-benefit ratio for children in resource-poor countries, as the contemporaneous trials in Asia (Bangladesh and India) and Africa (Ghana and South Africa) were stopped at the time of its withdrawal from the U.S. market. Although still licensed in the U.S., the vaccine has neither been tested since nor licensed in other parts of the world.

The decision to withdraw RotaShield sparked an outcry among international health experts, who felt they were being deprived of a potent weapon against a disease that killed millions of children annually. Kapikian argued for a permissive recommendation that would enable U.S. physicians to use the vaccine at their discretion, believing that this would send a powerful message to the developing nations and perhaps spur adoption of rotavirus vaccine in those settings. However, health ministers from developing countries declined to adopt the vaccine at a pivotal WHO-sponsored strategy meeting in 2000.

RotaShield's demise prompted serious consideration of ending rotavirus vaccine programs at pharmaceutical giants Merck and GSK. Both manufacturers had already

invested millions in their rotavirus vaccines, but with encouragement from the WHO, the Centers for Disease Control and Prevention (CDC), and other public health agencies, both opted to proceed with their rotavirus vaccine programs. Current human-animal reassortant rotaviruses for use as vaccines include either human VP7 or VP4 genes. Because VP7 was initially thought to be the most important antigen in inducing protection, human-animal reassortant rotavirus vaccines, such as RRV-TV, included only human VP7 genes. More recently, as VP4 has also been considered to be important in protection, human-animal reassortant rotaviruses now include either of these human genes to optimize protective immune responses.

While Kapikian was developing RRV-TV at NIAID, Clark, Offit, and Plotkin were conducting laboratory studies at CHOP on another candidate reassortant vaccine based on their bovine strain WC3. Genes encoding the VP4 and VP7 neutralization proteins from human rotaviruses were introduced into WC3 by gene reassortment to make the bovine strain vaccine more serotypically related to human strains. By co-infecting cells in culture with the WC3 strain and five different human rotavirus strains (G1, G2, G3, G4, and P[8]), the investigators were able to create a pentavalent, human-bovine, reassortant vaccine containing five live reassortant rotaviruses. Four express the VP7 protein (G1, G2, G3, or G4) from the human rotavirus parent strain and the VP4 attachment protein (P[5]) from the bovine rotavirus parent strain WC3. The fifth reassortant virus expresses the attachment protein (P[8]) from the human rotavirus parent strain and the outer capsid protein G6 from the bovine rotavirus parent strain.

Merck licensed the technology from Wistar and CHOP in 1992 and moved towards clinical development and testing of the vaccine, named RotaTeq. Throughout the 1990s, Merck conducted a number of in vitro and pre-clinical studies of RotaTeq leading to the REST trial in 2001, a phase III, multinational clinical trial involving 70,301 infants, with the U.S. and Finland accounting for more than 80% of all enrolled subjects (Vesikari et al. 2006a). The 4-year study, one of the largest vaccine trials ever performed by a pharmaceutical company, was primarily designed to evaluate vaccine safety with respect to intussusception but also to evaluate the immunogenicity and efficacy of the vaccine with respect to the severity of illness and prevention of hospitalizations or emergency department visits due to rotavirus gastroenteritis.

The risk of intussusception was evaluated for 42 days after each vaccine dose, the period of the highest risk for the previously licensed RRV-TV vaccine. The data failed to demonstrate an increased risk of intussusception in vaccine recipients relative to placebo; there were no confirmed cases of intussusception among vaccinees within the observation period. The overall rate of intestinal intussusception in the study was consistent with the expected background rate; additionally, there was no evidence of clustering of cases within a 7- or 14-day window period of observation after any dose. Pooled data from the large trial and two smaller phase III trials showed that in the week following the first dose of RotaTeq, the incidence of fever and irritability did not differ between vaccine and placebo recipients, although diarrhea and vomiting occurred more frequently among the former (10.4% vs. 9.1% and 6.7% vs. 5.4%, respectively).

The efficacy of RotaTeq was evaluated in the REST and one other phase III trial (Vesikari et al. 2006a; Block et al. 2007). Efficacy after completion of a three-dose

regimen was 74% for rotavirus gastroenteritis of any severity and 98% for severe disease. RotaTeq also demonstrated efficacy in preventing rotavirus gastroenteritis of any severity caused by the predominant G1 (75% efficacy) and G2 serotypes (63% efficacy). While there was a trend toward efficacy for the remaining serotypes, patient numbers were too small to show statistical significance. The impact of RotaTeq on the number of office visits was evaluated among 5,673 subjects in whom it showed an 86% reduction; reductions in emergency department visits and hospitalizations for rotavirus gastroenteritis were evaluated in 68,038 subjects over the first 2 years of life and were 94 and 96%, respectively (Vesikari et al. 2006a). Its efficacy against hospitalizations for gastroenteritis of any etiology was 59%.

RotaTeq was licensed in February 2006 by the U.S. Food and Drug Administration (FDA) for use in infants and is routinely recommended as a three-dose schedule at 2, 4 and 6 months of age (Parashar et al. 2006b; Cortese and Parashar 2009). Post-marketing vaccine safety monitoring by the government occurs through reports to the Vaccine Adverse Event Reporting System (VAERS) and via active surveillance using data from the Vaccine Safety Datalink (VSD). Merck is also conducting a post-marketing observational study to monitor for the occurrence of intussusception within 30 days of vaccination in 44,000 infants in the U.S. Currently available data do not support an association of RotaTeq with intussusception (Haber et al. 2008). As of May 2008, applications for licensure of RotaTeq have been filed in more than 100 countries including Australia, Canada, the European Union, and selected countries in Asia and Latin America. Through its partnership with the Rotavirus Vaccine Program at the Program for Appropriate Technology in Health (PATH), Merck is conducting clinical trials in Africa and Asia.

Development of a second reassortant rotavirus vaccine based on the bovine U.K. strain was undertaken by Kapikian and colleagues at NIAID in parallel with the RRV-TV program. The strain was developed in Belgium by SKB/RIT, predecessor of the vaccine branch of GlaxoSmithKline (GSK). Similar to the RRV strain, it was found to be well-tolerated but provided *unreliable* protection against rotavirus disease in children. The NIAID group subsequently developed a tetravalent vaccine, BRV-TV, incorporating four reassortant viruses with a single VP7 gene of either G1, G2, G3, or G4 human serotype and ten genes from the G6P[5] bovine rotavirus U.K. strain. Phase II data demonstrated favorable immunogenicity and no adverse interference with concomitantly administered childhood vaccines (Clements-Mann et al. 2001).

Before the withdrawal of RRV-TV vaccine, placebo-controlled trials of BRV-TV versus RRV-TV vaccines were conducted in 510 infants in Finland (Vesikari et al. 2006b). Two doses of study vaccine or placebo were administered at ages 3 and 5 months. The first dose of RRV-TV vaccine, unlike that of BRV-TV vaccine, was followed by a significant excess rate of febrile reactions. Both vaccines were similarly immunogenic and effective, with nearly 70% efficacy against any rotavirus gastroenteritis and 88–100% efficacy against severe disease during the first epidemic season.

With the emergence of the G9 serotype as an epidemiologically important serotype and the importance of the G8 serotype in focal areas, vaccine developers at NIAID are planning to add human-bovine (U.K.) reassortants with G8 and G9

specificity to the tetrivalent vaccine, thereby formulating a hexavalent vaccine for use in developing countries (Kapikian et al. 2005). In 2004, the BRV-TV vaccine was licensed to Aridis Pharmaceuticals, Bharat Biotech, and Bhutantan of Brazil for further development.

### **20.2.3 An Alternative Approach: Human Rotavirus Vaccines**

Although most of the work on rotavirus vaccines to date has involved animal rotaviruses or reassortant animal-human rotaviruses, human rotaviruses attenuated by passage in cell culture have recently been developed and tested. Additionally, rotaviruses recovered from asymptomatic human neonates that may be naturally less virulent are being developed as vaccine candidates. All of these live virus vaccines are delivered orally.

The first-licensed human rotavirus vaccine is a live attenuated human strain originally known as 89-12. The vaccine, developed by virologist Richard Ward and David Bernstein, a pediatric infectious disease specialist, both at the University of Cincinnati School of Medicine, was an outgrowth of their investigations of rotavirus protective immune responses. The 89-12 virus is a G1P[8] strain and thus represents the most common of the human rotavirus VP7 and VP4 antigens. Bernstein and Ward isolated the 89-12 virus from an infant with rotavirus gastroenteritis and attenuated it by serial passages in cell culture (Bernstein et al. 1998; Bernstein and Ward 2006). The 89-12 strain was observed to provide protection from rotavirus even if the initial infection was asymptomatic and preferentially induced neutralizing antibodies to the VP4 protein over the VP7 protein, with evidence of neutralizing antibodies to at least the four major rotavirus serotypes (G1–4). Recognizing that developing a vaccine from a human rather than an animal rotavirus strain might have certain advantages, Bernstein and Ward decided that this strain would form the basis for a vaccine candidate.

Early studies showed the 89-12 vaccine was safe, although it induced a low grade fever in 19% of recipients (Bernstein et al. 1998). Two doses provided 89% protection against any rotavirus disease and complete (100%) protection from more serious disease (Bernstein et al. 1999). The vaccine was further developed by Avant Immunotherapeutics and licensed to GSK Biologicals, who further modified the vaccine by cloning and cell culture passage of the parent strain. Subsequent large-scale studies have shown this evolved strain (RIX-4414) to be well-tolerated with no significant excess fever or diarrhea. Initial trials of the resulting vaccine, Rotarix, showed it to be safe, immunogenic, and efficacious (Vesikari et al. 2004).

In a double-blind, placebo-controlled trial of more than 63,000 infants enrolled in 11 Latin American countries and Finland, Rotarix was administered in two oral doses at 2 and 4 months of age and was well-tolerated with a reactogenicity profile similar to the placebo in terms of fever, diarrhea or vomiting (Ruiz-Palacios et al. 2006). During a 31-day observation period after each dose, there was no increased risk of intussusception among vaccinees as compared with placebo.

A subset of 20,000 infants in this large trial followed for efficacy demonstrated a protection rate of 85% against severe rotaviral gastroenteritis and complete protection against the most severe, dehydrating episodes (Ruiz-Palacios et al. 2006). The vaccine also proved highly efficacious in preventing rotavirus gastroenteritis of any severity caused by the predominant G1 (92% efficacy) and G3, G4, or G9 serotypes (88% efficacy). Although efficacy against the G2 serotype (41%) was not significant in the phase III trial, significant cross-protection of Rotarix against non-G1 and non-P[8] strains, including P[4]G2, was shown using a meta-analysis of efficacy trials. The aforementioned findings were confirmed in a European trial of Rotarix (Vesikari et al. 2007).

Rotarix was initially licensed in Mexico and the Dominican Republic in 2004. As of May 2008, it has been licensed in more than 100 countries worldwide, including the U.S., and more than 25 million doses have been distributed. The vaccine is recommended in a two-dose schedule beginning at the age of 6 weeks (Cortese and Parashar 2009). Clinical data from efficacy and safety trials of Rotarix in Asia and Africa are expected to become available during the next few years. Smaller studies in HIV-infected infants, pre-term infants, and twins are ongoing.

Neonatal strains are being explored as vaccine candidates because they appear to be naturally attenuated; a natural history study has shown that asymptotically infected neonates had a reduced frequency and severity of subsequent rotavirus diarrhea (Bhan et al. 1993). Strains obtained from asymptotically infected newborns in Delhi (116E) and Bangalore (I321), comprising G9P[10] and G10P[11] antigens, respectively, have been assessed as vaccine candidates. Each strain is a naturally occurring human-bovine reassortant: 116E is a human rotavirus with a single gene segment encoding VP4 derived from a bovine rotavirus; I321 is a bovine strain with two non-structural gene segments derived from a human strain (Cunliffe et al. 1997). These vaccine candidates are under development in India through a consortium with partners from the U.S. CDC and the Children's Vaccine Program at PATH (Glass et al. 2005).

A phase I trial of a single dose of either vaccine candidate or placebo in 8-week-old infants in Delhi demonstrated that while both vaccines were safe and well-tolerated, strain 116E was associated with superior immunogenicity when compared with strain I321 or placebo (Bhandari et al. 2006). In a recent study in three urban slums in Vellore, South India, neonatal G10P[11] infection with a strain resembling the I321 vaccine candidate failed to confer protection against subsequent rotavirus infection or diarrhea of any severity (Banerjee et al. 2007). These findings suggest that the 116E strain may be preferred to I321 for further evaluation as a vaccine candidate.

A human neonatal G3P[6] strain, RV3, developed by Ruth Bishop and her colleagues in Australia, has been evaluated as an oral vaccine in 3-month-old infants and found to be safe and well-tolerated. Although a small Phase II study indicated it has relatively low immunogenicity, those who developed an immune response were protected against clinical disease in the following year (Barnes et al. 2002). Further immunogenicity studies are planned with a higher dose of the vaccine.

## 20.3 Future Challenges

Post-marketing surveillance studies to monitor the impact of rotavirus vaccine on circulating viral strains will be important to screen for possible vaccine selection pressure and strain replacement. Studies to measure the extent of cross-protection against different rotavirus serotypes, including serotype G9, which is becoming increasingly important across Asia and Africa, and G8, which is gaining prevalence in parts of Africa, will also be needed to ensure that vaccines protect children in the developing world where such strains are prevalent.

The development and introduction of rotavirus vaccines for children in resource-poor countries of the world has been given high priority by the WHO. Vaccine efficacy, already demonstrated in children in industrialized and middle-income countries, needs to be proven in countries in Africa and Asia also. The availability of rotavirus vaccines in these areas will ultimately depend on distribution, including the need for a cold chain. The WHO Initiative for Vaccine Research intends to provide funding for the development of liquid or dry powder formulations to facilitate the production of rotavirus vaccines that are logically simple to administer in resource-poor countries, occupy minimal space in the cold chain, can be stored outside of the cold chain for reasonable time periods without loss of activity, and are compatible with multi-dose vial formats.

In 2003, the Global Alliance for Vaccines and Immunizations (GAVI) sponsored a new public-private organization, the Rotavirus Vaccine Program at PATH, whose role is to accelerate the development and introduction of rotavirus vaccines in the developing countries. Despite this support, implementation of rotavirus immunization programs in the developing world will require substantial input from the international donor community. Novel financing strategies will be needed to ensure that new vaccines are affordable and available in the developing world. Decision makers and parents in developing countries need to be informed about rotavirus disease since currently few have heard of the virus, and rotavirus infection is rarely diagnosed. Finally, in order for the global effort to be successful, special efforts will be required in India, China, and Indonesia, because one-third of all deaths due to rotavirus disease occur in these countries, which depend almost entirely on vaccines manufactured domestically.

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# Chapter 21

## Human Papillomaviruses

Ian H. Frazer

### 21.1 Papillomavirus-associated Diseases

#### 21.1.1 Cervical Cancer: A Historical Perspective

Cervical cancer is one of the few human cancers entirely attributable to infection with a virus, human papillomavirus (HPV). It is one of the commonest cancers among women worldwide, responsible for over 250,000 deaths each year. Although chronic disease of the uterus was known in ancient times, the concept of cancer as a disorder of cell growth is relatively recent and was only confirmed with the advent of technologies for the microscopic examination of human tissue in the first half of the nineteenth century, as systematized by Virchow and others (Bracegirdle 1977).

In 1842, Rigoni-Stern, a pathologist from Verona, observed that uterine cancer was a disease most commonly seen in married women and rarely in single women and nuns, in contrast to most other cancers he examined, which were more common in the latter groups (Scotto and Bailar 1969). He thus defined a possible role for sexual activity in the etiology of the disease, although he stated that uterine cancer was associated with “nervous irritability rather than licentiousness”. He also noted that unlike breast cancer, uterine cancer was a disease of younger women.

The clinical management of cervical cancer advanced throughout the nineteenth and twentieth centuries with improvements in surgery and radiotherapy; prevention through screening was championed by Papanicolaou in many countries in the 1950s, but the search for a presumed etiological agent of cervical cancer was less successful. A number of bacterial and viral causes were proposed, particularly herpes viruses, but with limited scientific tools available for causality research, the field had not advanced significantly until the introduction of molecular technologies to characterize DNA in the late 1970s enabled a link to be drawn with HPV.

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### 21.1.2 Papillomaviruses and Human Cancer

Papillomaviruses were among the first identified viruses. In the early part of the twentieth century, it was shown that skin warts in animals could be transmitted by a “filterable agent”, the accepted definition of a virus at that time. Rous, who early in his career had been the first to postulate a link between viruses and cancer with sarcoma virus in chickens (refer to Chapter 9), demonstrated in 1935 that Shope’s papillomavirus was the cause of warts in rabbits and that it could also promote skin cancer in these animals. “Experimentation carried out in my laboratory... showed the ‘warts’ produced by the virus to be genuine tumors, benign epidermal papillomas in which the virus persists although eliciting an antibody capable of neutralizing it on direct exposure” (Rous 1966). Additionally, Rous demonstrated that a virus could cause cancer in mammals; these benign tumors of lagomorphs could become malignant if exposed to cancer-promoting chemicals–carcinogens.

No cancer in humans had as yet been attributed to infection, although a causal link had been widely sought. Human genital warts, referred to as “figs” in Greek medical treatises, had long been known as a sexually transmitted disease, but the visible lesions only very rarely turned cancerous. Further, because the viruses responsible for warts could not be propagated in the laboratory, there was no awareness of the “zoo” (Galloway 1994) of over 200 HPVs now recognized by conventional serotyping methods. The literature before 1975 acknowledged that epidemiological observations were consistent with perhaps two or three different HPVs causing skin or genital warts.

The molecular biology revolution of the 1970s enabled physical mapping and eventually cloning and sequencing of genetic information, albeit not with the ease with which this can now be achieved, and the search for papillomavirus DNA in diseased human tissue accelerated. In the mid 1970s, two independent investigators, Gérard Orth at the Pasteur Institute in Paris, whose laboratory had a particular interest in skin cancer and animal papillomaviruses (Favre et al. 1975; Orth et al. 1977, 1978), and Harald zur Hausen, Chair of the Institute of Virology at Freiburg, whose laboratory pursued the association of viruses and human cancer (Gissmann and zur Hausen 1976; zur Hausen 1976; zur Hausen et al. 1974), demonstrated a plurality of HPV in human skin and genital lesions and showed an association of some of these with human epithelial cancers.

Subsequent work by Gissmann, Dürst, and others in zur Hausen’s lab established the presence of papillomavirus DNA and expressed viral protein in cervical cancer and in cervical cancer-derived cell lines (Gissmann and zur Hausen 1976; Durst et al. 1983; and Boshart et al. 1984). This observation, in concert with work by many laboratories establishing that animal papillomavirus genes were partial oncogenes able to immortalize cell cultures in vitro (Lowy et al. 1980; Watts et al. 1984; Tsunokawa et al. 1986; and Yasumoto et al. 1986), supported the hypothesis that HPV contribute to the induction of cervical cancer. This hypothesis has subsequently been confirmed through epidemiological studies (Mitchellet al. 1986; Daling et al. 1987; Lorincz et al. 1987; Munoz and Bosch 1992; and Bosch and de

Sanjosé 2003) that have described the association of HPV infection with cervical cancer and with the recognized premalignant condition, cervical intraepithelial neoplasia (CIN), leading to the eventual conclusion that over 99% of cervical cancer is a consequence of HPV infection (Bosch and de Sanjosé 2003).

### ***21.1.3 Papillomavirus and Other Human Diseases***

Although cervical cancer is the major, global public health problem attributable to infection with HPV, this family of viruses is responsible for a considerable additional burden of the disease, accounting for at least 5% of all worldwide cancer (Parkin 2006). The papillomaviruses can be broadly divided clinically into two groups: a group that has tropism for mucosal surfaces, particularly of the genital skin; and another with tropism for nongenital skin. At each site one subgroup, typified by genotypes 1 and 2 in nongenital skin and types 6 and 11 in genital skin, is responsible for evident warts and generally not associated with cancers. Skin warts caused by the common cutaneous HPV are universal in childhood and resolve spontaneously over months to years in immunocompetent individuals. HPV types 6 and 11 cause genital warts, which are extremely common in young adults and a major public health burden in many countries. They are also generally self-limited over months to years in immunocompetent adults, though they generally persist in immunosuppressed subjects.

A further subgroup of HPV can be distinguished at both mucosal and nonmucosal sites, typified by types 5 and 8 in nongenital skin and types 16 and 18 in genital skin, producing flat lesions and generally latent or subclinical infection. This phenotype in nongenital skin comprises a small number of skin cancer-associated viruses and a much larger number of viruses that are rarely, if ever, associated with disease but can be isolated from hair-root bulbs in healthy individuals (Boxman et al. 1997). It is believed that they initiate squamous epithelial cancers only in patients who are chronically immunosuppressed, or who have the rare genetic disorder epidermodysplasia verruciformis, due to a recessive mutation in a keratinocyte-associated transmembrane protein of uncertain function (Ramosz et al. 2002). However, a possible role for this group of viruses in squamous cell skin cancers in immunocompetent individuals is suggested by the aforementioned and by their isolation in normal-appearing skin surrounding certain epithelial cancers and by their oncogenic properties (Pfister 2003).

The “no wart” HPV subgroup tropic for genital skin includes over 10 HPV types associated with a high risk of cancer and a number of types unassociated with cancer. Two of the former, types 16 and 18, are strongly associated with cancer of the cervix and also with other anogenital cancers in men and women, some tonsillar epithelial cancers, and a small percentage of other cancers. In each of these neoplastic lesions viral genetic information is integrated within the cancer cell’s genome, usually allowing expression of two viral genes, E6 and E7, which are sufficient to immortalize epithelial cells (Jewers et al. 1992). Links between high-risk genital types of HPV and other squamous cancers, especially of the esophagus, are

also postulated. Although these tumors do not demonstrate integrated viral genomes, there is evidence from the association between bovine papillomavirus and esophageal cancer in cattle that at least some papillomaviruses can initiate cancer by a “hit and run” mechanism (Campo et al. 1985).

### ***21.1.4 The Natural History of Human Papillomavirus Infections***

The natural history of HPV infection, though well-defined for some genotypes, generally remains somewhat controversial. The majority of epithelial samples from which HPV DNA can be isolated in the genital tract of young and older women appears histologically normal and with little evidence of viral gene transcription or viral protein expression, suggesting that a state of latency can exist long-term, a view supported by evidence that immunosuppression can activate the virus to a state of productive infection leading to disease. However, viral clearance of the well-studied genital tract types, at least as measured by current DNA detection methods, has been shown to occur, with a mean time to clearance of about 12 months in immunocompetent subjects and longer in immunocompromised subjects (Koshiol et al. 2006).

Acute infection is histologically apparent within weeks to months of virus acquisition, presenting as persistent warts or flat lesions with abnormal keratinization. Persistent infection of the cervix with high-risk HPV types is strongly associated with premalignant epithelial change; such transformation of infected cells is now thought to occur early in the infection and become more obvious with time as clones of transformed cells persist and expand. While immunocompromised states, smoking, and the use of the oral contraceptive agents are all weakly associated with persistence and progression of HPV infection to cervical cancer (Bosch and de Sanjose 2007), most of the risk factors for progression remain undefined and may comprise genetic factors or stochastic events associated with acute infection. Perhaps the most important impetuses for the development of vaccines to prevent HPV infection were the revelations that infection with high risk types is extremely common (Koutsy 1997), with a point prevalence of up to 25% among women with normal cervical cytology (Clifford et al. 2005), and that cervical cancer was the most common female cancer in many countries in the developing world.

## **21.2 Approaches to and Development of the Papillomavirus Vaccines**

### ***21.2.1 Preclinical Studies***

Demonstration of an association between persistent high-risk HPV infections and cervical cancer in the 1980s, and of an association between HIV-associated immunosuppression with increased risk of HPV-associated premalignancy and cancer, led to considerable interest in the development of vaccines to prevent

HPV-associated disease. Evidence from animal studies suggested that prophylactic vaccination against HPV infection might be possible, as cattle (Jarrett et al. 1990) and dogs (Bell et al. 1994) immunized with purified inactivated papillomavirus virions could be protected against live virus challenge. Additionally, data from studies in rabbits (Evans et al. 1962) and cattle (Spradbow et al. 1977) with papillomavirus-associated tumors had suggested a possible role for therapeutic, autologous viral vaccines; sporadic attempts using immunotherapy for recalcitrant HPV-associated disease had shown some evidence of efficacy (Pass 1974).

### **21.2.2 Challenges for the Development of a Human Papillomavirus Vaccine**

There were several conceptual and practical hurdles to the development of HPV vaccines. The most significant was that there was no system for production of papillomaviruses in cell culture. Papillomavirus life cycle within the epithelium is closely linked to epithelial cell differentiation (Doorbar 2007). Viral episome replication occurs in undifferentiated basal epithelial cells under the control of the two nonstructural proteins E1 and E2; episomal amplification occurs along with cell division in the intermediate epithelial layers and is promoted by the block to epithelial differentiation produced by the E6 and E7 nonstructural proteins; and episomal packaging occurs in differentiated cells permissive for expression of the viral late proteins L1 and L2.

A lack of methods for growing the virus in vitro along with the relative scarcity of authentic virions in infected lesions, hindered studies of the natural immune response to papillomavirus infection and precluded large-scale production of virus for inactivation or attenuation to become the basis of a vaccine. The inability to study host immune responses made it difficult to determine whether the diverse HPV genotypes were immunologically distinct, particularly as the major capsid protein was more than 85% conserved between types at the amino acid sequence level (Chan et al. 1995). The persistence of HPV infection raised concern that these viruses were unable to induce a protective immune response. There were also nagging uncertainties concerning the strength of the association between HPV infection and cervical cancer. The epidemiology and natural history of HPV infection remained at that time poorly defined; it was widely held that cancer was likely to be a relatively *common consequence* of a relatively *rare infection* rather than, as is now recognized, a relatively *rare consequence of a common infection*. It was not clear that a vaccine could be effective against a predominantly mucosal infection, as the field lacked precedent in this arena. Finally, concerns were voiced as to whether a vaccine against an oncogenic virus could be safe.

### **21.2.3 HPV Vaccine Development in the Molecular Era**

Despite these many concerns, molecular techniques developed in the mid-1980s provided a basis for optimism. The availability of cloned HPV coding sequences and the development of prokaryotic expression vectors allowing for the production of the

smaller nonstructural viral proteins encouraged several research groups to study immune responses to papillomavirus proteins in animals and in humans. Early studies with the E6 and E7 nonstructural proteins demonstrated that they were immunogenic in animals. Although they did fail to elicit strong immune responses in HPV-infected humans, weak responses to the E7 protein of HPV16 were demonstrated in some patients with cervical cancer (Jochmus-Kudielka et al. 1989).

In the late 1980s, the advent of PCR technology allowed production of full-length sequence for the larger viral structural proteins L1 and L2, and the availability of eukaryotic expression systems enabled the *in vitro* production of sequence-authentic capsid proteins. Initial efforts with vaccinia as an expression vector (Zhou et al. 1991) yielded only low level protein expression but demonstrated that capsid proteins could self-assemble into empty capsids, or virus-like particles (VLPs). Research by several groups (Ghim et al. 1992; Kirnbauer et al. 1992; Rose et al. 1993; and Sasagawa et al. 1995) using baculovirus vectors, insect cells, immortalized cell lines, and recombinant yeast, produced larger amounts of VLPs and demonstrated that at least one circulating clone of the L1 gene of HPV16 had a mutation-preventing VLP assembly (Kirnbauer et al. 1993). VLPs produced *in vitro* resembled the native virion immunologically and stimulated antibody that could bind native virions, and where infection systems existed, could also neutralize virus (Christensen et al. 1994). VLPs were produced for several animal papillomaviruses and were shown to induce host-protective immunity against live virus challenge in dogs (Suzich et al. 1995) and rabbits (Jansen et al. 1995), providing proof-of-concept for the development of HPV prophylactic vaccines.

#### ***21.2.4 Commercial Development of HPV Vaccines***

While doubts still remained about whether a vaccine would be effective in providing durable protection against challenge with HPV at mucosal surfaces, a number of commercial companies launched research programs to scale up the technology for production of HPV VLPs and to plan clinical trials. Several challenges remained. It was recognized by the early 1990s that there were many different HPV genotypes, and these were likely to be immunologically distinct. However, the nature of the risk associated with each type was uncertain.

Fundamental gaps in the laboratory technology for the study of HPV were recognized at the time. There was a dearth of reliable serological markers of infection, as the existing assays were technically cumbersome and lacked type specificity. Further, there were no assays for infectious virus, and those for HPV in tissues lacked standardization, were not type specific, and were generally based on DNA-amplification methods, increasing the likelihood that a positive result reflected sample contamination or passive deposition of virus without infection. These also represent persistent problems today but at the time impacted on the determination of specific end points and in selection of a suitable candidate population for clinical trials of vaccine efficacy. The absence of simple animal models in which to assess protection against HPV challenge magnified the dilemma.

The two HPV types most commonly associated with cervical cancer, HPV16 and HPV18, responsible for about 70% of these cancers, were chosen as the best candidates for vaccine development. Within industry scientists, such as Alan Shaw and Katherine Jansen at Merck and Garry Dubin and Dominic Moncef-Slouai at Glaxo SmithKline championed vaccine development programs that subsumed the development of necessary supporting assays from the research arena. Scaling up of vaccine production (Inglis et al. 2006) resulted in a manufacturing process which required disassembly and reassembly of the recombinant VLP (Mach et al. 2006) to ensure acceptable purity, or truncation of the L1 protein to reduce its DNA-binding capacity (Schäfer et al. 2002).

### ***21.2.5 Clinical Trials of HPV Vaccines***

Phase I clinical trials established that HPV VLP were immunogenic in humans, producing antibody capable of neutralizing virus in vitro (Emeny et al. 2002; Ault et al. 2004; Brown et al. 2004; and Fife et al. 2004). These findings enabled phase II studies of monovalent vaccines, which confirmed safety and immunogenicity and demonstrated that vaccines could prevent persistent HPV infection in individuals in type-specific manner (Koutsky et al. 2002; Villa et al. 2005; and Harper et al. 2006).

A spirited debate meanwhile was going on within such entities as the U.S. FDA and the World Health Organization (WHO) as to what would be an acceptable measure of efficacy for licensure of an HPV vaccine designed to prevent cancer. Clinicians and epidemiologists won out over virologists, arguing strongly that it was necessary to have as a study end-point a high grade, anogenital precancerous lesion such as CIN 3 that would normally require treatment. This decision set a high bar for trial design. Several large clinical trials were subsequently required to demonstrate that previously uninfected women immunized with vaccines containing HPV16 and HPV18 immunogens were effectively protected with 95% efficacy for periods of at least 3 years against the development of CIN 2/3, and also against vulval and anal intraepithelial neoplasia attributable to these HPV types (Ault 2007; The FUTURE II Study Group 2007; Garland et al. 2007; Joura et al. 2007; and Paavonen et al. 2007).

Three further critical findings emerged from these studies. HPV vaccines were not useful as immunotherapy for existing infection; the natural history of HPV infection was neither accelerated nor modulated in women already infected at the time of vaccination (Hildesheim et al. 2007). Second, although there was some evidence of protection against disease caused by HPV types not in the vaccine, it was insufficient to reduce the incidence of type-specific cervical disease among HPV naïve women to below 30% that of the rate in placebo recipients and therefore, did not mitigate the need for continued screening as a secondary preventative measure against cervical cancer (Stanley 2007). Finally, regardless of the age of vaccination up to forty-five years, women were protected against HPV infection and associated disease for any type that was included in the vaccine, if they were not infected at study commencement. As expected, the incidence of new HPV infection in the placebo groups was lower in older women, and, as in the pivotal

trials, women with evidence of persistent infection at recruitment were not benefited by vaccination.

### ***21.2.6 Vaccine Deployment Issues and Controversies***

Clinical data from the phase III vaccine studies provoked significant debate about the optimal target population for community vaccination against HPV infection and about the community benefit to be derived from immunizing older women. Equally fierce, though less rational, has been a debate in some communities about whether immunization of young women before the onset of sexual activity would increase the incidence of early and extensive sexual activity. Despite these debates, programs for general immunization of preteen girls have been established in many countries. Considerable interest is now focused on vaccine safety. Available data suggest that HPV vaccines are safe, with only the expected local reactions to adjuvant administration, some injuries consequent upon vaccination associated fainting episodes, and very infrequent allergic reactions (Slade et al 2009). Some consumer groups have however misinterpreted reported adverse events, including deaths, in recently vaccinated subjects as vaccine associated morbidity and mortality. Additionally, conversion illnesses have been reported in the lay press from several sites of mass vaccination, and there have been sporadic allergic reactions, albeit at similar reported incidence to those observed with other adjuvanted vaccines.

Attention has also focused on the duration of protection following vaccination. There is a lack of standardized assays for VLP-specific or neutralizing antibody, though a reference standard serum is being developed (Ferguson et al. 2006). As there is no evidence that antibody of any particular specificity or titer reliably measures vaccine-induced protection, duration of efficacy will likely be judged on clinical grounds. Postmarketing surveillance studies linking vaccination to cytology and cancer registries are underway in several locations. Although universal immunization will impact the specificity and sensitivity of cytology-based screening, the use of extant screening programs as a component of the measurement of long-term vaccine efficacy requires that population-screening strategies are not altered as a consequence of vaccine introduction. Longer-term surveillance also faces the challenge that there are no licensed, type-specific HPV tests; while alteration in the incidence of abnormal cervical smears can be assessed, the frequency with which these are associated with vaccine as opposed to non-vaccine HPV types will be difficult to establish.

### ***21.2.7 One Target, Two Products?***

Debate about which patents validly define the technology on which the vaccines are based was somewhat clarified in the U.S. following prolonged patent court litigation, through an appeal against the findings from a four-way interference action in 2007 (US Court of Appeals for the Federal Circuit 2007). Notwithstanding the interference

process, an out-of-court settlement between Merck and GSK in the preceding year on cross-licensing and royalty payments gave each company freedom to operate as producers of VLP-based vaccines to prevent cervical cancer (Grimes 2006).

The near simultaneous availability of two different vaccines to protect against HPV infection has naturally led to some tension between the two manufacturers. Gardasil, incorporating HPV 6, 11, 16, and 18 types, has been on the market in many jurisdictions for 1 to 2 years longer than Cervarix, which incorporates HPV types 16 and 18 only. The vaccines also differ in method of manufacture: Gardasil is made in recombinant yeast with full-length L1 and incorporates a proprietary alum adjuvant used over many years; Cervarix is made in insect cells infected with recombinant baculovirus, using a truncated L1 protein sequence for HPV16 and employs one of the newer and more potent adjuvants, monophosphoryl lipid A, which increases the level of antibody to VLP after immunization. Both vaccines have proven equally efficacious to date at preventing HPV16 and 18 associated disease, although Gardasil has also been shown to protect against HPV6 and 11 associated genital warts. Gardasil is licensed worldwide in over 90 countries and Cervarix in about 20 at the time of writing. Government-sponsored programs for universal immunization of young women using Gardasil have been initiated in Australia, several European countries, Canada, and by several health maintenance organizations and states in the U.S. Cervarix has been selected for the government-sponsored program in the U.K., a decision that has prompted some professional debate because of the increasing burden of HPV 6/11-associated genital wart disease in younger people there (Kmietowicz 2008).

### ***21.2.8 Alternate Vaccines for HPV***

While VLP based on HPV L1 protein have become the dominant paradigm for protection against infection, clinical studies of alternative strategies for vaccination are ongoing. Vaccines based on the minor L2 capsid protein of HPV (Kawana et al. 2003; Gambhir et al. 2006; and Kondo et al. 2007) have entered preclinical trials, and least one major immunogenic epitope may be able to offer wide cross-protection across different HPV types (Alphs et al. 2008). Vaccines using a pentameric L1 protein produced in *E. coli* may provide a substantial subset of the epitopes displayed on the complete L1 VLP (Yuan et al. 2001), thus potentially offering protection from a simpler vaccine manufacturing technology, are also in preclinical trials.

### ***21.2.9 Unanswered Questions***

There remain several unanswered questions regarding deployment of the vaccine that must be resolved through further research. Particularly, it would seem important to determine the efficacy of the vaccine for males, both for prevention of HPV-associated

warts and for reduction of transmission. The case for universal vaccination against HPV would be strengthened if vaccination reduces the incidence of other HPV-associated cancers, including tonsillar cancer, or of recurrent respiratory papillomatosis. While universal immunization of women past the age of peak incidence of new HPV infections will probably not be cost effective, one group that might benefit would be those presenting with a clinical lesion attributable to a high-risk HPV infection that requires treatment, as there is a significant incidence of recurrent disease after treatment, which might be due to reinfection since unresolved infection does not appear to be associated with immunity.

### 21.3 HPV Vaccines Today

Rapid uptake of the currently available HPV vaccines among young women in many developed countries over the last 2 years should result in a gradual reduction in the frequency of abnormal pap smears requiring surgery over the next decade and in the incidence of cervical cancer over the next half century (Smith et al. 2008). Until vaccines are developed which protect more comprehensively against the spectrum of HPV types relevant to cervical cancer, screening programs will continue. Immunization of older women may give further insight into the natural history of HPV infection. The global challenge will be to deliver vaccines in the developing world as these areas have the highest incidence of the disease. Until this has been achieved, HPV vaccines cannot be said to have achieved their full potential.

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# **Chapter 22**

# **The Future of Vaccine Discovery and Development**

**Adel Mahmoud**

## **22.1 Introduction**

The science and practice of vaccination were initially based on multiple historical observations that related the acquisition of protection against a specific microbe to a previous exposure to the same agent (Hilleman 2003). Although, in modern history, the practice was confined to inducing protection against infectious diseases, its historical roots extended to exposures to toxins, and its current conceptual framework encompasses many more pathological conditions, such as cancer (Finn 2008). Fundamentally, vaccination aims at inducing host immune responses to mount preventive or therapeutic defenses against diseases of varying etiologies, including those of infectious, and potentially autoimmune or neoplastic origin. This chapter defines our current understanding of the challenges facing the discovery and development of new vaccines for a variety of disease conditions; the aim is to identify the critical steps in exploring future imperatives and directions.

## **22.2 A Fresh Look at the Human–Microbe Interplay**

We currently use approximately 30 vaccines against infectious diseases of humans. Their impact is well-documented globally but most significantly in developed countries, where many of these infections have become experiences of the distant past (Plotkin 2005). However, it is sobering to examine in detail the composition of these vaccines. Tables 22.1 and 22.2 summarize our current, preventative, vaccine armamentaria against the infectious diseases of humans. The most striking observation is that most are constructed of whole organisms or relatively defined extracts

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**Table 22.1** Currently utilized whole cell vaccines

Vaccine	Organism(s) used for vaccination	Source	Vaccine type
Smallpox	Vaccinia virus	Bovine <sup>1</sup>	Live virus
Rabies	Rabies virus	Human	Inactivated
Typhoid	<i>Salmonella typhi</i>	Human	Inactivated, live attenuated, or purified polysaccharide
Cholera	<i>V. cholerae</i> with (live) or without (killed) deletion in toxin gene	Human	Live attenuated or inactivated + toxin subunit
Tuberculosis	BCG	Bovine	Live attenuated mycobacteria
Yellow fever	Yellow fever virus	Human	Live attenuated
Influenza	Influenza A and B	Human	Inactivated or cold-adapted live virus
Poliomyelitis	Poliovirus types 1, 2, 3	Human	Live attenuated or inactivated virus
Measles	Measles virus	Human	Live attenuated
Mumps	Mumps virus	Human	Live attenuated
Rubella	Rubella virus	Human	Live attenuated
Adenoviral acute respiratory disease	Adenovirus serotypes 4,7	Human	Live virus
Varicella	Varicella-zoster virus	Human	Live attenuated
Japanese encephalitis	JEV	Human, mosquito (live vaccine)	Inactivated or live attenuated (China)
Hepatitis A	Hepatitis A virus	Human	Inactivated
Rotavirus	Rotavirus	Bovine, human	Bovine-human reassortant

<sup>1</sup>Refer to Chapter 2 for a complete discussion of the postulated origins of vaccinia

**Table 22.2** Currently utilized vaccines comprising products or extracts of microbial origin

Vaccine	Organism(s)	Product
Anthrax	<i>B. anthracis</i>	Purified bacterial protein
Diphtheria	<i>Corynebacterium diphtheriae</i>	Toxoid
Tetanus	<i>Clostridium tetani</i>	Toxoid
Pertussis	<i>Bordetella pertussis</i>	Whole cell extract or acellular extracts
Pneumococcal	<i>Streptococcus pneumoniae</i> (23 serotypes)	Purified capsular or conjugated polysaccharide*
Hemophilus	<i>Hemophilus influenzae</i> type b	Purified capsular and conjugated polysaccharide*
Meningococcal	<i>Neisseria meningitidis</i> serogroups A,C,W135,Y	Purified capsular or conjugated polysaccharide*
Hepatitis B	Hepatitis B	Recombinant subunit**
HPV	Human papillomavirus types 6, 11, 16, 18	Virus-like particle**

\* Conjugate vaccines include protein carriers

\*\* Molecularly-defined antigens

except for hepatitis B and human papillomavirus (HPV) vaccines, which are based on molecularly defined molecules.

Historically, several principles guided the discovery and development of vaccines. The Jennerian approach used in original smallpox vaccines and still used in newer generation smallpox products involves the utilization of an infectious agent of animals that relates to one of humans but is less pathogenic (refer to Chapter 2). In an extension of this empiric principle more than a century later in the early 1900s, an effective vaccine against tuberculosis, *BCG*, was developed by repeated passage of a bovine pathogen in culture (refer to Chapter 8); nearly 100 years later, an effective rotavirus vaccine based on reassorting bovine and human viruses (refer to Chapter 20) was developed in a “modified Jennerian” approach (Heaton and Ciarlet 2007). In selected, other situations, von Behring’s toxoid approach was used to develop successful vaccines for diphtheria and other toxin-based infections (refer to Chapter 7).

The first and most significant challenge for future vaccine discovery is to define, isolate, and characterize components of microbes that comprise potential protective immunogens. With such limited experience based on available vaccines, research to identify protective immunogens has been the central theme in the discovery of new vaccines in recent years. Heretofore, the fundamental approaches were to harness the host immune response to identify components of the invading organisms that represent possible targets or to raise antibodies, polyclonal or monoclonal, to use as immunological probes. Unfortunately, the yields from these attempts have been modest.

An example of the difficulty facing the identification of potential protective antigens is illustrated by our experience with HIV/AIDS (Mahmoud 2007). Sera and T-cells from infected individuals with varying degrees of host responses to the virus have immunologically defined possible protective viral antigens (Barouch 2008; Walker and Burton 2008). Data from experimental animals have provided evidence for the potential ability of the envelope protein gp120 or other proteins encoded by the viral genes *gag*, *nef*, and *pol* to induce varying degrees of protection. These observations led to clinical trials that demonstrated neither protection against the acquisition of HIV infection nor the moderation of viral load in vaccinated individuals who acquired the infection. While there may be several explanations for the inability to induce protection using this strategy, immunogen identification is clearly problematic and remains the basic challenge facing the HIV vaccine research community. Alternative approaches are urgently needed, especially in the realm of this and other chronic infections (Fauci et al. 2008).

What undermines our approaches to define microbial immunogens? The answer likely involves examining human–microbe interplay in evolutionary biology terms. Microbes have evolved as coinhabitants of the earth; their interaction with humans spans a broad-spectrum from commensalism to mutualism to pathogenesis (Lederberg 2000). Induction of disease in a host may represent a unique and limited aspect of the human–microbe interaction (Dethlefsen et al. 2007). If pathogenesis is essential for the survival of microbes from an evolutionary perspective, it is highly unlikely, from the same evolutionary vantage point that microbes would benefit from their most susceptible components being exposed to host defense mechanisms.

Our dependence in the discovery of potential protective immunogens on what is recognized by host immune responses or on what experimental animal models may recognize by polyclonal antibodies, therefore, appears to be less than optimal. Equally flawed is the assumption that raising monoclonal antibodies against microbes will result in easy identification of potential protective components, as the success of this approach is still predicated on a chance occurrence that such products will identify a potential protective immunogen. In spite of the specificity of antigen–antibody reactions, identifying single molecules that will drive vaccine discovery has been challenging.

An alternative approach to immunogen identification is to examine microbial constituents that perform crucial survival functions and then to induce host immune responses that recognize these molecules and interfere with their function. Translating this proposed principle into practice compels a deeper molecular understanding of microbial organization, but it would pave the way to identify susceptible target components of invading organisms that may interfere with their invasive or pathogenic potential. Recent work has established precedent for this strategic approach to vaccine development.

## 22.3 Discovery of a Human Papillomavirus Vaccine Candidate

The association of HPV infection with carcinoma of the cervix was initially demonstrated by epidemiological observations that infection with certain types of HPV was consistently found in patients suffering from cervical cancer (zur Hausen 2008). Pathological examination of tumors confirmed these observations and led to the groundbreaking work of Nobel laureate zur Hausen and colleagues demonstrating HPV DNA integrated in the genome of most cervical carcinoma samples obtained from several geographic areas (Durst et al. 1983). These seminal observations led to intensive investigations of the virus genome, elucidation of the function of its proteins, and cloning of all open-reading frame segments of its DNA sequence.

Several of the encoded, early viral proteins are involved in HPV oncogenesis. The viral oncogene E6 interferes with the function of the tumor-suppressor gene p53; E7, another HPV gene, disrupts the function of the tumor-suppressor retinoblastoma gene (Dyson et al. 1989; Scheffner et al. 1990; and Werness et al. 1990). Independent research groups engaged in cloning the conserved viral DNA segments encoding the HPV capsid proteins L1 and L2 in Australia and the U.S. observed, nearly simultaneously, that the cloned proteins undergo a process of self-assembly into virus-like particles (VLPs) following *in vitro* expression (Zhou et al. 1991; Kirnbauer et al. 1992). This observation was reminiscent of that involving the only other molecularly expressed, defined protein that had demonstrated effectiveness as a vaccine – against hepatitis B virus (Hilleman 2003). VLPs proved to be the inaugural discovery leading to the development of two effective vaccines against HPV (Mahmoud and Levin 2007).

What can we learn from this experience? HPV vaccine discovery was not based on examining viral antigens recognized by host immune responses or monoclonal

antibodies, standard immunological methods for vaccine discovery. Instead, the approach to the identification of protective immunogens derived from a fundamental understanding of the HPV genome structure and the functions of its gene products. While this may not be the only future path to the successful discovery of potential protective immunogens against other pathogens, it highlights the utility of this approach. Without this level of understanding, our chances of defining potential targets are probably compromised.

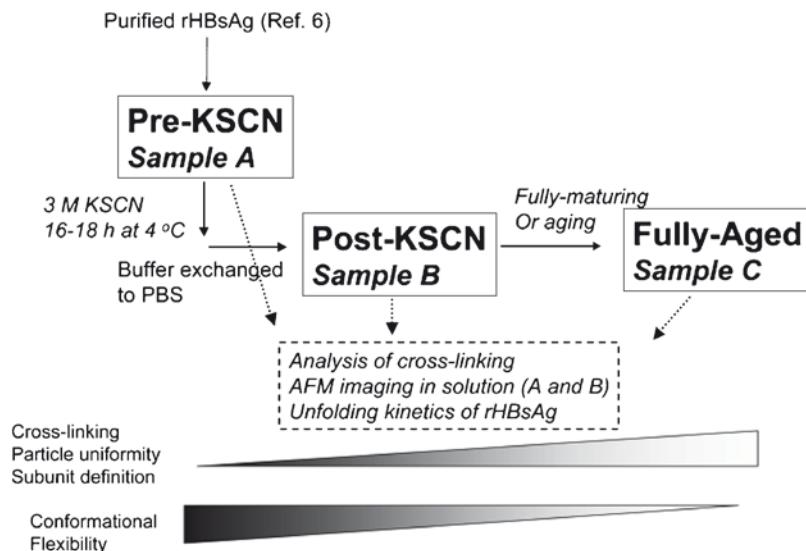
## 22.4 Immunogenicity of Recombinant Proteins

Success with the first, molecularly defined, recombinant vaccine – hepatitis B – owed to circumstances surrounding the development of the first plasma-derived subunit vaccine against this pathogen in the early 1980s (refer to Chapter 17). The latter vaccine comprised purified, 22-nm particles obtained from the blood of chronic hepatitis B carriers (Hilleman 2003). However, once the donor population contemporaneously became one of the recognized, sentinel groups with the then newly described Acquired Immunodeficiency Syndrome, caused by a transmissible agent of uncertain etiology, the plasma-derived vaccine faced major public health challenges and scrutiny. Cloning of the viral surface antigen and the development of a recombinant product were, therefore, innovative responses of the scientific and pharmaceutical communities engendered by public health exigencies and made possible by the availability of the requisite molecular technology. Within 5 years, the current generation of vaccines was launched.

Why did recombinant vaccines succeed? Early studies that defined the structure of the hepatitis B surface antigen (HBsAg) isolated from blood samples of chronic carriers demonstrated the presence of two major virus-specific components: a 25 kD polypeptide and a 30 kD glycoprotein. Immunologic studies on these molecules demonstrated their remarkable immunogenicity in mice compared to a denatured pool of the two polypeptides, but equally significant at that early stage of vaccine development was the observation that the reduction of disulfide bonds and alkylation of the free cysteine groups abolished the immunogenicity of these molecules, thus confirming the necessity of structural features in the determination of immunological properties.

The successful molecular cloning of HBsAg significantly augmented the understanding of the association between its structure and immunogenicity. Recombinant HBsAg particles produced in yeast had similar amino acid composition and sequence and were immunologically similar to those isolated from the blood of chronic viral carriers (Yamaguchi et al. 1998); however, the yeast-produced particles comprised mainly nonglycosylated polypeptides and were slightly larger than the human-derived particles. Furthermore, the former was composed of dimers of disulfide-bonded polypeptides. From these and other studies it became evident that maturation and self-assembly of HBsAg were critical steps in producing immunogenic material.

A recent, detailed characterization of recombinant HBsAg (Fig. 22.1) summarizes the steps used for evaluation of maturation of recombinant HBsAg particles in



**Fig. 22.1** Summary of steps used to complete the formulation of recombinant HBsAg in vitro. Sample A is the in vitro product before exposure to KSCN. Sample B refers to product after treatment with 3M KSCN for inducing cross linking; an essential step for stability and immunogenicity. Sample C is the product after storing at 37°C for approximately 1 week, followed by storage at 4°C. This step is intended to complete the maturation and epitope development. Depicted in the lower part of the illustration is visualization of the progress of cross linking and conformational flexibility (Dr. Q. Zhao and Landes Bioscience)

relation to cross-linking and conformational flexibility (Zhao et al. 2006). While it is known that limited cross-linking of recombinant HBsAg is initiated spontaneously, the process is completed in later steps. The downstream purification steps include treatment with 3M KSCN, enhancing disulfide bond formation, essential for better stability and immunogenicity of the molecule and shown to be associated with reduction of relative monomer content of the molecule and a corresponding increase in its trimer content. The next step in the maturation of recombinant HBsAg involves heat treatment to enhance cross-linking and results in particles that are completely resistant to SDS-induced denaturation. These and other studies associate the changes in the conformational flexibility of HBsAg particles with enhancement of their antigenicity and immunogenicity as vaccine products.

Analogous observations on the expression, crystallization and assembly of HPV L1 VLPs were obtained in in vitro expression systems (Chen et al. 2000). For example, expression of HPV16 L1 in *E. coli* results in pentamers assembled into capsid-like structures. The T-1 icosahedral assembly with 12 pentamers forms a tightly-linked ring. The shape of these morphological units resembles those pentamers seen by electron microscopy; the detailed structure of L1 particles formed the basis of vaccine development. In studies of HPV L1 proteins expressed in yeast, it was found that the resulting particles demonstrate type-specific properties.

HPV18 L1 protein forms uniformly assembled VLPs with a mean size of 60 nm in diameter; whereas VLPs from HPV6 L1 and HPV16 L1 are more irregular in form and uniformly smaller in size.

In order to develop an efficient and scalable process for vaccine development, procedures for disassembling and reassembling these VLPs were developed, such as their exposure to high salt concentrations, that result in more homogenous particles (Mach et al. 2006). These disassembled/reassembled particles demonstrated improved thermal stability and remarkable enhancement of their in vitro antigenicity. This was confirmed by in vivo immunogenicity studies in mice. Recent investigations on the kinetics and dynamics of HPV16 L1 VLP formation demonstrate that the assembly reaction proceeds under energy constraints; assembly at pH 7.2 is proportional to its ionic strength (Mukherjee et al. 2008). These assembly studies provide detailed evidence of additional requirements, such as disulfide bond formation and quaternary structure, for optimal HPV vaccine development.

## 22.5 Vaccines Against Malignancy

Oncogenesis represents a multifaceted challenge to host immune and inflammatory reactions. Immune recognition of neoplastic processes is evidenced by a myriad of immune responses detected in diseased animals and humans, including those directed against tumor-specific antigens, tumor-associated antigens, or in the case of oncogenic viruses, viral products (Finn 2008). There is robust evidence for the localized inflammatory recognition of tumors at their sites of tissue invasion (Ji et al. 2006). Two opposing forces that may permit tumor growth involve failed or compromised immune surveillance and the impact of tumors on host immune responsiveness as manifested by systemic or localized suppression (Finn 2008). The outcome of these interactions raises the question of whether we can employ therapeutic or preventative approaches to cancer based on the general principles of immunization.

The repertoire of therapeutic monoclonal antibodies against tumor-specific antigens and tumor growth factors has greatly expanded over the past decade (Mendelsohn 1997; Finn 2008); these products are becoming important components of cancer therapy. Furthermore, attempts are currently underway to define the possible use of tumor-specific antigens or -T-cells to suppress tumor growth, although the approach of using tumor-specific antigens as immunogens is in an earlier stage of development. Currently, studies are underway using the HER2 antigen for breast cancer and anti-idiotype for B cell lymphomas (Inoges et al. 2006), none of the previous studies for immunization against malignancies has been conclusive. In contrast, immunization against hepatitis B or HPV using viral protein-based vaccines has demonstrated remarkable success in preventing hepatocellular or cervical carcinoma, respectively (Blumberg 1997; Lowy and Schiller 2006). Experimental studies underway in mice aiming to induce prophylactic immunity targeting selected tumors, such as prostate (Garcia-Hernandez Mde et al. 2008), may open the way to new approaches for cancer prevention.

## 22.6 Lessons Learned and Future Challenges

The history of vaccine discovery and development has produced a wealth of information but only a few lessons that guide the future. Among them is the difficulty in identifying potential protective immunogens in infectious organisms or specific cancer targets. After more than two centuries of scientific effort, we have very few well-defined immunogens. In the two examples in which the immunogen has been molecularly defined, hepatitis B and HPV, discovery was neither dependent on using the host immune response to identify viral target antigens nor was it a result of raising polyclonal or monoclonal antibodies against the pathogens. The denatured polypeptides of both vaccine preparations are neither immunogenic nor protective. Furthermore, these antigens contain microbial polypeptides and host cell lipids; immunogenicity is dependent on the conformational and thermal stability of the VLPs. It is clear from these observations that optimal immunogenicity of these molecules is due to their quaternary structure and not their polypeptide sequences and, as with many other vaccines, the final product includes an adjuvant to augment the induction of robust protection. The aforementioned should dictate a fundamental change in our future approach to immunogen discovery.

The future of vaccine discovery has to be based on a more fundamental and detailed understanding of the molecular structure and organization of target organisms and tumors. As we define their genomics and proteomics, we will be better able to determine which of their constituents may be used as targets against which to direct protective immune responses. Future successes also will depend on a basic transformation of the science of immunology and how it applies to host protection, shifting the paradigm away from qualitative descriptions of immune responsiveness to a much more quantitative and coordinated understanding of how the entire system functions in host protection.

The publication of DNA sequences of the complete genomes of many microbes opens a new way to explore their genetic organization and gene functions (Rocha 2008) and promises to elucidate unique opportunities for vaccine discovery. Furthermore, genomic studies using DNA and phenotype microarrays may help to discern events in microbial pathogenicity that could serve as a step toward identifying targets critical to microbial survival or virulence (Bochner 2009). Along with the expanding fields of defining human microbiota, metagenomics and metabolomics of microbial communities, a new horizon is being pursued in the arena of microbial ecology and pathogenicity (Turnbaugh and Gordon 2008). The future of vaccine discovery is, therefore, intimately linked to the identification of factors crucial to microbial survival. It is an era best described by the metaphor coined by Joshua Lederberg in 2000: “Our wits against their genes”!

The pace of expansion of our immunological knowledge base, beginning with the landmark works of Metchnikoff, von Behring, and Ehrlich in the late nineteenth century (refer to Chapter 3) and continuing today has been exponential, but a fundamental switch to a more quantitative level of understanding is needed. Monitoring the components of the immune responses to microbes or tumors is no

longer sufficient; rather, it is essential to quantify and evaluate the dynamics and regulation of these responses and to determine their mechanisms of protection. Information acquired from immunologic surrogates through the latter half of the twentieth century allowed us to monitor host responses to immunization; however, these data contributed little to our understanding of protective mechanisms, a necessary step forward toward clearer pathways to vaccine discovery and development. This is especially true in the realm of chronic infections, inflammatory, and neoplastic states.

The aforementioned paradigm shifts begin to address a number of challenges to future vaccines, such as productive strategies for dealing with intracellular pathogens, as current assumptions are mainly based on host interactions with extracellular invaders; and an appreciation of the relative roles of innate and adaptive immunity in order to optimize vaccine protection (Pulendran 2007). The identification of novel pathogen-recognition receptors, such as those of the Toll-like receptor system or other newly recognized families of pattern recognition molecules, and defining their respective roles in initiating protective responses are critical in this process.

Other aspects of vaccine immunology must be harnessed to enhance future development. Because most defined polypeptides of microbial origin are only modestly immunogenic, effort is needed to understand and design antigen presentation strategies that result in significantly enhanced immune responses. In this particular context, VLPs deserve considerable attention. Protein assembly into VLPs has been achieved with many infectious agents (Roy and Noad 2008). The most important step is to link such technology to the evolution of function, immunogenicity, and protective capabilities of such assembled proteins. VLPs may also have potential use as carriers for unrelated molecules that, by themselves, are ineffective in inducing protection, a concept illustrated by recent malaria vaccine work (Mettens et al. 2008).

Finally, the development of new adjuvants and immunostimulants is of particular importance for future vaccine discovery. These molecules are frequently essential in order to harness host innate and adaptive immunity to produce sustainable protection in vaccinated individuals and will be of particular significance as we move into the era of defined immunogens. Currently, there are only two approved adjuvants for human use, but the development of new molecules and formulations is expanding (Reed et al. 2008). Excellent experimental studies demonstrate candidate molecules that specifically target components of innate immunity and that significantly enhance immunogenicity, although the approval of new adjuvants remains challenging from a regulatory perspective because of concerns of autoimmunity and other rare but possible side effects. As with new immunogens, the more we understand the mechanisms of actions of these novel molecules the more likely we will be able to effectively evaluate their efficacy and safety.

Clearly, the history of vaccinology is still evolving. The early nineteenth century witnessed the acceptance of the concept through the smallpox vaccine experience. The development of microbiology and immunology as distinct disciplines in the latter portion of that century laid the foundation for the discovery of vaccines against many of the major bacterial scourges of the time. But it was Pasteur's work with rabies followed by a series of incremental and significant advances in the laboratory

that led to the monumental discoveries in the Enders laboratory and subsequently the “golden age” of vaccines of the later twentieth century. We are perhaps poised for the next such series of breakthroughs that will allow further chapters to be written.

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