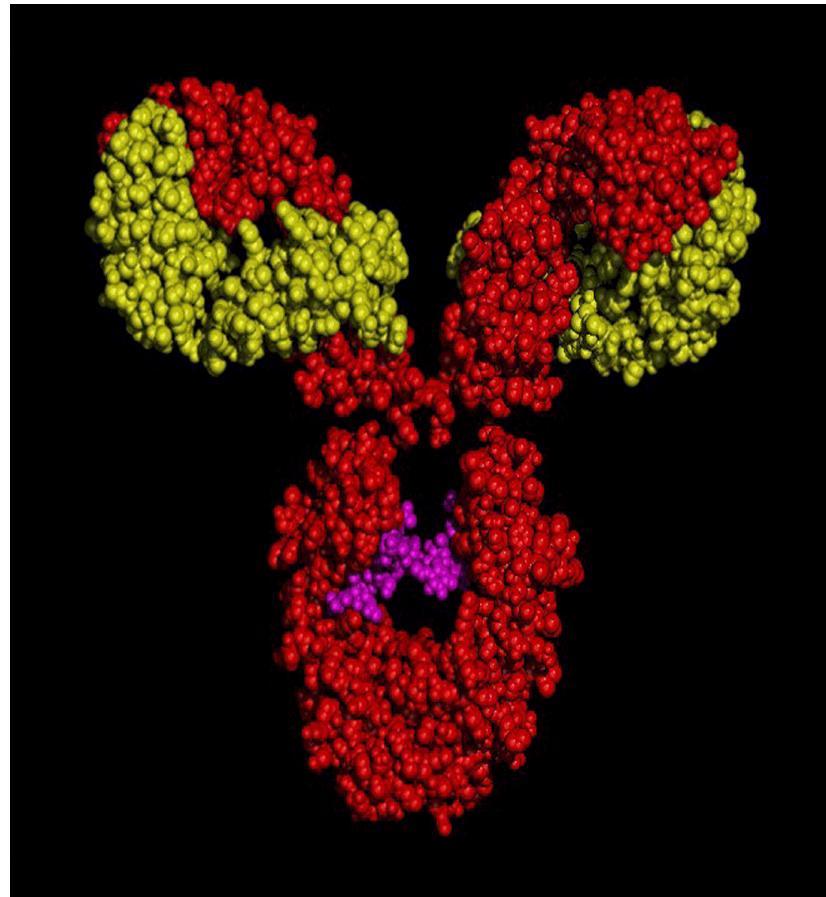


Local application of human IgG to prevent biomaterial-centered bacterial infection



Rijksuniversiteit Groningen

**Local application of human IgG to prevent
biomaterial-centered bacterial infection**

Proefschrift

ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
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In memory of Dr. Anthony G. Gristina, M.D.

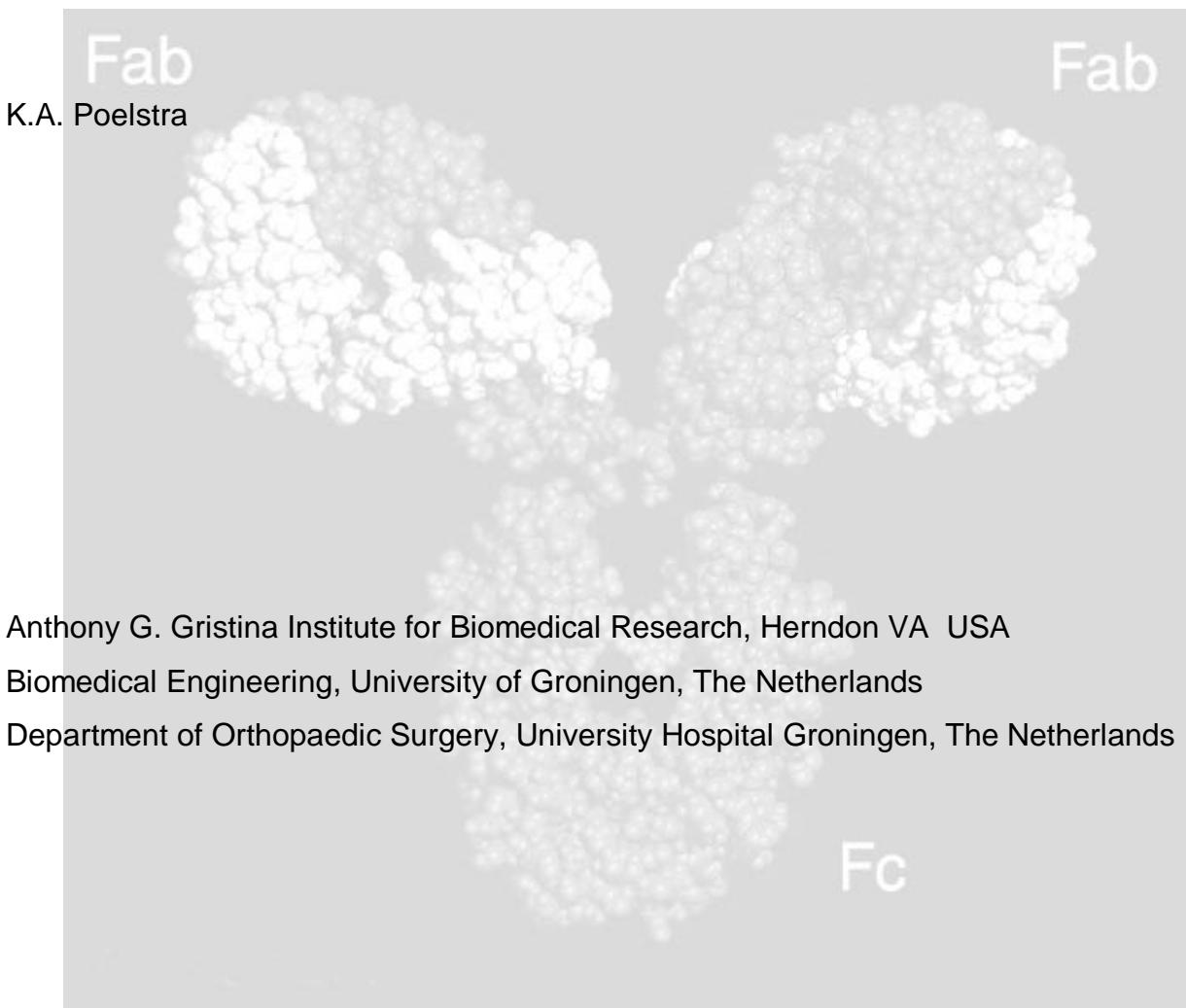
Voor Angelique

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Introduction and research objectives



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History of immunotherapy

In the 1890s, researchers first determined the efficacy of passively administered antibodies against pneumococcal infections in animals. This observation led scientists to vaccinate animals and use the resulting anti-sera to treat tetanus, diphtheria, rabies or vaccinia.^{4,16,26,29,46} Hericourt examined the protective capabilities of serum therapy by raising “specific” antibodies in animals to treat people with certain forms of cancer. While a short-term remission was realized, this was not yet a radical anti-cancer therapy, although results suggested that antibody therapy could be an important adjuvant therapy following surgery to prevent infection and therefore promote healing.²⁵

Just prior to World War I, pneumococcal pneumonia was epidemiologically the single most important infectious disease. The development of anti-pneumococcal sera received considerable attention and, by the early 1930s, became the standard treatment for lobar pneumonia.⁸ Immediate adverse reactions of serum therapy were fevers and chills, while more delayed, severe reactions referred to as “serum sickness” included rash, proteinuria and arthralgia.⁹ These problematic side-effects could be reduced by using human sera instead of sera raised in animals. In the treatment of measles, for example, even less severe reactions could be avoided by concentrating the protective elements comprising sera using ammonium sulfate precipitation³² and preparation of the immunoglobulin from pools of sera rather than serum from single donors.²⁸ Development of a fractionation process for isolating purified immunoglobulin based on pH, ionic strength, temperature and cold ethanol fractionation (Cohn fractionation)¹¹ set the stage for large scale fractionation of pooled sera and has constituted the basis for most modern preparations of commercial immunoglobulin ever since.

The first clinical use of these fractionated immunoglobulin products was in the prevention and mitigation of measles and as a prophylactic agent against hepatitis A via intramuscular injections. Later, gamma-globulin from donors with high titers against hepatitis B, rabies, tetanus, diphtheria and varicella-zoster was used for prophylaxis of these respective diseases.^{10,22} Immunoglobulin preparations for intramuscular use

(IMIG) produced by Cohn fractionation contained almost exclusively IgG with small amounts of IgM and IgA, but intravenous administration of this product gave rise to frequent and serious side effects resulting both from complement activation and also from dimeric forms of IgG. A number of different modifications to reduce IgM and IgA fractions and therewith the complement activation of the original product resulted in improved preparations for intravenous use (IVIG).^{6,15} The initial rationale for developing IVIG was the relatively low amount of IgG that could be administered intramuscularly due to pain at the injection site and limited muscle mass in some patients. Further therapeutic exploitation, however, has resulted in hundreds of clinical reports on IVIG treatment regimens and commercial product lines dedicated to specific IVIG formulations worth millions of dollars annually.

The immune system

The immune response system of humans and that of other higher animals provide mechanisms for specific responses to the invasion of pathogenic microorganisms and foreign materials. This response protects the host largely against disease. The immune response is characterized by specificity, memory and the acquired ability to detect foreign substances.

The specificity of this response allows for the recognition of even very slight biochemical differences between molecules. Consequently, the macromolecule coating on specific microbial strains can elicit a different response during infection than the macromolecules of even a very closely related strain of the same species. The immune response is adaptive and acquired: having responded once initial exposure of a particular recognized macromolecule, called an antigen, a memory system is established through cell communication that permits a rapid and specific secondary recognition response upon re-exposure to that same antigen. Because of this memory mechanism, humans can develop immunity against specific diseases and as a consequence, usually suffer from many diseases, like chicken pox, only once. To artificially acquire or prompt a state

of immunity, vaccines have been developed that intentionally expose the body to specific non-infective microbes, fragments, macromolecules, or other antigens, with the intent of generating immunity against the antigen upon repeated re-exposure. A well-known early example is vaccination by exposure to cowpox in the late eighteenth century by the English physician Edward Jenner, to prevent the acquisition of the deadly smallpox.³⁵ Since that time, major world-wide vaccination efforts have eradicated many of the most deadly diseases. More advanced vaccination strategies are now being commercialized against many remaining infection problems.³³

Two forms of immunity

There are two different forms of mammalian immunity. The first, called antibody-mediated immunity, elicits cellular production of specific antibodies when foreign antigens are detected. Plasma cells derived from white blood cells (B-lymphocytes or ‘B-cells’) synthesize antibodies in response to the detection of foreign macromolecules with antigenic properties. This type of immunity is called humoral immunity, because after release by the B-cells, the antibody molecules circulate extracellularly through the body fluids. The second form of immune response is cell-mediated immunity, whereby certain cells of the body acquire the ability to destroy other cells that are recognized as foreign or abnormal. This type of immunity depends upon T-lymphocytes or ‘T-cells’.

Both B- and T-cells originate from the bone marrow and become differentiated during maturation. Precursors for T-cells pass through the body to get processed in the thymus gland, located apical to the heart. They remain mostly inactive until fully matured in other lymphoid tissues. B-lymphocytes also develop in the bone marrow but the name ‘B-cell’ refers to bursa-dependent lymphocytes because, in birds and chickens, these cells differentiate in the bursa of Fabricius before starting to produce antibodies. Humans do not have a bursa of Fabricius; the designation B-lymphocyte is applied to lymphocytes that can differentiate into antibody-synthesizing cells in humans. Once stimulated by antigenic recognition, these B-cells generate antibody-secreting plasma cells that produce a variety of classes of antibodies that collectively are called immunoglobulins.

To effectively battle foreign antigens and infection, the immune system is equipped with a complex immunological tolerance mechanism. The most important aspect is host self-tolerance that prevents the body from mounting an immune attack against its own tissues. Since the immune system randomly generates a vast diversity of antigen-specific antibodies over time, a potential risk exists for such an attack that has to be prevented incrementally by self-check processes over a lifetime rather than being genetically pre-programmed. Next to the basic structures of macromolecules that elicit antigenic “foreign” recognition responses, other factors including the time and the site of encountering epitopes are also important in building host tolerance. For example, in one tolerance process, B-cells that express surface receptors with high binding affinity for self membrane-bound antigens are deleted immediately after leaving the bone marrow to prevent an auto-immune attack on host tissues.

Antibody molecules as immunoglobulins

Antibody molecules or immunoglobulins comprise a large mass fraction of circulating, soluble proteins found in the serum (26mg/ml) of the blood, extravascular fluids, mucosal membranes, and some tissue surfaces. Synthesis of antibodies in B-cells or plasma cells occurs after antigenic stimulation. Five different classes of antibodies can be produced: IgG, IgA, IgM, IgD and IgE. The various classes serve different functions in host immune response with their basic properties outlined in Table 1.

All immunoglobulin molecules have the same basic structure consisting of four peptide chains, two identical heavy chains and two identical light chains, joined by multiple disulfide bridges linking the chains and stabilizing the tertiary and quaternary protein structure eliciting function (Figure 1). Differences in the heavy chains are responsible for the five major classes of immunoglobulins. The light chains always comprise kappa (κ) and lambda (λ) peptide sub-units that allow the immune system to combine atorally endless arrays of different versions of immunoglobulin binding sites capable of recognizing encountered antigens.

Property	IgG	IgA	IgM	IgD	IgE
Molecular weight	1.50×10^5	1.62×10^5	9.50×10^5	1.85×10^5	2.00×10^5
Heavy chain	γ	α	μ	δ	ε
Light chains	κ or λ	$\kappa + \lambda$	$\kappa + \lambda$	$\kappa + \lambda$	$\kappa + \lambda$
Valency for antigen binding	2	2 (4)	5 (10)	2	2
Concentration in Normal serum	8-16mg/ml	1.4-4mg/ml	0.5-2mg/ml	0-0.4mg/ml	17-450 μ g/ml
% total immunoglobulins	70-75	15	10	0-1	0.002
Complement fixation					
Classical:	++	-	+++	-	
Alternative:	-	\pm	-	-	
Cross placenta	+	-	-	-	-
Fix to mast cells	-	-	-	-	+
Macrophage/neutrophil binding	+	\pm	-	-	-
Major characteristics	Most abundant Ig in body fluids; combats infecting bacteria and toxins	Major Ig in sero-mucous secretions: protects external body surfaces	Effective agglutinator produced in early immune response	Mostly present on lymphocyte surfaces	Protects external body surfaces; responsible for atopic allergies

Table 1:

Properties of the five classes of immunoglobulins

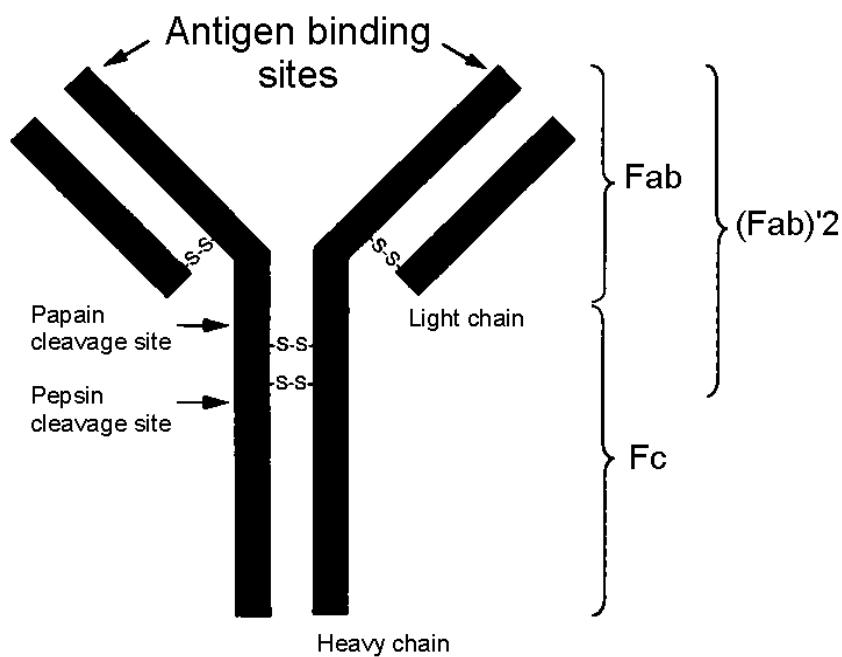


Figure 1:

General structure of an immunoglobulin unit: two heavy chains (50,000Da) and two light chains (25,000 Da) arranged in a Y-shape, stabilized by multiple disulfide bonds.

Either of the light chain types may combine with any of the heavy chain types, but in any one immunoglobulin molecule, both light chains are identical, as are the heavy chains. This allows the antibody to bind simultaneously with both 'arms' bivalently to antigens. This important bivalent function permits antigen cross-linking, ultimately leading to immunoprecipitation, membrane patching, capping, signaling and subsequent complement activation to neutralize the antigenic entity.²⁴

The fundamental basis of antibody-mediated or humoral immunity is derived from the reactions of antigen with antibody molecules. The interactions of antibodies with surface antigens of bacterial cell walls render many pathogenic bacteria susceptible to phagocytosis and cell based enzymatic destruction (neutralization). Many ingestive processes (e.g., bacteria, latex or polystyrene particles, etc.) require antigen-antibody reactions before phagocytic engulfment can take place. This facilitating process, activated by antigen-antibody reactions, is called opsonization. This process can be initiated by non-specific antibody-binding but for many pathogenic, virulent organisms, opsonization can only be established by specific antibodies with Fab fragments directed against characteristic epitopes on the bacterium surface.^{1,2,17,45} Destruction of these pathogens inherently resistant to opsonophagocytosis --especially those armed with a polysaccharide protective capsule-- requires antigen-antibody reactions to overcome and prevent severe infections. The Fc portion of the IgG molecule contains sites that are recognized by a class of cellular receptors called Fc_yR responsible for cell-mediated recognition of antibody-antigen complexes. These receptors are found primarily on polymorphonuclear cells (PMN's), monocytes, macrophages and natural killer (NK) cells, and assist (along with C3b complement receptors) in the phagocytosis (clearance) of immune complexes and antibody-coated foreign substances like pathogenic bacteria. Once combined with an antigen, the Fc portion of the IgG molecule can also be recognized and bound by complement C1 to form an antigen-antibody-complement complex that activates the complement cascade, ultimately leading to pathogen lysis.

The immunoglobulin molecule can be split by the enzyme, papain, into Fab binding fragments (ab=antigen binding) and the Fc tail fragment (c=crystallizable or constant).

The enzyme, pepsin, cleaves immunoglobulins in another location forming divalent Fab fragments $[(\text{Fab})_2]$ but not yielding Fc fragments. The structural subunit flexibility and compositional variability of the immunoglobulin molecules permit the specificity of their reactions with antigens and signaling to cellular immune elements (e.g., T-cells, NK cells). Additionally, allosteric changes in immunoglobulins result in a three-dimensional conformation that allows the antigen- and antibody molecules to fit properly together after an “adapted fit” model. The Fab regions are hypervariable, allowing for diversity of antibody molecules that provide the necessary basis for wide, protective immune response against many different antigens, including bacterial toxins, together with the infinite different structural variations resulting from genetic variation.

Immunoglobulin G - IgG

IgG is the largest mass fraction of the body's soluble immunoglobulin molecules, comprising generally 70-75 percent of total antibody circulating mass.

Research described in this dissertation is focused on the local delivery of immunoglobulin G to prevent bacterial infection, a characteristic property of IgG discovered around 1900. Reviewing the capabilities of other immunoglobulin classes or cellular immunity is beyond the scope of this study and will therefore not be addressed in further detail.

The IgG immunoglobulin group consists of four protein subclasses all sharing the common structure shown in Figure 1 (IgG_1 , IgG_2 , IgG_3 and IgG_4), with IgG_1 being the largest fraction (~65%). The different subclasses vary slightly in chain composition, number and arrangement of their disulfide bonds and function. IgG_2 antibodies are opsonic and are produced in the human body in active response against pathogen toxins. IgG_1 and IgG_3 antibodies activate complement most effectively and bind best to monocytes and macrophages to induce phagocytosis of opsonized bacteria. IgG_4 antibodies function as skin-sensitizing immunoglobulins.

Stimulated by chemoattractants in peripheral tissues, IgG can pass readily through the vascular endothelium into the extravascular, extracellular spaces (extravasation) where it can react with antigens to stimulate the attraction of phagocytic cells to invading microorganisms. IgG reactions with surface antigens on bacteria also activate the complement system and attract additional neutrophils to the site of infection. IgG is also the only immunoglobulin that passes through the placenta to confer passive immunity to the fetus and neonate.

Current IgG clinical preparations

Safety

Currently, intravenous IgG (IVIG) is used in millions of doses annually to treat many specific pathologies in immunocompromised patients, resulting from immune-depleting therapies or diseases. IVIG is produced from pools of purified human IgG collected from thousands of donors. Depending upon the population from which the plasma is derived, IgG titers against different pathogens vary. To ensure the quality of the product to treat infection, titers against a number of different bacterial, viral and fungal antigens are usually determined. Four recent seminal reviews written within this decade have focused on most of the important issues (clinical and economic) surrounding the use of IgG to treat various infectious pathologies.^{7-9,22} The consensus view of IVIG from these reviews is summarized below:

For many years, Cohn fractionation was thought sufficient to eliminate viral contamination in IgG clinical preparations. Although this was proven true in 1986 for HIV by both the Food and Drug Administration (FDA) and the Centers for Disease Control (CDC), transmission of hepatitis B was still regarded as possible. A 1996 study of the aggregated risk of viral transfer (HIV, HBV, HCV and HTLV) in all donor blood products available (e.g., blood, plasma, immunoglobulins, clotting factors) was one in 34,000 per unit transfused.²⁷ Individual donors are therefore screened for the presence of HbsAg (hepatitis B surface antigen) in their blood to eliminate viral spread.

Unfortunately, hepatitis C (non-A, non-B hepatitis) screening was also required since outbreaks of this disease occurred both among immune thrombocytopenic purpura patients (ITP) and immunocompromised individuals who received IVIG preparations.¹⁴ All currently available evidence indicates that the new solvent/detergent (SD) treatment eliminates the risk of transmission of the major pathogenic viruses in blood --HIV, HBV and HCV-- as well as other enveloped viruses.²⁷ Between 1985 and 1997, the New York Blood Center recorded the administration for the US of approximately 5,790,000 doses of SD treated IMIG-IVIG (\approx 28,951,000g IgG), excluding anti-D IgG (1,000,000 doses), HB-IgG (117,000 doses), CMV-Ig (121,000 doses), RSV-Ig (199,000 doses) and other Ig products (anti-tetanus etc., 486,000 doses) without reported toxicities. Even SD-treated plasma from HIV-infected men, pooled to produce hyper-immune anti-HIV immunoglobulin preparations, did not transmit HIV or hepatitis and the immunoglobulin has now been proven to be safe in clinical trials.^{14,27} There is a significant difference in the patterns of clinical use of human polyclonal IgG between the United States and Europe where IgG use is significantly less attributed in part to the patient paranoia and commercial stigma of blood-derived products. Unfortunately, European blood-banks are not sharing their data with third parties about the use of IgG which makes a direct comparison between the two continents impossible (personal communication Dr. van Aken, CLB Amsterdam, The Netherlands).

Efficacy

The therapeutic efficacy of IVIG has been studied against a number of viral diseases including HIV, herpes simplex, Epstein Barr virus (EBV) and varicella zoster. Viral disease occurs by viral particles that successfully enter (transfect) a host-cell, multiply using the host replicating machinery, lyse the host cell and disperse to invade other host cells. Specific IgG antibodies neutralize viruses by attaching to viral particles and preventing the viral transfection processes by inhibiting the virus to enter the host-cell. In this case, the viral binding titer of IgG can approximate the neutralization titer, since IgG-virus complexation limits infectivity.

Although numerous animal data suggest that viral illness can be modified with passive immunization,^{19,34,36,37} the best human results have shown that only some symptomatic improvement is achieved, or that viral shedding was reduced.³⁹

In January 1996, a specific respiratory syncytial virus immune globulin intravenous (human) (RSV-IGIV) product (RespiGam®, Medimmune, Inc., Gaithersburg MD) was introduced to the United States clinical market. This originally IVIG product pooled from vaccinated, hyperimmune human donors, contains a high concentration of neutralizing and protective antibodies directed against RSV,³⁸ the main cause of severe sinusitis and pneumonia in young children. Clinical studies have been successful in proving this product's efficacy as being the first anti-viral immunoglobulin product.²¹

The rationale for using IVIG against bacterial infection is just as compelling as its use against viruses. Supplementing broad spectrum immunoglobulins will supply a patient with sufficient opsonic antibodies to fight present and potentially pathogenic bacteria. IVIG is reported to synergize with β -lactam antibiotics since IVIG contains anti-lactamase antibodies against antibiotic resistant pathogens.¹³ Although encouraging results from randomized blinded studies, especially in newborns and intensive-care patients, were achieved with IVIG,^{5,12,18,23,39-41} antibody therapy is currently only indicated in relatively few clinical infection situations. Chemically produced antibiotic formulations have proven over the years to be more efficacious, less expensive and more versatile. Only in situations where immunoglobulin-deficient patients are involved or post-exposure prophylaxis is needed against different viruses (e.g., rabies, measles, hepatitis A and B, varicella) including toxin neutralization (diphtheria, tetanus, botulism), can IVIG provide measurable benefit.

Additionally, IVIG treatment is very expensive for several reasons. First, IVIG requires intravenous infusion and hospitalization, unlike other, simpler, effective antibacterial products that have been on the market for decades (e.g., oral antibiotics). Secondly, many antibiotics are now off patent, and can be produced inexpensively under a generic name, while the cost of IVIG treatment has increased due to a current world-wide

shortage of donated blood, and enhanced screening and safety requirements in its preparation from serum. The price for one vial of IgG (10grams; one dose) in 1994 averaged approximately \$300 while the manufacturer for the same vial in April, 1999, charged roughly \$900 (Baxter Healthcare). Because of the still unproven clinical efficacy when applied intravenously, high cost, low availability and associated stigma as a blood-derived product, IVIG has not competed well against antibiotics.

However, in the prevention and treatment of infections in contaminated wounds (e.g., abdominal surgery, burns, traumatology) the use of broad-spectrum protective agents provide a major advantage that remains attractive. This has been the rationale supporting the use of broad-spectrum antibiotics as prophylaxis and as first-line of defense to treat these infections (e.g., penicillin, later cephalosporins). Unfortunately, widespread antibiotic resistance, the emergence of new pathogens and the increase in immunocompromised patients leaves many anti-microbial drugs less effective than ever before.

Also, the increased use of biomaterial implants and their propensity to promote the formation of an overlying glycocalix by adhering microorganisms (=biofilm-forming infections)²⁰ call for the development of new anti-infective strategies in the race against virulent and therapy-resistant bacteria. By reducing bacterial adhesion to a biomaterial implant during surgical intervention and therewith reducing the risk for biofilm formation, host tissue integration of the implant could be increased. Improved tissue integration and wound healing, not complicated by immediate postoperative wound infection, are also important in the prevention of latent infections caused by hematogenous spread of bacteria that can enter the bloodstream during e.g., dental restoration procedures or a penetrating trauma and colonize the biomaterial even years after initial implantation. Neutralizing contaminating organisms intra-operatively before the 4-6h “decisive period” is over^{48,49} could bring reduction in postoperative, biomaterial centered infection with virulent (antibiotic-resistant) bacteria.

Non blood-derived, recombinant custom monoclonal antibodies (Mabs) are currently being improved for specific therapies, but highly specific preparations of Mabs are very expensive and are only slowly becoming more abundant and available for clinical application. The use of intravenous IVIG may be appropriate in some indications but continues to be both costly and scarce. Moreover, considerable variation between different lots of the same product can occur, and an *in vitro* titer may not correlate to an *in vivo* titer or neutralization or therapeutic efficacy, because of differing antibody affinity, epitope restriction and IgG subclass composition.

Since infectious complications leading to the dangerous state of septic shock frequently systemize from local infections (e.g., i.v. catheter, urinary tract infection (UTI), orthopaedic implant infection), local anti-infective therapy as an alternative to systemic antibiotic prophylaxis or treatment, applied directly to sites of infection could prove very useful. However, in the face of increasing antibiotic resistance, increased antibiotic use should be discouraged,³ especially in local applications that could encourage resistance.⁴⁴ By potentiating the body's own intrinsic ability to overcome bacterial invasion, preventing systemic spread of infection and the cause of sepsis, infection incidence could be reduced, ultimately leading to a reduction in antibiotic use and increasing antibiotic resistance. Several immuno-modulatory paths can be stimulated locally at infection sites to work in tandem with or without antibiotics. In particular, humoral immunopotentiation involves supplementation of antibodies using immunotherapy. Such an approach side-steps antibiotic resistance mechanisms since humoral immunity works by a distinctly different mechanism. Importantly, bacterial antibiotic resistance does not alter their susceptibility for opsonization and phagocytosis. Hence, immunotherapy using IgG should function just as well against resistant organisms. Since IVIG has demonstrated limited clinical appeal, a shift in the use of immunotherapy from systemic treatment to local application represents a new route to provide humoral immunopotentiation at sites of high infection risk. Such an approach could be effective using reduced IgG doses and sophisticated delivery strategies to reduce infection incidence, severity, and antibiotic resistance complications.

Research objectives

The primary aim of this thesis research is to investigate the efficacy of locally applied pooled polyclonal immunoglobulins (IgG) to prevent bacterial infection with different bacterial challenges in different *in vitro* and *in vivo* models.

Specifically, the hypothesis is that locally applied, pooled polyclonal IgG represents a useful prophylactic agent to prevent post-surgical bacterial infection by reducing bacterial adhesion and biofilm formation in the wound, and stimulating phagocytic clearance, ultimately leading to increased host survival.

This hypothesis was assessed using several experimental designs:

- First, an *in vitro* study was conducted using a parallel plate flow chamber to study the adhesion of *Pseudomonas aeruginosa* IFO3455 to glass in the presence and absence of IgG;
- Secondly, a closed murine peritonitis model was used to study the efficacy of locally applied IgG against *P. aeruginosa* IFO3455 and other clinically relevant bacteria that infect the abdominal cavity;
- Thirdly, in a newly developed animal model for investigating postoperative spinal implant infection, the anti-infective efficacy of locally applied IgG was tested against methicillin resistant *Staphylococcus aureus* (MRSA);
- Fourth, in both a murine burn- and a closed peritonitis model, IgG was applied locally together with systemic, prophylactic but sub-optimal doses of a third generation cephalosporin to study the additive or synergistic effects when IgG is administered in tandem with a currently clinically relevant antibiotic;
- Lastly, an open peritonitis implant model in mice was developed to study the efficacy of the local application of IgG in a controlled-release gel-carrier in tandem with systemic prophylactic cefazolin or vancomycin to prevent biomaterial-centered infection by MRSA and *P. aeruginosa*.

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Pooled human immunoglobulins reduce adhesion of *Pseudomonas aeruginosa* in a parallel plate flow chamber

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Abstract

*The influence of pooled polyclonal immunoglobulin (IgG) interactions with both bacteria and model substrates in altering *Pseudomonas aeruginosa*-surface adhesion is reported. Opsonization of this pathogen by polyclonal human IgG and pre-adsorption of IgG to glass surfaces both effectively reduce initial deposition rates and surface growth of *P. aeruginosa* IFO3455 from dilute nutrient broth in a parallel plate flow chamber. Polyclonal IgG depleted of *P. aeruginosa*-specific antibodies reduces the initial deposition rate or surface growth to levels intermediate between exposed and non-exposed IgG conditions. Bacterial surface properties are changed in the presence of opsonized IgG. Contact angle analysis via sessile drop technique shows a drop in *P. aeruginosa* surface hydrophobicity after IgG exposure consistent with a more hydrophilic IgG surface coat. Zeta potential values for opsonized versus non-opsonized bacteria exhibit little change. X-ray photoelectron spectroscopy measurements provide surface compositional evidence for IgG attachment to bacterial surfaces. Surface elemental ratios attributed to IgG protein signals versus those attributed primarily to bacterial polysaccharide surface or lipid membrane change with IgG opsonization. Direct evidence for antibody-modified *P. aeruginosa* surface properties correlates both with reduction of bacterial adhesion to glass surfaces under flow in nutrient medium reported as well as previous reports of IgG efficacy against *P. aeruginosa* motility in vitro and infection in vivo.*

Advances in the development of bioengineered implants and devices now provide clinicians with an enormous range of prostheses that improve the quality of life for millions of patients. Unfortunately, this progress has been accompanied by clinical challenges involving implant-associated infections and host foreign body responses that compromise healing and often resist conventional antibiotic treatment.¹⁻⁴ Infectious organisms preferentially target synthetic, implanted materials, eliciting serious and costly infections that frequently require removal of the colonized device.⁴⁻⁷

Pathogen colonization and maturation into biofilms has been documented on numerous biomedical devices used in orthopaedics, urology, cardiology and other fields of surgery, even those that survive treatments with first-line antibiotics.^{4,8} Initial microbial adhesion is a primary determinant of biomaterial-centered infection, because initially adhering microorganisms link the final mature biofilm to the biomaterial surface and elicit device-centered infection.^{4-6,9}

Prevention of microbial surface colonization on implants is one possible approach to reduce biomaterial- and implant-centered infection. For decades, many different strategies have attempted to redesign, surface-modify or coat implant materials to block initiation of this process.^{5,10-17} Many bacteria-surface interactions have been reported. Correlations between bacterial surface hydrophobicity and surface adhesion have been studied well, and are now well-established.¹⁸⁻²¹ However, design of surfaces that resist colonization and reduce infection remains problematic. In addition to surface chemistry approaches, antibiotic-releasing coatings have not been fully successful in overcoming clinical presentation of biofilm formation and subsequent implant failure.^{8,22-24} Both systemic and local antibiotic therapies are frequently ineffective in reducing infection incidence on implants, are complicated by the array of possible pathogens involved in implant-centered infection, or encounter antibiotic resistant infections.^{1-3,8,25,26}

Because surgical sites are often immunocompromised as a result of surgical- and implant-induced trauma, the restoration, stimulation or supplementation of endogenous immune responses are alternatives to bolster host defenses against infection. Both clinical presentations -- acute, immediate postoperative infection of newly implanted biomaterials and longer-term, latent implant site infection -- could benefit from local potentiation of the host's immune system to defeat contaminating indigenous and exogenous pathogens during and after surgical implantation procedures. Because infection often interferes with wound healing, local wound site immuno-stimulation may also improve subsequent healing processes and long-term tissue integration, but such benefits have not yet been reported.²⁷

As a predominant host immune component, pooled human polyclonal IgG comprises millions of opsonizing antibodies specific to microorganism epitopes, facilitating endogenous pathogen killing by stimulating phagocytosis, complement lysis and oxidative responses by macrophages and neutrophils.^{20,21,28} Of the array of immunoglobulins produced for host defense, immunoglobulin G (IgG) subclasses comprise the largest mass fraction of circulating antibodies. Direct elucidation of IgG opsonization on the adherence of bacteria to a substratum have not yet been reported although it is often asserted that opsonization hinders bacterial tissue interactions. *In vitro* assays have recently shown that pooled polyclonal human IgG limits motility of several flagellar pathogens, a factor linked to virulence.²⁹ Recent *in vivo* studies have used locally delivered pooled polyclonal human IgG to reduce *P. aeruginosa* infection severity and incidence in several animal infection models.³⁰⁻³³ These new data provide evidence for the protective benefit of exogenously administered polyclonal antibodies to overcome lethal infection from several different virulent bacteria strains.

In order to further understand aspects of the basic mechanisms surrounding bacterial-surface interactions and the influence of antibodies to moderate biomaterial-centered infection, the efficacy of pooled human polyclonal IgG in the direct prevention of bacterial adhesion and surface-growth was investigated. In this study, we compare the initial adhesion rates and surface-growth dynamics for a proven virulent IgG-opsonized and non-opsonized *Pseudomonas aeruginosa* on glass surfaces *in situ* using a published parallel plate flow chamber system.^{9,34} Surface adhesion and growth characteristics of both IgG-opsonized and non-opsonized bacteria were correlated with bacteria surface properties determined via contact angle, zeta potential and X-ray photoelectron analytical measurements.

Methods

Bacteria and growth conditions: The flagellar pathogen, *Pseudomonas aeruginosa* IFO3455, was used in this study.^{29,35,36} The strain was streaked and grown for 24h from a frozen stock on nutrient agar (Oxoid). The plate was then kept at 4°C for not more than one week, at which time a new plate was streaked. A ‘preculture’ was inoculated and incubated at 37°C in ambient air for 24h. This preculture was used to inoculate a second culture in 100ml 100% nutrient broth (100%NB) that was grown for 18h. Bacteria from the second culture were harvested by centrifugation at 10,000g for 5min at 10°C and washed twice with Millipore-Q water. Subsequently, bacteria were sonicated on ice for 10s to separate cell clusters and then resuspended at a concentration of 3×10^8 cells per ml in sterile 2%NB in isotonic PBS (pH 7.0) with or without 0.2wt% commercial pooled human polyclonal IgG (Baxter Gammagard S/D, Lot 98F03AB11).

Microbial cell surface characterization: Bacterial cell surfaces were characterized by zeta potential and water contact angle measurements in the presence and absence of opsonizing IgG. Zeta potential measurements were conducted on *P. aeruginosa* cultures harvested and washed as described above. Bacteria were resuspended (3×10^7 cells per ml) in either sterile PBS, or sterile PBS supplemented with 0.2wt% pooled polyclonal IgG, both at pH 7.0. The electrophoretic mobility of each suspended bacterial sample was measured at 150 V using an automated Lazer Zee Meter 501 (PenKem Inc., Brookhaven Instr., Worcestershire, UK) and converted into zeta potentials, assuming that the Helmholtz-Smoluchowski equation is valid.³⁷

Sessile drop contact angle measurements on washed *P. aeruginosa* IFO3455 filter ‘lawns’ were performed as described previously³⁸ to determine the intrinsic cell surface hydrophobicity in the absence and presence of adsorbed human polyclonal IgG. Briefly, *P. aeruginosa* harvested from an overnight culture in nutrient broth was suspended either in sterile PBS or sterile PBS supplemented with 0.2wt% IgG. Bacteria were subsequently washed three times to remove soluble polysaccharides, proteins and

salts, resuspended in Millipore-Q water and deposited on 0.45 μ m pore-size cellulose acetate filters to produce an even, confluent bacterial ‘lawn’ of approximately 50 stacked bacterial layers. The *P. aeruginosa* IFO3455 lawns were air-dried until so-called ‘plateau contact angles’ could be measured using sessile water droplets, followed by analogous contact angle measurements with formamide, methylene iodide and α -bromonaphthalene as described previously.³⁸

X-ray Photoelectron Spectroscopy: X-ray photoelectron spectroscopy (XPS) was used to determine the overall microbial cell surface composition and performed as described previously.³⁹ In short, freeze-dried, copiously rinsed freshly cultured bacteria in Millipore water, either pre-incubated with polyclonal IgG or untreated, were pressed into small stainless steel cups and placed on the sample stage of the spectrometer (S-probe; Surface Science Instruments, East Sussex, UK) equipped with an aluminum anode (10kV, 22mA) and operating at 10⁻⁹ Torr. Survey spectra were collected using a pass energy of 150eV and spot size of 250 x 1,000 μ m. High resolution spectra were acquired at a pass energy of 50eV for selected peaks in the sequence C_{1s}, O_{1s}, N_{1s}, P_{2p} and again C_{1s} to account for contamination or deterioration of the samples under X-ray flux. The binding energy for all spectra were referenced to the C1s C-H peak at 284.8 eV. The area under each peak, after linear background subtraction, was used to calculate peak intensities, yielding elemental surface concentration ratios for oxygen/carbon, nitrogen/carbon and phosphorus/carbon after correction for instrument sensitivity factors as supplied by the manufacturer. The O_{1s} peak was fit using a least-squares program into two Gaussian components assigned to 531.4eV (O1) and 532.8eV (O2). These two O_{1s} components were representative of oxygen species involved in C-O-C and C-OH chemistry (O2), and remaining collective oxygen chemical environments (O1).

Parallel Plate Flow and Adhesion Chamber: The parallel plate flow chamber (internal dimensions: 76 x 38 x 0.6 mm), associated microscope and image analysis system have been described previously.^{19,34} This system comprises a phase contrast microscope (Olympus BH equipped with Olympus ULWD-CD Plan 40 PL long working distance

objective) with a CCD-MXR camera (High Technology, Eindhoven, The Netherlands) coupled with a TEA (Difa, Breda, The Netherlands) image analyzer. Initial adhesion rates, surface growth and detachment were directly observed and recorded on the bottom glass plate *in situ* under laminar flow (flow rate = 1.5 ml/min) without any additional shear forces acting on the adhering bacteria. Glass was chosen both for its optical transparency necessary for observation by phase-contrast microscopy, cleaning convenience and ease of sterilization by autoclaving. Just prior to sterilization, glass plates and spacers were thoroughly washed using sponges, water and surfactant, sonicated for 30min and thoroughly rinsed multiple times with Milli-Q water.

Bacterial Adhesion Experiments: Prior to each experiment, the entire flow system was autoclaved, and subsequently filled with 2%NB in PBS (pH 7.0), ensuring that all air bubbles were removed from the system. Once filled, and prior to the addition of bacterial suspension with or without IgG, additional nutrient broth was flowed through the system for 60min at a flow rate of 1.5 ml/min, and subsequently switched to the bacterial suspension ($\sim 3 \times 10^8$ /ml) at the same flow rate. All experiments were performed at 37°C. In one experimental setup, this flow was changed to 1wt% IgG for deposition onto the glass surface for 60min prior to exposure to a bacterial suspension in 2%NB. Subsequently, bacteria were perfused through the system for 75min without recirculation, and images were captured using phase contrast microscopy.

The number of initially adhering bacteria was expressed as a so-called ‘initial deposition rate’, i.e., the increase in the number of adhering *P. aeruginosa* per unit area per time, extrapolated to $t=0$ (9,34), according to:

(1)

$$j_0 = \frac{dn(t)}{dt} \Big|_{t=0}$$

where $n(t)$ is the number of bacteria adherent at a given time, t , extrapolated to $t=0$.

Following the 75min exposure to bacterial suspension, flow was switched to 100%NB and continued for 18h to study bacterial surface growth (generation and doubling times) and biofilm development. Fifteen successive images were recorded every three min during this surface-growth mode, from which the number of adhering organisms, the generation time of individual adhering bacteria and the bacterial surface coverage were determined under different conditions.^{9,34} Images are automatically tracked sequentially by computerized image analysis and subject, based on frame-by-frame optically discriminated differences, to computer algorithms that calculate doubling times of surface coverage^{9,34} according to:

$$n_t = n_0 * 2^{*(t/t_d)} \quad (2)$$

where t_d is the doubling time sought, n_t is the number of bacteria on the surface at time t and n_0 is the original number of bacteria observed.

Generation times in 100%NB were calculated by visually timing the division speed of 30 different adhering, duplicating bacteria per experimental setup during the growth phase.

In two flow chamber adhesion experiments, *P. aeruginosa* was first exposed to 0.2wt% pooled but modified human IgG solutions for 60min. This IgG had been previously depleted of antibodies specific for this *Pseudomonas* strain by incubation for two hours with 10^{12} CFU *P. aeruginosa* IFO3455.²⁹ After this incubation, bacteria were spun down for 15min at 10,000g and discarded with adhering specific antibodies, while the remaining IgG-containing supernatant was collected and filtered (0.2μm) to remove possible bacterial contamination and subsequently used in this flow chamber experiment as ‘depleted IgG’.

Results

Bacterial adhesion and surface growth under flow: Table 1 shows results determined directly from the flow chamber image analysis for the deposition of *P. aeruginosa* IFO3455 suspended in either 2% nutrient broth (NB) in PBS or 2%NB in PBS supplemented with 0.2wt% IgG, both on either bare glass surfaces in NB or on glass surfaces pre-adsorbed with 1wt% IgG. As the first column reflects, initial deposition rate in the absence of IgG is 258/cm²/s. Initial adhesion rates observed both for bacteria pre-opsonized with IgG (120/cm²/s) and for non-opsonized bacteria on the pre-adsorbed IgG glass surface (106/cm²/s) were significantly lower (student t-test) when compared to the initial adhesion rate in the absence of IgG (both p-values<0.01). The combination treatment of both bacterial opsonization and glass pre-adsorption with IgG did not further reduce the initial adhesion rate (136/cm²/s). Interestingly, the adhesion rate observed for bacteria incubated with 0.2wt% depleted IgG is also lower (186/cm²/s) than the initial adhesion rate for bacteria in NB on bare glass lacking IgG (258/cm²/s).

Pseudomonas surface generation times for adhering bacteria in 100%NB, presented in Table 1, are significantly longer in the presence of IgG (43-45min) compared to generation times in the absence of IgG (39min, p-value<0.01, student t-test). The surface doubling time (i.e., time to double bacterial numbers adhering to the surface) is presented in Table 1 as well. No significant difference exists between the different experimental groups. On average, approximately 60min is required to double the bacterial surface coverage in 100%NB either in the presence and absence of IgG when no planktonic bacteria deposition is present.

Finally, bacterial surface coverage after 2 and 5h of 100%NB flow is reported in Table 1 for the different experimental conditions. The observed characteristic clustered bacterial surface coverage shown in Figure 1A after 2h is a more direct reflection of initial adhesion rate and generation time than the 5h time point.

Table 1:

*Adhesion and surface growth of P. aeruginosa IFO3455 in the parallel plate flow chamber, including initial deposition rates, generation times for bacteria adhering to the surface, time to double surface coverage and percent surface covered after 2h and 5h in 100%NB.*¹

P. aeruginosa (PA) adhesion conditions	Initial deposition	Generation time (SD)	T double (SD)	Surface coverage (%)	
	rates (SD) (cm ⁻² s ⁻¹)	(min)	(min)	(2h)	(5h)
Control (bare glass, no antibodies)	258 (12)	39 (6)	61 (4)	33	92
PA pre-opsonized with IgG ²	120 (36)	45 (3)	56 (4)	15	85
Glass pre-adsorbed with IgG	106 (3)	44 (6)	60 (2)	11	65
PA pre-opsonized and glass pre-adsorbed with IgG	136 (22)	45 (10)	60 (3)	25	92
PA pre-opsonized with PA-depleted IgG ²	186 (7)	43 (4)	58 (4)	20	90

¹All experiments performed in triplicate.

²Represents the mean of two separate experiments.

After 5h, however, bacterial desorption kinetics and reorganization of the newly adherent, and surface-resident organisms result in a more homogeneous, evenly spaced surface coverage, shown in Figure 1B. The observed surface redistribution of bacteria occurs both in the absence and presence of IgG (data not shown). For both the 2 and 5h time point, the slowest surface growth was observed with pre-adsorbed IgG on the glass surface (11% and 65% coverage, respectively). Under other conditions, full surface coverage is nearly achieved after 5 hours.

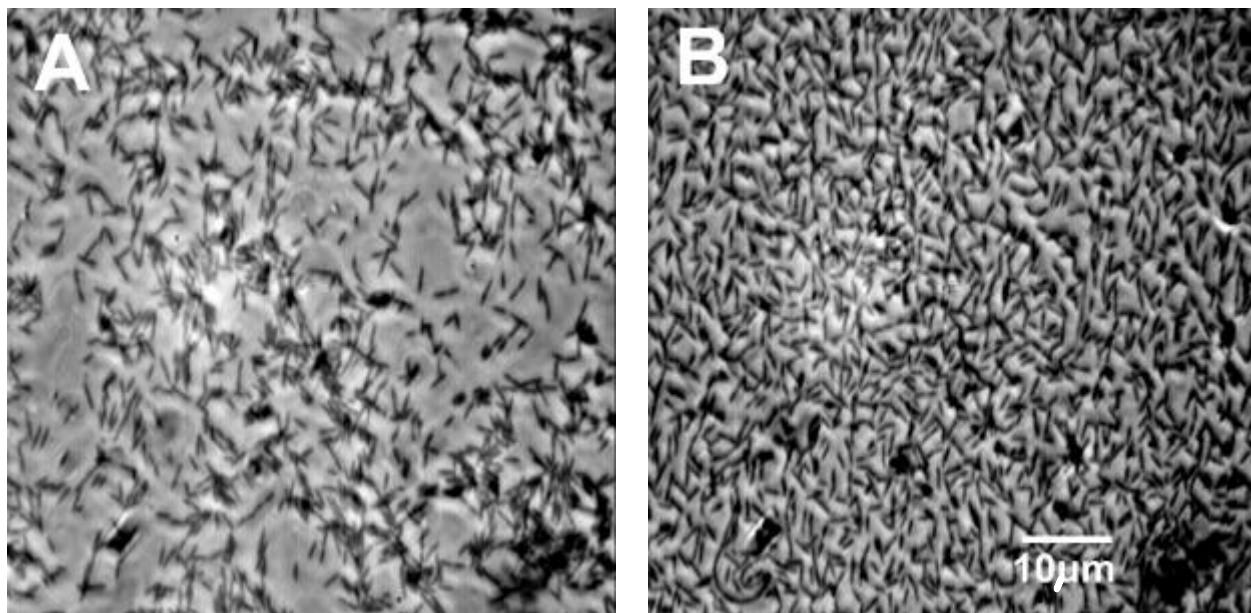


Figure 1:

*Video microscopy images *in situ* in the parallel plate flow chamber showing deposition patterns for P. aeruginosa after (A) 2h and (B) 5h in 100%NB at 37 °C. Redistribution of initially clustered microorganisms to more homogeneously spaced, denser packed coverage of the surface is observed over this time frame. This effect is independent of the presence or absence of polyclonal human IgG.*

Bacteria zeta potential: *P. aeruginosa* IFO3455 exhibits a narrow zeta potential distribution for freshly cultured bacteria in buffer with a mean value of -21 mV +/- 4mV. The negative value is attributed to the ubiquitous, poly-anionic glycocalix polysaccharides and peptidoglycans that comprise the surface coats of these bacteria. After incubation with 0.2wt% IgG in buffer, the bacterial zeta potential becomes slightly more negative with a wider distribution (-24 mV +/- 9 mV). This difference lacks statistical significance.

Contact angle analysis: Bacterial contact angles are summarized in Table 2 and demonstrate that *P. aeruginosa* IFO3455 is an unusually hydrophobic organism. Water contact angles on ‘lawns’ of this organism approximate 120 degrees, indicative of high non-wetting. Exposure of bacteria to 0.2wt% IgG in PBS, followed by copious rinsing as described above, produced a remarkable decrease in bacterial cell surface hydrophobicity, exhibiting water contact angles of 64 degrees, a trend also reflected in the reduced formamide probe liquid contact angles. Contact angles measured with lower surface energy methylene iodide and α -bromonaphthalene liquids, probing dipolar and apolar cell-surface characteristics⁴⁰ were also significantly reduced after treatment with IgG.

X-ray photoelectron spectroscopy (XPS): Results for XPS analysis of freeze-dried *P. aeruginosa* samples with or without IgG exposure are shown in Table 2. XPS elemental ratios shown for nitrogen, oxygen and phosphorus each compared to carbon support significant surface compositional changes after, compared to before, IgG exposure. Specifically, nitrogen contributions increase substantially after IgG exposure, consistent with the adsorption of antibodies (e.g., amide groups) to the bacterial cell wall surfaces. Reductions in oxygen and phosphorus elemental contributions support these data. Oxygen generally comprises a higher atomic compositional percent of polysaccharides and peptidoglycans in bacterial cell walls than in proteins. IgG opsonization contributes new oxygen signals unique to the IgG protein layer but attenuates those signals derived from the buried bacterial cell wall components, leading to a net oxygen signal reduction (see Table 2). Closer examination of the high-resolution O_{1s} XPS signal at 532eV produces two distinct oxygen signals, representing two different oxygen species distributions before and after IgG exposure, at 532.2eV and 532.8eV. Table 2 shows a quantitative breakdown of these contributions attributed to carbonyl oxygens (i.e., from amides, aldehydes, ketones, and esters) at the lower O_{1s} binding energy, and the ester and alcohol oxygens at higher O_{1s} binding energies.⁴⁶ A significant shift in the relative contributions of each oxygen species to lower binding energy (e.g., to protein carbonyl oxygens) is observed after IgG adsorption. This is consistent with both addition of an

overlayer of protein (IgG) and a masking of underlying bacterial cell wall saccharide chemistry. Phosphorus signals, attributed to phospholipids in the bacterial cell membrane, drop nearly 50% after IgG exposure, also attenuated by the overlying IgG protein layer.

Table 2:

Surface hydrophobicity and XPS-determined elemental surface composition ratios for bacterial ‘lawns’ of *P. aeruginosa* IFO3455 alone or *P. aeruginosa* IFO3455 pre-adsorbed with polyclonal IgG.

<i>P. aeruginosa</i> incubated with:	Contact angle [*] (degrees)				XPS elemental ratio			Oxygen species [#] (percent)	
	Water	Form- amide	Methylene iodide	α -Bromo- naphthalene	N/C	O/C	P/C	O_1 (531.4eV)	O_2 (532.8eV)
No IgG	120	55	58	40	0.096	0.347	0.015	36.7	63.3
Polyclonal IgG	64	38	48	23	0.130	0.280	0.009	49.7	50.3

^{*} n=8, static sessile drop, room temperature (see ref. 38).

[#] percent of total XPS O_{1s} peak centered at 532.2eV; two Gaussian contributions for two oxygen binding environments.

Discussion

In this work, the adhesion of *P. aeruginosa* IFO3455 to glass was studied in a parallel plate flow chamber in the absence and presence of pooled polyclonal human IgG antibodies. This bacterial strain has been previously studied with regard to its *in vivo* virulence and pathogenicity.^{30-33,35,36} Additionally, recent *in vitro* data indicate that commercial pooled human polyclonal IgG preparations contain measurable titers and binding activity to slow its flagellar motility and growth.²⁹ *In vitro* flow conditions reported herein used a documented adhesion buffer (2%NB in PBS) that maintains bacterial metabolic activity while promoting minimal growth.⁴⁰ Subsequent bacteria surface-growth dynamics were assessed in full medium (100%NB) that supports bacterial proliferation.

Flow chamber bacterial deposition kinetics observed under different conditions in this study demonstrate that *P. aeruginosa* IFO3455 adherence to glass is repeatedly, significantly reduced in the presence of IgG pre-adsorbed to the glass surface, and by the presence of IgG opsonization of bacteria in the adhesion buffer. Combinations of both IgG exposures did not further decrease initial deposition rates. IgG depleted of *P. aeruginosa*-specific fractions reduced bacteria deposition rates, although this decrease over the control was not nearly as significant as the adhesion reduction observed with undepleted polyclonal IgG. However, the *P. aeruginosa*-specific depleted polyclonal IgG exhibits a drastically reduced yet small, measurable binding titer²⁹ against IFO3455 (data not shown). This could explain the reduced, but still measurable decrease in surface deposition of bacteria observed with depleted IgG. Additionally, non-specific bacterial binding ability to adsorbed IgG on glass should also contribute to this effect.^{20,21} The presence of IgG adsorbed to the flow chamber glass surface also reduced the initial deposition rates of intrinsically hydrophobic, ‘non-opsonized’ *P. aeruginosa*. Since excess, non-opsonized IgG is immediately removed from the flow chamber as soon as adhesion buffer flushes it away, *P. aeruginosa* IFO3455 is less able to actively adhere to glass surfaces in the presence of IgG, whether it is pre-adsorbed on the glass surface, or opsonized on the cell surface.

Generation times of adhering bacteria in 100%NB were significantly longer for *P. aeruginosa* adhering on glass in the presence of IgG, whether preadsorbed to the flow chamber glass surface or first directly opsonized onto planktonic bacteria prior to adhesion. Similar to complete IgG, *P. aeruginosa*-depleted IgG extended the generation time of adherent bacteria significantly compared to nutrient broth lacking any IgG. These results suggest again that some IgG influence on adhesion may be non-specific. Bacterial adhesion to polyclonal IgG-adsorbed polymer surfaces has been shown to be non-specific.^{20,21,41,42} Visual observations of individual bacteria to determine the generation time suggest that bacteria were physically inhibited from ‘stretching’ or cell body expansion prior to separation after their division in the presence of adsorbed immunoglobulins. The time necessary for adhering bacteria to double their surface

coverage is comparable between all experiments performed in this study in the absence and presence of IgG (~60min). These data indicate that while initial adhesion of bacteria is influenced by IgG, eventually the log-order proliferation rates of adhering bacteria compensate to equalize all initial conditions over time.

Inhibitory influences of IgG on bacteria post-adhesion (i.e., surface generation- or doubling-times) should be limited because only the original bacterial population in the flow cell interacts with IgG in solution prior to rinsing. IgG initially bound to the glass surface remains after rinsing the flow cell, but is expected to undergo time-dependent changes (i.e., denaturation, desorption, exchange with NB) over time. Fractions of IgG initially capable of adhering bacteria may lose this capability over time on the glass surface. Because generation- and doubling times are measured in IgG-free 100%NB, new, dividing populations of adherent bacteria produced do not necessarily undergo opsonization or IgG-associated effects. Hence, eventual proliferation rates can readily mask initial IgG influences on bacteria over time. Since initial rates of adhesion are higher and generation times are shorter for bacteria in the control, IgG-free, condition (more rapid proliferation) but doubling time is comparable with IgG-containing experiments, the desorption rate for generations of proliferating *P. aeruginosa* IFO3455 must be more rapid in the absence of IgG over time.

Bacterial zeta potentials remained approximately the same value prior to and after exposure to IgG, leading to the conclusion that electrostatic properties probably do not contribute significantly to differences observed in the bacteria deposition and surface growth in the different experiments. The pI for immunoglobulin is 6.3-7.3⁴³, similar enough to the buffered pH used in these flow experiments to ensure little new surface charge by IgG opsonization. Opsonization of *P. aeruginosa* presumably neutralizes, masks or physically covers considerable glycocalix surface area of each bacterium. Antibodies are known to occupy surface areas of approximately 2200Å²/molecule under close-packed conditions,⁴⁴ sufficient area to cover many individual hydrophobic or charged bacterial surface residues. Estimates of 10³ - 10⁴ IgG molecules per opsonized

bacterium^{20,27} indicate that substantial surface modification of bacteria can occur by exposure to IgG. Zeta potential measurements indicate that surface charge remains generally unaffected, however. Contact angle data showing rather extreme bacterial hydrophobicity prior to IgG exposure support an absence of high surface charge density for this strain: relatively few polar or charged residues must be surface resident to produce these high contact angles. Other bacteria exhibit more wettable surfaces, although *P. aeruginosa* as a gram-negative species, and here possibly due to rough bacterial lawn surfaces, are generally more hydrophobic.⁴⁵

Two interpretations therefore appear to be likely: (1) opsonization by IgG is sparse, leaving the majority of charge on the original glycocalix and its double layer intact, and the zeta potential, therefore, largely unaffected, or (2) the IgG binding effectively substitutes equivalent charge and double layer contributions to compensate for masked bacterial surface charge, while contributing more polar protein chemistry producing higher wettability. Based on contact angle and XPS data, plus previous *in vitro* motility²⁹ and *in vivo* infection efficacy data^{30,31,36} known for this virulent pathogen strain in IgG-binding scenarios, it appears likely that the latter explanation is more appropriate. Since pooled IgG is known to exhibit considerable, measurable *in vitro* and *in vivo* influence on *P. aeruginosa*, IgG opsonization capacity must be considerable. This effect is consistent with contact angle and XPS data (Table 2) showing significant, quantitative changes in bacterial physical and chemical compositional properties after IgG exposure, consistent with IgG binding.

As described above, *P. aeruginosa* IFO3455-specific IgG buries glycocalix surface chemistry under the IgG hydrophilic protein hydration shell, leading to improved, opsonized aqueous wettability. But, IgG-specific binding cannot be solely responsible for the entire observed reduction in water contact angles because specific opsonizing IgG populations are a relatively minuscule fraction of the complete IgG pool.^{20,29} The large observed reductions in bacterial surface hydrophobicity for *P. aeruginosa* IFO3455 (Table 2) incubated with IgG could more logically be attributed to non-specific

adherence of hydrophilic IgG components to the bacterial surface, ultimately leading to reduced water contact angles. Substantial non-specific opsonization is consistent with long-standing analysis in the literature for IgG.^{20,21}

In summary, this paper demonstrates that human polyclonal IgG reduces the deposition and surface growth dynamics of *Pseudomonas aeruginosa* IFO3455 in a parallel plate flow chamber in several conditions of relevance to device-centered infection. Implant-associated or biomaterial-centered infection is phenomenologically linked to bacteria-surface interactions. While molecular aspects of adhesion mechanisms and methods to influence them are not well-documented, technologies directed at reducing initial bacterial surface adhesion events continue to develop. The use of selected antibodies against adhesive bacterial components has allowed new insight into both mechanisms of surface-mediated virulence as well as development of new anti-microbial approaches useful to fighting infection in implant sites.⁴⁷ However, outside of such ‘customized’ or ‘designer’ antibody approaches, the ability to effectively address more generic issues surrounding biomaterial-centered infection will rely on broader understanding of bacterial-implant surface interactions in serum, plasma and physiological milieu. The results reported here attempt to address the interactions of bacteria and surfaces with pooled polyclonal IgG as a step toward understanding more complex aspects of biomaterial-centered infection.

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Efficacy of locally delivered polyclonal immunoglobulin against *Pseudomonas aeruginosa* peritonitis in a murine model

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Abstract

*Infectious peritonitis results from bacterial contamination of the abdominal cavity. Conventional antibiotic treatment is complicated both by the emergence of antibiotic-resistant bacteria and by increased patient populations intrinsically at risk for nosocomial infections. To complement antibiotic therapies, the efficacy of direct, locally applied pooled human immunoglobulin G (IgG) was assessed in a murine model (strains CF-1, CD-1 and CFW) of peritonitis caused by intraperitoneal inoculations of 10^6 or 10^7 CFU *Pseudomonas aeruginosa* (strains IFO-3455, M-2, and MSRI-7072). Various doses of IgG (0.005-10mg/mouse) administered intraperitoneally simultaneous with local bacterial challenge significantly increased survival in a dose-dependent manner. Local intraperitoneal application of 10 mg IgG increased animal survival independent of either the *P. aeruginosa* or murine strains used. A local dose of 10 mg IgG administered up to 6 hours prophylactically or at the time of bacterial challenge resulted in 100% survival. Therapeutic 10 mg IgG treatment given up to 12 hours post-infection also significantly increased survival. Human IgG administered to the mouse peritoneal cavity was rapidly detected systemically in serum. Additionally, administered IgG in peritoneal lavage fluid samples actively opsonized and decreased bacterial burden via phagocytosis at 2 and 4 hours post-bacterial challenge. Tissue microbial quantification studies showed that 1.0 mg of locally applied IgG significantly reduced the bacterial burden in the liver, peritoneal cavity and blood and correlated with reduced levels of interleukin-6 in serum.*

Peritonitis is often caused by ulcers, appendicitis, diverticulitis, ileus (bowel obstruction), gunshot or stab wounds, and disturbances during abdominal surgical procedures,⁸ allowing the escape of indigenous bowel bacteria into the peritoneal cavity.^{28,45} Nosocomial peritonitis is caused by exogenous pathogenic bacteria, including *Pseudomonas aeruginosa*,^{7,24} *Staphylococcus aureus*,³⁶ and *Staphylococcus epidermidis*,^{28,39,44} that gain access to the abdominal cavity during prolonged surgical procedures, or via a port of entry such as that created for continuous ambulatory peritoneal dialysis (CAPD).⁴⁵ These pathogens cause nosocomial peritonitis at even higher rates in immunocompromised⁴⁶ and geriatric populations when compared to

typical patients,⁴⁴ resulting in a significant, growing medical problem impacting both patient mortality and rising healthcare costs.³⁸

The current treatment regimen for peritonitis relies on the use of intravenous antibiotics: penicillin, third- and fourth-generation cephalosporins, or quinolones.^{3,24,28,33,45} Selection of antibiotics is complicated by uncertainties surrounding the identification of infecting pathogens in a mixed contaminating flora and a documented lack of correlation between *in vitro* antibiotic studies of pathogen susceptibility and antibiotic efficacy in clinical settings.^{13,14,24} However, initial antibiotic therapy for severe intra-abdominal infection fails in 20-40% of all cases, leading to additional antibiotic use.³⁴

Antibiotic resistance occurs at a significant rate³³ among intra-abdominal infections, and this condition is frequently associated with clinical failure.⁹ Increasing emergence of antibiotic resistant bacteria coupled with increasing immunocompromised and elderly patient populations are significant incentives prompting development of new anti-infective therapies. Among many therapeutic approaches, the use of systemic intravenous immunoglobulins (IVIG) has shown promising but inconsistent results in preventing *P. aeruginosa* and other bacterial infections.^{4,5,7,20,25,26,29,42,43} Early studies reported therapeutic benefit against CAPD-associated peritonitis by using pooled human IgG added directly to dialysate fluid.^{17,25,26} No other local applications of immunoglobulins to treat peritonitis are known, although a recent publication supports local use of injected IVIG subcutaneously in treating *P. aeruginosa* burn infection.¹⁰

This study explores the feasibility of using locally delivered pooled human IgG directly to the peritoneal cavity as a potential therapeutic complement or alternative to the antibiotic treatment of peritonitis. IgG delivered to a contaminated tissue site immediately opsonizes invading bacteria, promoting subsequent pathogen agglutination and, stimulated by cytokines and chemotactic factors, killing by invading macrophages and neutrophils.^{11,22,23} Major advantages of locally delivered polyclonal IgG include its application in controlled dosage formulations directly to infected sites and its ability to clear infection independently of antibiotic resistance mechanisms.

The aim of this study was to determine the prophylactic efficacy of locally applied, pooled human IgG against intra-abdominal challenges of different *P. aeruginosa* strains. Both *in vitro* and murine *in vivo* data support the use of pooled polyclonal IgG to neutralize *P. aeruginosa* in the host peritoneal cavity, preventing the systemic spread of bacteria as well as sepsis and mortality.

Materials and Methods

Animals. Female CF-1, CD-1 and CFW mice (22-24 g) were purchased from Charles River Laboratories (Raleigh, NC). All animals were acclimated for seven days, given food and water *ad libitum* and kept on a twelve hour light-dark cycle. The Gristina Institute's animal care and use committee (IACUC) approved all of the animal procedures in this study.

Bacteria. *Pseudomonas aeruginosa* strains (IFO-3455, obtained from Dr. A. S. Kreger (27); M-2, obtained from Dr. I. A. Holder (30); and MSRI-7072, a local hospital clinical isolate) were grown for 18 hours in 20 ml of trypticase soy broth at 37°C while agitated at 150rpm in a benchtop incubator shaker. Cultured bacteria were twice sedimented by centrifugation at 7649 \times g for 10 minutes, washed, and diluted in saline to obtain a concentrated bacteriae suspension. Serial bacterial dilutions were plated on Trypticase soy agar (TSA) and colonies were counted after 24 hours incubation at 37°C to determine initial colony forming units (CFU) per ml. In parallel, the optical absorbance of these dilutions was measured with a Beckman DB-GT grating spectrophotometer ($\lambda=650\text{nm}$, visible filter). Standard curves plotting optical absorbance versus CFU concentrations were then constructed. Typically, bacterial suspension absorbance ranges of 0.46-0.9 resulted in $\sim 10^9$ CFU/ml. Heat-killed *P. aeruginosa* M-2 was produced by incubating these bacterial cultures at 56°C for 3 hours and plating 100 μl of the 10^7 CFU/ml stock solution on TSA to confirm non-viability.

Murine peritoneal infection model. The peritonitis model involved injecting mice with either live or heat-killed *P. aeruginosa* in 500 µl (IFO-3455, LD₉₀ = 10⁷ CFU; M-2, LD₉₀ = 10⁷ CFU; MSRI-7072, LD₅₀ = 10⁷ CFU) intra-abdominally by using a syringe with a 30G needle. The infectious challenge was followed immediately by a separate 500 µl co-localized abdominal injection of IgG (therapy) or either human serum albumin (HSA, Lot# 66H9306, Sigma, St. Louis, MO), 0.2 M glycine or 5% dextrose as placebo treatments. Mortality studies involved the intra-abdominal injection of *P. aeruginosa* where animal survival was assessed over a 10-day period post-challenge and survival outcomes in the treatment and control groups were compared.

Immunoglobulin therapy. Commercially pooled human IgG (Lot# 2620M039A, Gammagard®, Baxter International Inc., Deerfield, IL) was diluted in 5% dextrose (recommended by the manufacturer) to obtain the varying IgG concentrations used in these trials. An anti-human IgG ELISA¹⁸ was used to determine polyclonal human IgG titers against three different *P. aeruginosa* strains. Titer numbers express the inverse log dilution of IgG concentration at 50% ELISA optical absorbance (450nm) from the inflection mid-point on each IgG-bacteria binding curve. Higher titer numbers reflect increased IgG binding to each bacteria strain. A second ELISA using a capture mouse anti-human IgG and detection peroxidase-conjugated anti-human IgG (Jackson Immuno Research Laboratories, Inc.; products 209-005-088 and 209-035-088) was used to detect human IgG (optical absorbance at 450nm) in mice serum and peritoneal lavage as described below.

Quantitative Microbiology. At various times post-infection, mice were anesthetized with Metofane® (Mallinckrodt Veterinary, Inc., Mundelein, IL) and blood withdrawn via cardiac puncture. Following euthanization (cervical dislocation), a saline lavage of the peritoneal cavity was performed using 3 or 5 ml of sterile saline and lavage fluid (~2-4 ml) was collected. Livers were excised, weighed in 10 ml saline and homogenized (Omni-International GLH Homogenizer, Marietta, GA). Blood, peritoneal lavage fluid, and homogenized livers were serially diluted and plated on TSA and bacterial colonies were enumerated after 24 hours incubation at 37°C.

Serum IL-6 and human IgG assay. Serum was separated from the blood (obtained via cardiac puncture) using a benchtop HN-SII centrifuge (10 min. at 3000rpm, IEC, Needham Heights, MA) and assayed with a commercial ELISA (Pharmingen, Inc., San Diego, CA, $\lambda=450$ nm) to determine the levels of interleukin-6 (IL-6) and human IgG. The detection range for the IL-6 assay was between 15 - 2000 pg/ml, and for the human IgG between 5 - 5000 ng/ml. Standard curves were constructed from known amounts of murine IL-6 contained in the ELISA kit and from commercially pooled human IgG (Lot# 2620M039A, Gammagard®, Baxter International Inc., Deerfield, IL), respectively. Murine serum IL-6 and human IgG levels were determined by comparing the experimental absorbance values from serum or peritoneal lavage to standard curves.

In vitro opsonophagocytic assay. Murine peritoneal lavage fluid, collected 2 hours after bacterial challenge and human IgG treatment, was assayed to determine the opsonizing activity of the applied IgG. Fixed volumes of peritoneal fluid (2 ml in test tubes) were incubated *in vitro* at 37°C and agitated at 150 RPM. The bacterial burden in peritoneal lavage fluid was assayed immediately upon collection and after 2 hours of incubation by plating 100 μ l of serially diluted peritoneal fluid on TSA. Colonies were enumerated after 24 hours incubation at 37°C.

Statistical analysis. Data in this study are expressed as the mean \pm the standard error of the mean (SEM). Student T tests were used to compare the control and therapy groups of the bacterial burden enumeration studies while Z tests and analysis of variance (=ANOVA) tests were used to compare mortality. All probabilities of less than 5% were considered significant. Datum outliers, defined as any datum outside of the range of the mean \pm 2 times the standard deviation, were excluded.

Results

Polyclonal human IgG titer determination against *Pseudomonas aeruginosa* strains.

Titers of commercial pooled human IgG were determined against three strains of *P. aeruginosa* by using a published ELISA method.¹⁸ Titers of 355, 501, and 398 were calculated for this IgG lot against *P. aeruginosa* IFO-3455, M-2, and MSRI-7072, respectively. These titers represent a significant IgG binding activity against the pathogens.

Local intraperitoneal delivery of IgG. Various doses of locally delivered IgG were tested against a lethal dose of *P. aeruginosa* (IFO-3455, 10^7 CFU) in four separate experiments with CF-1 mice to determine the dose benefit range. Survival of IgG-treated groups increased from the control dose of 0.005 mg and higher in a dose-dependent manner. As shown in Figure 1, the highest percentage of survival resulted from the highest concentration of IgG (96% with 10.0 mg) delivered directly to the peritoneal cavity. A step wise threshold of IgG efficacy is observed over a narrow therapeutic dose range beginning at approximately 0.5 mg IgG per mouse. All IgG doses applied intraperitoneally that were given higher than this produced significant improvements in mouse survival (ANOVA with Tukey's test, $p<0.008$ comparing survival with doses of 0.5 and 10mg). An optimal efficacious dose of 10 mg IgG per 22-24 g mouse (strains CF-1, CD-1 and CFW) was chosen for the survival studies to provide the most consistent results in lower numbers of mice with less variance and greater reliability.

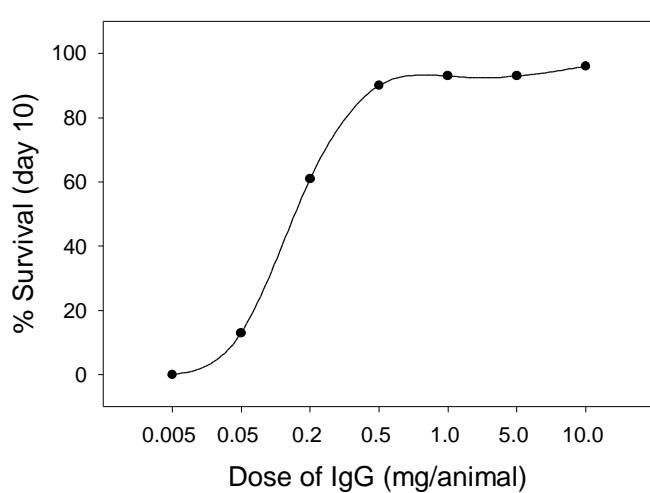
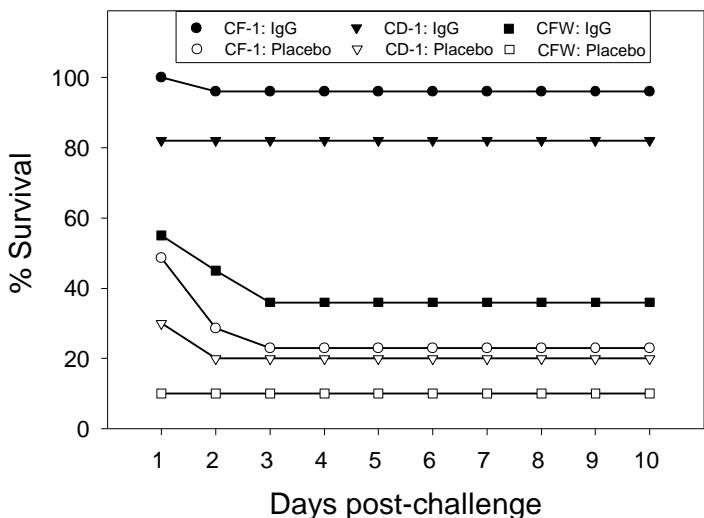


Figure 1:

Dose-response curve for locally applied intra-abdominal IgG against *P. aeruginosa* IFO-3455: CF-1 mouse survival ($n=10-25/\text{group}$) at day 10 post-challenge with 10^7 CFU i.p. injected simultaneous with a separate injected dose of IgG (0.005, 0.05, 0.2, 0.5, 1.0, 5.0 or 10 mg per animal). IgG therapy increased survival in a dose dependent-manner. Data represent the mean survival of IgG-treated mice from four different experiments. Differences between 0.5mg/animal and 10mg/animal IgG doses is statistically significant (ANOVA with Tukey's test, $p<0.008$).

**Figure 2:**

CF-1, CD-1 and CFW mouse strain survival assessed over 10 days in the peritonitis model using a single local intraperitoneal injection of 10 mg IgG or placebo treatment (5% dextrose) against a lethal dose of 10^7 CFU *Pseudomonas aeruginosa* (strain IFO-3455) injected intraperitoneally ($n=10-35/\text{group}$). IgG treatment resulted in significantly increasing survival compared to placebo (5% dextrose) treatment in all three mice strains (ANOVA with Tukey's test, $p<0.001$).

Mortality studies were conducted with CF-1, CD-1 and CFW mice to determine the efficacy of locally delivered IgG on bacterial challenges in different mouse strains. The results in Figure 2 show the 10-day survival of mice challenged with IFO-3455 and given either a single local 10mg IgG dose or a placebo (5% dextrose) treatment. Statistical differences were assessed by using an ANOVA with Tukey's test.

The 96% survival of IgG-treated CF-1 mice is significantly higher than the 23% survival of the placebo-treated group ($p<0.001$). The 80% survival of IgG-treated CD-1 mice is significantly higher than the 10% survival of the placebo-treated group ($p<0.001$). The IgG-treated CFW mice showed reduced but still significantly improved percentage of survival over the ten-day period compared to the placebo-treated group ($p<0.001$).

CF-1 murine mortality studies were conducted by using single 10-mg local IgG treatments against lethal doses of three different *P. aeruginosa* strains (IFO-3455, M-2 and MSRI-7072) to determine whether protection imparted by locally delivered IgG was dependent on bacterial strain. The results presented in Figure 3 show bacterial strain-dependent survival with or without local IgG protection. Mice challenged with a lethal dose inoculum of the IFO-3455 strain and treated with a single local 10 mg IgG dose exhibited a 90% survival, which was significantly higher than the observed 20% survival

of the placebo-treated group (z test, $p<0.01$). Figure 3 also shows that 100% of the mice injected with a lethal dose of the M-2 strain survived with a single local 10-mg IgG treatment, whereas the placebo-treated group survival rate was only 6% ($p<0.001$). Furthermore, mice inoculated with the clinical MSRI-7072 strain and treated with a single local 10 mg IgG dose exhibited 100% survival, a value significantly higher than the 50% survival seen in the placebo-treated group ($p<0.05$). Figure 3 also shows that the control experiment with 10^7 CFU heat-killed *P. aeruginosa* (strain M-2) inoculum with or without IgG treatment produced 100% survival ($n = 6$ mice), whereas, without IgG treatment, live M-2 at the same inoculum dose produced little survival. In addition, control experiments with single local 10-mg HSA doses produced no significant differences between 5% dextrose-treated and HSA-treated control groups in either the mortality studies or the tissue bacterial quantification (data not shown).

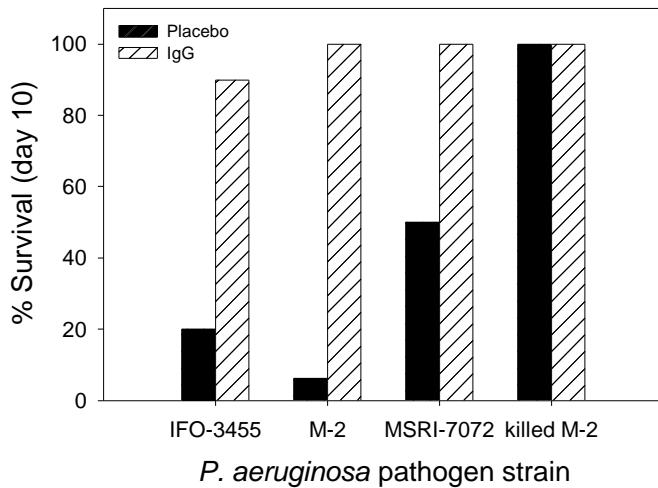
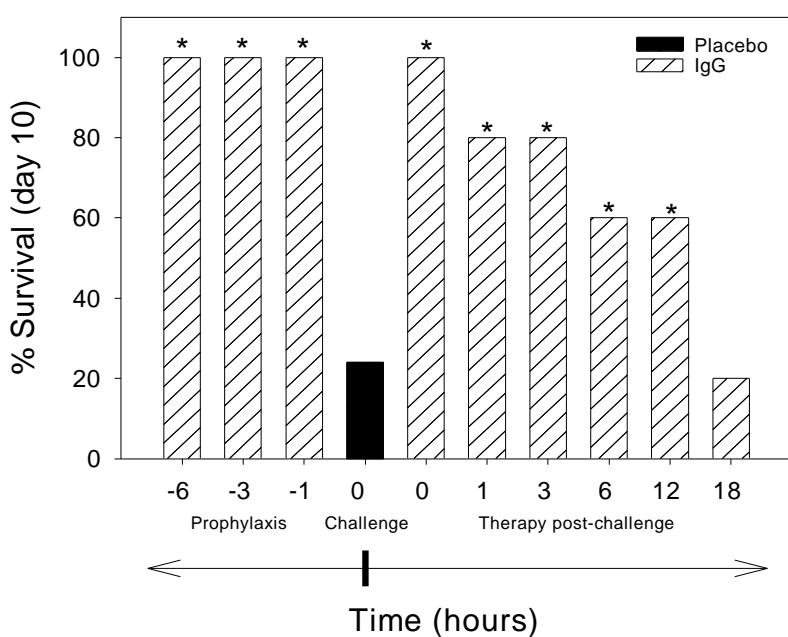


Figure 3:

*Pathogen strain influence on mouse survival (day 10) in a peritonitis model using local IgG administration. Three strains of *Pseudomonas aeruginosa* were each separately injected intraperitoneally (dose= 10^7 CFU) simultaneously with separate, single injections of 10 mg pooled human IgG, or placebo (5% dextrose, $n=10$ /group). IgG treatment significantly increased survival compared to placebo treatment (z test, $p<0.05$)*

Efficacy of local IgG application pre- and post-challenge. To investigate the prophylactic and therapeutic properties of locally applied IgG, 10 mg IgG doses were delivered intraperitoneally in CF-1 mice one, three or six hours before, at the time of, and one, three, six, twelve or eighteen hours following bacterial challenge (IFO-3455, 10^7 CFU). Figure 4 shows results for these studies. IgG administered one, three or six hours prior to bacterial challenge and simultaneously with bacterial challenge produced 100% survival ($p<0.05$ compared to placebo-treated group). Mice treated with locally injected IgG one, three, six and twelve hours post bacterial challenge exhibited significantly higher survival compared to the placebo-treated group ($p<0.05$) while mice treated at eighteen hours post-challenge showed no significant differences in survival.

**Figure 4:**

Survival in the mouse peritonitis model (day 10) influenced by time of local IgG administration relative to bacterial challenge. Single IgG injections intraperitoneally (10 mg) were administered prior to (prophylaxis), simultaneous with and after (therapy) lethal intraperitoneal injections of *Pseudomonas aeruginosa* (strain IFO-3455, 10^7 CFU, $n=10$ to 25/group). All IgG treated groups with an asterisk (*) significantly increased survival compared to placebo (5% dextrose) treatment (z test and ANOVA with Tukey's test, $p<0.05$).

Systemic and local IgG in vivo distribution over time. Serum and peritoneal lavage fluid were collected from groups of CF-1 mice treated with 10-mg IgG and euthanized at 0, 2, 3, 6, 9, 12, 24, 36 and 48 hours and every 24 hours thereafter up to day 7 after intraperitoneal challenge with IFO-3455 to compare systemic and local distributions of human IgG. Placebo (5% dextrose)-treated mice were only analyzed at 0, 2 and 3 hours and no human IgG was detectable (data not shown). As shown in Figure 5, the amounts of intraperitoneally resident human IgG decline sharply by between 2 and 3 hours post-administration (half-life ~2.5 hours) and decrease constantly over time. Simultaneously, human IgG levels in serum increase as peritoneal IgG decreases, spiking to almost 3 mg/ml at 9 hours and decreasing thereafter. Human IgG is detectable rapidly in serum after intraperitoneal administration and remains detectable by ELISA methods in both serum and peritoneal lavage for up to 7 days post challenge.

Quantification of bacteria in systemic tissues and IL-6 levels in serum. Tissue samples were collected from groups of CF-1 mice treated with 1.0 mg IgG or placebo (0.2 M glycine) 6, 24 or 72 hours after intraperitoneal challenge with IFO-3455 in order to compare the bacterial burdens in the liver, peritoneal cavity and blood. A lower, non-lethal 10^6 CFU challenge was used to ensure animal survival up to the 72 hour time point. Liver, blood and peritoneal lavage samples from placebo-treated control mice and

local IgG-treated mice were homogenized, serially diluted and plated, and the values for log CFU per tissue were compared. The results (Figure 6) show that IgG-treated mice have significantly reduced numbers of bacteria in the liver, blood and peritoneal lavage six hours post-challenge compared to the bacterial burden in control mice ($p<0.05$).

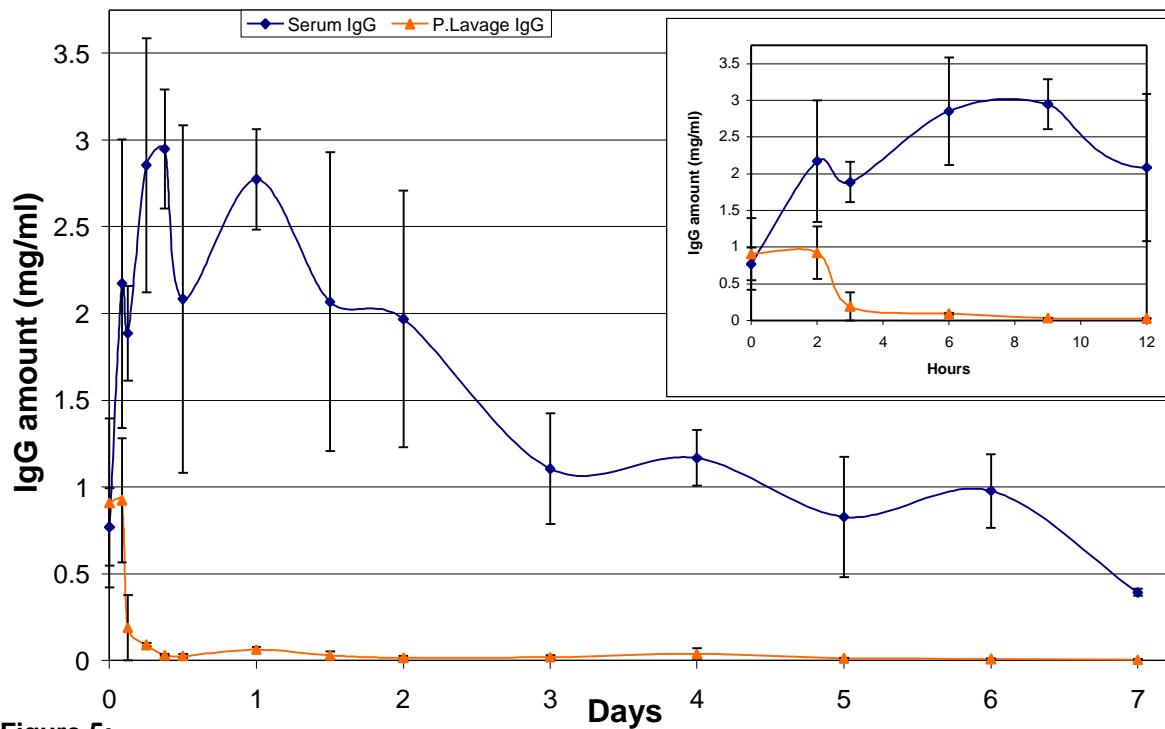


Figure 5:

ELISA detection of human IgG levels in murine serum (closed diamonds) and peritoneal lavage fluid (open triangles) post bacterial-challenge after intraperitoneal (i.p.) injection of IgG and i.p. lethal injections of *Pseudomonas aeruginosa* (strain IFO-3455, 10^7 CFU, $n=3-4$ /time point in each group) over 7 days. Human IgG is detectable rapidly in mouse serum after i.p. administration and remains detectable by ELISA methods in both serum and mouse peritoneal lavage for up to 7 days. Inset: Human IgG levels in mouse serum and peritoneal lavage in the first 12 hours post-administration.

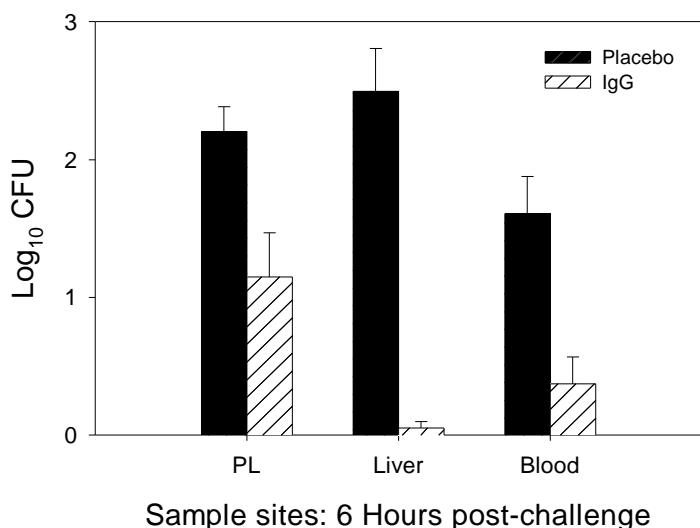


Figure 6:

Bacterial burden at various tissue sites assessed at 6 hours following intraperitoneal (i.p.) injection of 1.0 mg IgG against 10^6 CFU *Pseudomonas aeruginosa* (strain IFO-3455) i.p. Mice ($n=10$) peritoneal lavage (PL), liver homogenate, and blood analysis yielded CFU values that show IgG treatment significantly decreased bacterial burden compared to placebo (0.2 M glycine) treatment after 6 hours in all samples (t test, $p<0.05$). The PL and blood bar graphs represent the log₁₀ CFU/ml and the liver bar graph shows the log₁₀ CFU/g liver.

Bacteria were not present in the liver, peritoneal lavage or blood of IgG-treated mice by 24 hours post challenge. Additionally, ELISA was used to determine the murine serum levels of the inflammatory cytokine, IL-6. Figure 7 shows that IgG-treated mice had significantly lower levels of IL-6 at six hours post-challenge compared to the control groups ($p<0.05$). The low IL-6 levels at 24 and 72 hours post-bacterial challenge were comparable to normal circulating murine IL-6 levels and correlated with the low bacterial burden found in the peritoneal cavity, liver and blood (Figure 6).

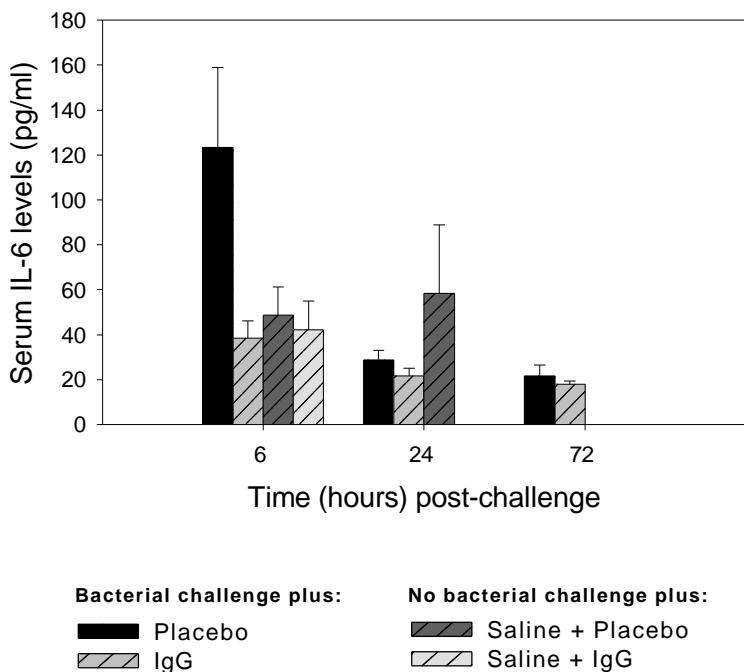


Figure 7:
Serum IL-6 levels following intraperitoneal (i.p.) injection of 1.0 mg IgG against a non-lethal i.p. dose (10^6 CFU) of *P. aeruginosa* (strain IFO-3455). Serum IL-6 levels of CF-1 mice ($n=5-25$) were determined using an ELISA. IgG treatment decreased IL-6 levels significantly compared to control ($p = 0.04$) at six hours post bacterial challenge. Saline + glycine placebo treatment without a bacterial challenge was used to determine normal background IL-6 levels in CF-1 mice. Saline + 10 mg IgG treatment without a bacterial challenge shows that IL-6 levels resulting from IgG treatment alone are not significantly different from normal background IL-6 levels (t test, $p<0.05$).

In vitro opsonophagocytic assay. Murine peritoneal lavage fluid was assayed *in vitro* 2 hours post-challenge with 10^7 CFU *P. aeruginosa* IFO-3455 to determine the opsonizing influence of applied human IgG. Peritoneal lavage fluid of mice treated with 10 mg IgG had significantly reduced levels of bacteria compared to placebo- (5% dextrose) treated mice both immediately after lavage and 2 hours later (Figure 8). The presence of human IgG facilitated the clearance of bacteria from lavage fluid whereas control-treated lavage exhibited bacterial growth during this incubation period.

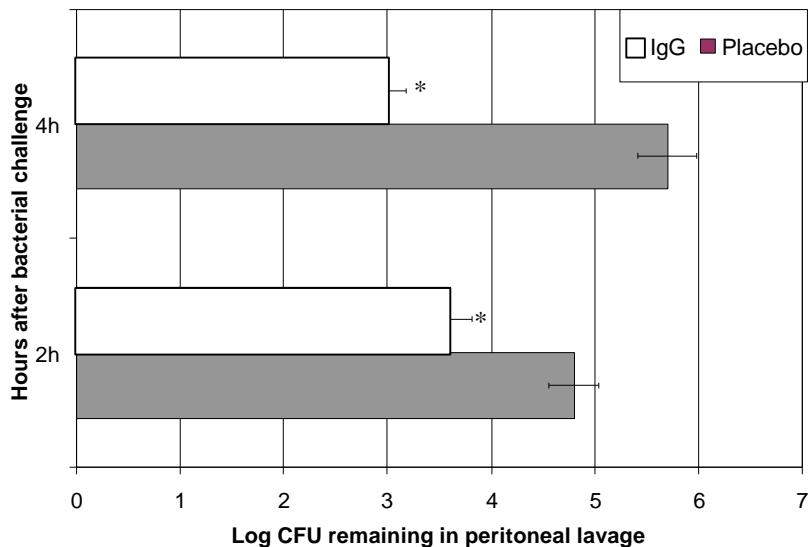


Figure 8:

*In vitro opsonophagocytic assay using mouse peritoneal lavage shows enhanced bacterial clearance with IgG. Murine peritoneal lavage fluid was assayed in vitro 2 hours after intraperitoneal (i.p.) dosing with human IgG and i.p. challenge with 10^7 CFU *P. aeruginosa* (strain IFO-3455). Peritoneal lavage fluid of mice treated with 10 mg IgG significantly reduced levels of bacteria compared to placebo- (5% dextrose) treated mice both immediately after peritoneal lavage and 2 hours later (t test, $p<0.05$). The presence of human IgG facilitated clearance of bacteria from lavage fluid while control-treated lavage exhibited bacterial growth during the incubation.*

Discussion

Local delivery of IgG directly to tissue and wound surfaces represents a potential alternative strategy against infections that is both independent of antibiotic resistance and complementary to current antibiotic treatment regimens. In this study, locally delivered IgG has been assessed in a murine peritonitis model to determine its efficacy alone against *P. aeruginosa*. This common nosocomial pathogen^{7,24,28,40,4} is responsible for 5-10% of CAPD-related and 24% of acute community-acquired perforating appendicitis infections,²¹ and it is a pathogen of particular clinical concern due to its increasingly frequent antibiotic-resistant forms that are emerging during treatment with broad spectrum antibiotics, its late complications, and its high morbidity.²¹ In 1986, Lamperi and co-workers reported that the local application of pooled human IgG (SRK-Ig [Swiss Red Cross]; pooled IgG from volunteers) as an intra-abdominal dialysate lavage treatment was beneficial against certain forms of peritonitis.^{17,25,26}

The current study shows that locally delivered pooled human IgG significantly increases the survival of all IgG-treated groups in a dose-dependent manner against different challenges of multiple *P. aeruginosa* strains and in different strains of mice compared to controls.

Recent studies linking the inhibition of *P. aeruginosa* motility and associated virulence to human pooled polyclonal IgG and its titers *in vitro* support a specific IgG mechanism that confers protection.³⁷ Since all *P. aeruginosa* strains used here are flagellate pathogens, and since commercial human polyclonal IgG is known to significantly hinder both flagellar pathogen motility *in vitro*³⁷ and infection *in vivo*,¹⁰ the observed efficacy of IgG against infection is attributed to these immunospecific modes of action. Treatment with HSA failed to either improve survival or decrease bacterial burden over placebo treatment, demonstrating that local IgG efficacy is due to specific polyclonal IgG antibody interactions with *P. aeruginosa* and not due to non-specific protein effects. The ELISA-based high IgG titers against the three *P. aeruginosa* strains are consistent with the observed reduction of burden and enhanced survival.

The observed success of this commercial IVIG preparation in enhancing prophylactic survival indicates that specific hyperimmune^{15,16} and monoclonal^{1,35} sera produced against gram-negative exo- and endotoxins may not be required for prophylactic efficacy. The observed decline of IgG therapeutic efficacy post-infection suggests that these alternative sera may prove useful for improving titers or efficacy for this late therapeutic condition.³² Higher survival rates of IgG groups against the lethal IFO-3455 strain in outbred cohorts of CF-1, CD-1 and CFW mice (Figure 2) together with the significantly increased survival of IgG-treated CF-1 mice against the M-2, MSRI-7072 and IFO-3455 pathogen strains (Figure 3), show that IgG efficacy is not strain dependent in either bacteria or mice. Differences observed in the survival of the three different mouse strains against IFO-3455 challenge (Figure 2) are not readily explained. All strains are outbred genetically, supporting some statistical variance in their immune responses. Otherwise, all strains are white albino breeds, with CD-1 and CFW strains originating overseas (e.g., Switzerland).

Abundant peritoneal macrophages and opsonins, including IgG and complement, are major endogenous constituents of the host's immune defense against peritoneal infection.^{20,22,23} Macrophages and neutrophils are chemotactically attracted to bacterial endotoxins and are signalled by cytokines. Therefore, the prophylactic presence of specific IgG pools should benefit the host against *P. aeruginosa* infections and peritonitis in general. Measurable IgG titers reflect extensive and rapid IgG binding to *P. aeruginosa* epitopes, limiting *P. aeruginosa* motility, sterically hindering peritoneal epithelial attachment, and enhancing phagocytic clearance. The data in Figure 8 support IgG-enhanced killing in peritoneal lavage isolates as a result of increased opsonic activity and bacterial opsonization by peritoneally applied exogenous human IgG. Locally administered IgG alone confers on the mouse the ability to survive infection by otherwise lethal bacterial challenges from the three *P. aeruginosa* strains.

Preventative (prophylactic) antibiotics are most effective against infection when therapeutic tissue concentrations are present at the time of bacterial contamination; antibiotic effectiveness is lost when administered three hours after tissue pathogen contamination.⁴¹ In this study, locally applied IgG was most beneficial as a prophylaxis when given prior to and simultaneously with bacterial challenge (Figure 4). This effect coincides with the detected rapid clearance of intraperitoneally administered IgG into mouse systemic circulation. That is, protection against infection appears to be a combination of IgG-mediated effects both locally and systemically. Figure 5 shows that locally delivered IgG is taken up systemically within 3 hours of injection into the peritoneal cavity. This result is consistent with extensive perfusion of the peritoneum and the use of intraperitoneal injection as an established method for giving systemic anesthetics to mice. Hence, a significant fraction of human IgG given locally is rapidly systemically available. Nonetheless, data from Figure 8 show that the fraction of human IgG still present in the peritoneal cavity maintains a substantial capability to facilitate bacterial clearance. The proliferation of bacteria from the site of initial abdominal infection leads to the infection of other organs, the over-production of endotoxins, the induction of cytokine cascades, the progression to septic shock, and sepsis.⁴⁰ Increases in circulating levels of inflammatory cytokines, including tumor necrosis factor- α ,

interferon- γ , IL-8 and IL-6^{2,4,21,31,47} are clinical indicators of peritonitis.⁴⁰ Reduced circulating IL-6 correlates with decreased host microbial load. Low levels of systemic bacteria detected at 6 hours (Figure 6) and decreased IL-6 levels (Figure 7) in locally IgG-treated groups compared to placebo-treated control groups are consistent with both local and systemic IgG opsonophagocytic activity. Opsonophagocytic data (Figure 8) support continued bacterial clearance in peritoneal lavage fluid containing human IgG, while the bacterial burden increases in this lavage without exogenous IgG. Human IgG is still detectable peritoneally for up to 7 days, with more substantial amounts circulating in blood (Figure 5). Extrapolation of the detected peritoneal human IgG bacterial clearance activity (Figure 8) to longer times in the presence of the remaining peritoneal human IgG (Figure 5) supports possibly prolonged local opsonophagocytic reduction of host bacterial burden along with systemic IgG protection to confer survival.

The data indicate that locally delivered IgG, applied most beneficially as a prophylactic measure, lowers the incidence and severity of infection by reducing the acute bacterial burden and systemically inhibiting sepsis. Because peritonitis is considered a compartmentalized inflammatory process, with much more significant cytokine production locally versus systemically, it has been suggested that anticytokine therapies would be most effectively directed locally at the peritoneal cavity.⁴⁰ The use of locally administered, pooled human IgG is also complementary to current antibiotic therapies. Combined IgG-antibiotic treatments are a potentially useful extension of therapy against infection. Additionally, this strategy is an option for combating bacteria that are resistant or may develop resistance to antibiotics,^{6,12,17,19,43} since IgG functions independently of resistance mechanisms. As a potential clinical prophylactic, pooled human IgG might be applied prior to closure during abdominal surgery as a topical lavage, as a treatment in CAPD dialysate fluid by using targeted delivery vehicles or controlled release strategies (e.g., microspheres, gels, or coatings). Tailored, optimization of IgG local dose and delivery kinetics is an anti-infective strategy that is entirely different from the use of IVIG. Such an alternative approach could suit a variety of infectious complications and clinical needs beyond the scope of peritonitis. Such approaches offer new possibilities for decreasing the risks of post-surgical infection, associated morbidity and for lowering mortality rates.

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A novel spinal implant infection model in rabbits

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Abstract

A new spinal implant model was designed to study device-centered infection with methicillin resistant *Staphylococcus aureus* (MRSA) in multiple non-contiguous surgical sites in the lumbar spine region of a rabbit. Large numbers of recent studies show that postoperative wound infection following spinal implant surgery, and the rise in antibiotic resistant bacteria, are a concern. Anti-infective strategies must be tested in relevant animal models that will lead to appropriate clinical studies. Eight anesthetized New Zealand white rabbits underwent completely isolated partial laminectomies and subsequent stainless steel K-wire implantations directly into the transverse processes of vertebrae Th13, L3 and L6. The middle sites (L3) were used as sterile control sites while the outer sites (Th13, L6) were challenged with different amounts of MRSA. Rabbits were sacrificed after seven days and biopsied to provide evidence for device-centered infection. Bacterial growth on the implant surfaces and in surrounding tissues and bone was assayed. Overall device-centered infection was established after seven days in 100% of the sites challenged with 10^3 CFU MRSA or higher. No infection was seen in any of the control sites located between infected vertebrae. Multiple blood and liver samples showed that the separate localized infections did not systemize after seven days. This new animal model demonstrates that multiple biomaterial implants can be evaluated in the same animal and provides a technique for investigating postoperative device-centered infection of the spine. Infection was demonstrated in non-contiguous lumbar sites of the spine, while adjacent control sites remained sterile. Since there was no cross-contamination or systemic spread of the infection, multiple anti-infective strategies or implant materials can now be tested for efficacy in a single animal to combat dramatic and costly postoperative implant infections.

Infection is a known and problematic complication in posterior or posterolateral reconstructive spine surgery. The introduction of spinal instrumentation in the 1960's increased the incidence of postoperative spinal infection^{14,16,22} and, although modern spinal surgical techniques have decreased the incidence of this complication, postoperative spinal implant infection still occurs at a significant rate. The average incidence under antibiotic prophylaxis can be 0.1 percent but is reported to be 8.2

percent and higher for complicated cases, depending on patient- and procedure-related risk factors.^{4,7,9,13,15,17,24,25} Geriatric, immunocompromised, diabetic and obese patients all have greater risks of infection.^{1,7,17,25} The most common organism isolated from postoperative spinal wound infections is *Staphylococcus aureus*.^{6,7,15,16,17,25}

Foreign bodies, dead space, devitalized or even necrotic tissue all contribute to a decrease in the body's ability to eliminate bacteria that inevitably end up inside the wound after long surgical procedures.²¹ Many pathogenic bacteria require adhesion-dependent colonization to establish infection. The formation of a surface-adherent and protective 'biofilm' is characteristic of a mature infection, particularly on implanted devices. Once established, this biofilm is very difficult to eradicate despite the use of antibiotics that are highly active in standard *in vitro* susceptibility tests.^{10,11,20} In addition, the unfortunate emergence of methicillin and vancomycin resistant pathogen strains and of a large immunocompromised patient population add significant magnitude to the surgical infection problem.^{8,10,23}

Apart from patient discomfort, the cost of treating spinal implant infections ranges from \$6,000 to \$900,000 per case, and in approximately 50% of these cases, operative intervention is required.^{9,15,19} Prevention of postoperative wound infection (prophylaxis) is therefore the first line of defense in the battle against rising healthcare costs^{4,7} with antibiotic prophylaxis as current routine.²¹

In order to direct new anti-infective therapies to spinal implant surgery, more appropriate *in vivo* animal models are required. Many animal models have been designed to study artificial joint infection or osteomyelitis in long bone, but none of these models accurately represent the local environment of current postoperative wound infections after spinal implant surgery.^{2,3,5,18} The model described herein is based on the separate implantation of three biomaterials in isolated laminectomy defect sites in the lumbar spine of a single rabbit, mimicking aspects of posterior spinal instrumentation used in lumbar fusion surgery. Individual sites were intentionally challenged with different amounts of methicillin resistant *Staphylococcus aureus* to establish a consistent, localized device-centered infection.

Materials and Methods

Animals. After approval of all protocols by the institutional animal care and use committee, eight New Zealand white (NZW) female rabbits were obtained, weighing 2.5 – 3.0 kg each.

Bacterial inoculum. One day prior to surgery, methicillin resistant *Staphylococcus aureus* (MRSA, ATCC 33593) was suspended in 20 ml trypticase soy broth and incubated at 37°C, shaking at 150 RPM. After 18 hours, the culture was two times diluted in sterile saline and centrifuged (8,000 RPM) for 10 minutes. Final concentrations of bacteria were obtained by making different dilutions in sterile saline. The final bacterial concentration (Colony Forming Units (CFU) per milliliter) was estimated using a spectrophotometric assay and determined by plating on Trypticase Soy Agar (TSA).

Surgical procedure. The entire back and major parts of the rabbit gluteal region were thoroughly shaved one day prior to the surgical procedure. The animals were fasted 12 hours before premedication with butorphanol tartrate (0.1 mg/kg) and injected intramuscularly with an anaesthetic cocktail comprising ketamine HCl (44 mg/kg), xylazine (5 mg/kg) and acepromazine maleate (0.75 mg/kg) thirty minutes later. The positions of the desired vertebrae, thoracic 13 (*Th13*), lumbar 3 (*L3*) and lumbar 6 (*L6*) were marked on the back of the animal, and 0.5 - 1.0 ml of antibiotic-free Marcaine® 0.5% was injected subcutaneously at every site as local anaesthesia. After preparing the back with povidone-iodine, sterile drapes were used to cover the entire animal, leaving a circular area open (5 cm diameter) above the desired vertebral surgical site.

A 2.5 cm dorsal skin incision was made longitudinally in the midline, followed by a single incision in the fascia to expose the spinous process (Figure 1a). Using a small rongeur, the entire spinous process, with surrounding musculature and ligaments, was excised from the base (weighing 0.08 – 0.10 g.), creating a hollow self-contained defect, mimicking a partial laminectomy defect (Figure 1b). The ligamentum flavum was not violated, and the dura was not exposed.

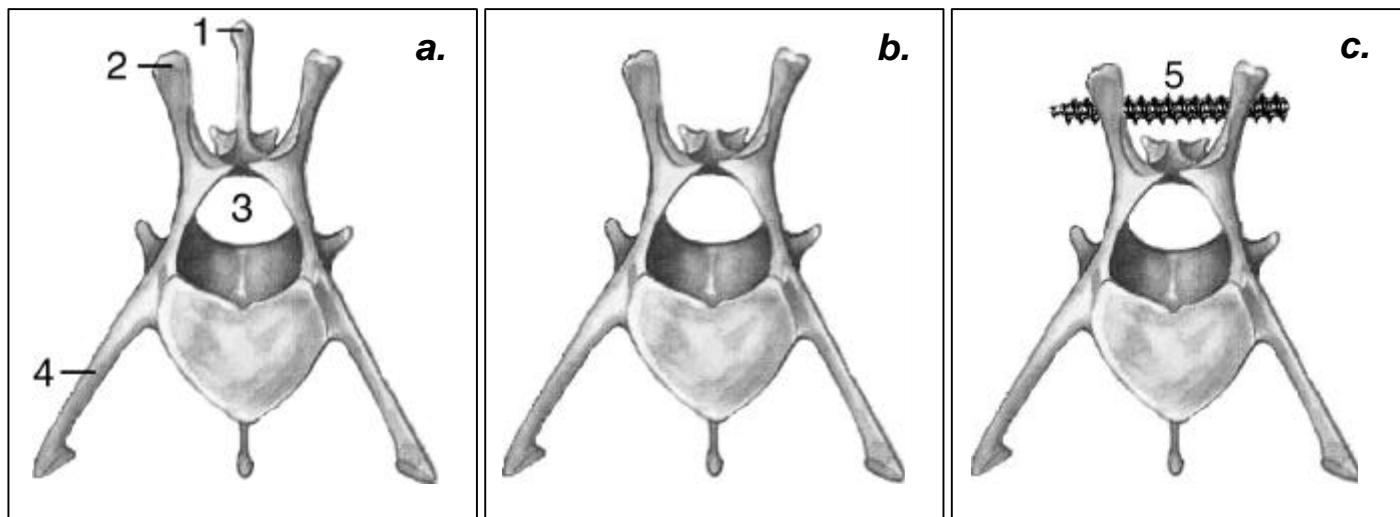


Figure 1:

- a. Cranial view on intact rabbit lumbar vertebra
(1. Spinous process; 2. Transverse process; 3. Spinal canal; 4. Costal process),
- b. After creating the laminectomy defect,
- c. After implantation of the stainless steel K-wire (5).

From the left side of the animal, a 0.85 mm diameter stainless steel threaded Kirschner wire (generously donated by Smith&Nephew-Richards, ASTM F138) was screwed through both transverse processes, crossing the laminectomy defect, and cut adjacent to the lateral wall of the left transverse process at the appropriate length (Figure 1c). Bacterial inoculum or sterile isotonic solution (both volumes: 100 µl) was squirted from a sterile syringe needle (30G) onto the implant and inside the defect pocket. The fascia was closed tightly by running sutures with braided, biodegradable Vicryl™ 2/0. The skin was closed with interrupted Ethilon nylon™ 2/0 sutures.

Immediately after uncovering the animal, the back was again prepared with povidone-iodine and covered with new sterile cloth. Using a new set of sterile instruments, the second implantation was performed at the next randomly selected vertebra, with approximately 4 cm separating adjacent incisions. The same procedure was repeated at the third vertebral site. The closed wounds were left uncovered to prevent bandage irritation of the skin.

To prevent unwanted contamination, the sterile control site was consistently operated on first, and positioned deliberately (L3) between the two inoculated sites (Th13 and L6) to investigate the possibility for any cross-contamination. Variability was minimized and using the same surgeon (KP) to perform all operations standardized surgical trauma. After the procedure, the animals were housed individually in standard cages, and provided with water and standard antibiotic-free rabbit chow. The animals were monitored daily, particularly with regard to their wounds, temperature, signs of sepsis and body weight.

Evaluation. According to the approved protocol, the animals were sacrificed after seven days, using an intravenous injection of pentobarbital (10mg/kg). Prior to euthanasia, blood was drawn from the ear vein of each animal, and cultured to determine systemic sepsis. Under sterile conditions, biopsies of the skin (suture area), the fascia and muscle (suture area), the vertebral lamina, the K-wire implants and both transverse processes were removed from all sites. Also, a piece of the right liver lobe (approximately 5 grams) was removed to monitor systemic infection. Harvested tissue samples were immediately homogenized (Omni-International GLH homogenizer) and implants were sonicated (NEY Ultrasonik 100) for 30 minutes to detach bacteria. Serial dilutions were plated on TSA and incubated for 24 hours at 37°C. Finally, the CFU burden was determined per gram tissue sample to enumerate bacterial amounts at every site. Biomaterial-centered infection was arbitrarily defined to occur at sites where MRSA was present on the K-wire, and a positive bacterial finding was evident in two or more tissue samples from that same site.

Results

Procedure, dose and observation time. No problems with either the anaesthetic or the surgical procedures were observed during the evaluation of these eight rabbits. The data for all eight rabbits are combined in Table 1. Blood and liver sample cultures were consistently sterile for all rabbits after 7 days, indicating that the separate localized infections did not produce systemic sepsis. Vital functions (weight, temperature, food and water intake) supported the observation of lack of systemic spread of infection.

Infection analysis. When a surgical implantation site was infected, bacterial growth was consistently found in all tissue samples from that particular site. Bacterial growth localized, for example, only on the K-wire implant or the bone, was never observed. In infected sites containing pus in the muscle area and in the defect, a translucent film covered the metal implant surface. Other than MRSA, no other bacterial growth was seen on any of the TSA culture plates post-harvest.

Evaluation. The eight animals were divided in two groups of two, and one group of four animals, respectively. In the two animals from Group 1, bacterial amounts of 10^4 and 10^5 CFU MRSA were randomly applied as local inocula in the spinal 'bacterial-sites' (Th13, L6). Subsequent bacterial growth in these sites created consistently large amounts of debris and pus, indicating high levels of infection. Post-mortem quantification of bacteria in these sites, shown in Table 1, indeed revealed extremely high bacterial burdens. No evidence for infection was, however, observed in the 'sterile control sites' (L3) located in between these contaminated sites (Th13, L6), and no bacteria were detected in liver or blood samples. Hence, all three implant sites (Th13, L3 and L6) were considered to have isolated, localized infection only. Subsequently, the next two rabbits (Group 2) were used to determine optimal inocula doses for this model using randomly applied 10^2 and 10^3 CFU MRSA.

As shown in Table 1, the lower dose (10^2 CFU MRSA) did not cause a consistent infection in these two animals after seven days. The higher dose (10^3 CFU MRSA)

produced a consistent localized infection as evidenced by pus, debris and post-mortem pathogen quantification. No evidence of systemic sepsis was found in liver or blood samples. We chose therefore to continue with a bacterial challenge of 10^3 CFU MRSA in the next four rabbits (Group 3). A sterile control site (L3) located between Th13 and L6 was again included to confirm that three completely isolated test sites could be established in a single animal spine without causing cross-contamination.

The overall infection rate for 'positive control sites', as calculated from the data in Table 1, was 89% (16:2) including the ineffective inoculum of 10^2 CFU MRSA. When only 10^3 CFU challenges in Group 3 are considered, a 100% infection rate after seven days was achieved. All control-sites (L3), lacking inoculum, remained sterile and post-mortem analysis of blood and liver samples showed no signs of systemic sepsis after seven days in all animals. Data in Table 1 for Group 3 animals show that biopsied tissue samples from the different infected sites post-mortem contained on average $\sim 1.8 \times 10^6$ CFU MRSA/gram tissue. No differences (t-test) were found between averaged CFU values post-mortem between the different spine sites (Th13, L6).

Discussion

Antibiotic resistant, biofilm-forming infection is a dramatic and costly complication of surgical procedures and limits the use of implanted biomaterial devices.^{4,17} Host defenses and administered antibiotics are often only partially effective against biomaterial-centered spinal infections, with complications sometimes mandating complete removal of the device. Rates for spinal infection increase up to three times when metallic spinal implants are used.^{12,25}

Many animal models have been designed to date to study surgical infection, but none link a spinal implant infection with the ability to test specific preventative treatments. Soft tissue damage and dead space areas characteristics of posterior spinal implant surgery are now combined as factors in a single model.

Table 1 Spinal Implant Infection Model Experimental Summary

Implantation site, CFU challenge, Operating time per site and CFU post-mortem per gram tissue, after 7 days implantation.

Animal	Spinal Implantation Site									
	Th13			L3			L6			post-mortem ^c CFU MRSA
	Inoculation ^a CFU MRSA (min)	time ^b post-mortem ^c	CFU MRSA	Inoculation ^a CFU MRSA (min)	time ^b post-mortem ^c	CFU MRSA	Inoculation ^a CFU MRSA (min)	time ^b post-mortem ^c	CFU MRSA	
Group 1										
1	10^4	12	5.0×10^7	None	11	Sterile	10^5	11	1.6×10^7	
2	10^5	11	6.3×10^6	None	13	Sterile	10^4	12	5.0×10^7	
Group 2										
3	10^2	11	sterile	10^3	9	7.9×10^5	10^2	7	sterile	
4	10^2	13	1.6×10^6	10^3	9	3.2×10^5	10^3	9	1.0×10^6	
Group 3										
5	10^3	11	1.2×10^6	None	9	Sterile	10^3	11	6.3×10^6	
6	10^3	12	1.0×10^6	None	9	Sterile	10^3	10	1.6×10^6	
7	10^3	9	3.1×10^6	None	11	Sterile	10^3	14	5.0×10^5	
8	10^3	10	2.0×10^5	None	10	Sterile	10^3	9	2.5×10^5	
Mean (Group 3)^d										
	10.30min		1.4×10^6			9.45min	Sterile	11.00min	2.2×10^6	

A. Total CFU MRSA applied to each site at the time of surgery (in 100 µl aqueous carrier).

B. Surgical time per site (min).

C. Average CFU determined from biopsied tissue samples and the harvested implant (CFU/g sample).

D. Average CFU values and operating time showed no significant differences between the infected spinal implant sites.

Dead space areas such as laminectomy defects tend to fill up with blood, providing an ideal medium for pathogens to multiply. In the presence of a foreign body, the risk of long-lasting antibiotic resistant infections in spinal surgery is high. Results from this three-site biomaterial-centered infection model in New Zealand white rabbits successfully demonstrate the utility of this novel animal infection model for use in spinal surgery research.

Multiple surgical sites remain isolated, despite substantial bacterial inoculation, indicating that cross-contamination is prevented. Lack of detectable systemic sepsis also provides evidence that sterile site contamination cannot occur via this route. The sterile sites in Groups 1 and 3 were operated on first by design to prevent inadvertent spread of pathogens from surfaces of inoculated sites to the sterile wounds. No correlation was found between the surgical site sequence and the post-mortem bacterial burden between Th13 and L6. Additionally, no correlation between the time the animal was under anesthesia and the CFU values found post-mortem in randomly operated infected sites could be detected. It is arguable whether host response to infection is different in this multi-site model over one in which only one site is challenged. However, the intrinsic advantage of comparing different sites within the same animal with the observed lack of site communication or systemic complications is highly significant because the localized inflammatory reactions observed at each site in one animal are then directly comparable. This design decreases the statistical variance and therewith the number of animals required in a given study, compared to one-site models.

Methicillin resistant *Staphylococcus aureus* (ATCC 33593) demonstrated a consistent capability to establish a device-centered infection after seven days in this model when inocula exceeded 10^2 CFU/site. Infection efficiency was targeted by design at 100% in inoculated sites in order to study cross-contamination and systemic spread of the infection. Consistent infection might well be feasible using lower doses of MRSA, but infection using 10^2 CFU/site was unreliable. Greater clinical relevance would be achieved using low doses of inoculum and monitoring progression of infection over longer time frames.

Future research using this multi-site biomaterial-centered model will focus on testing different pathogens and varying bacterial burdens in single animals with and without the use of a biomaterial implants. Different implanted biomaterials (metals, composites, polymers and tissue-engineered scaffolds) will also be analyzed for susceptibility to spinal infection, in tandem with application of systemic or local antibiotics and lavages to prevent and clear device-centered infections.

Further research is required to prevent the devastating and costly complications resulting from postoperative wound infections. This model provides an experimental vehicle to move forward with this testing. In the presence of implanted biomaterials and antibiotic resistant bacteria, routine use of antibiotics no longer protects against infection. In addition to improved surgical techniques and antibiotic prophylaxis, new methods to reduce device-centered infection must be studied in animal models relevant to clinical practice. Future work will consider these exact experimental scenarios using this model.

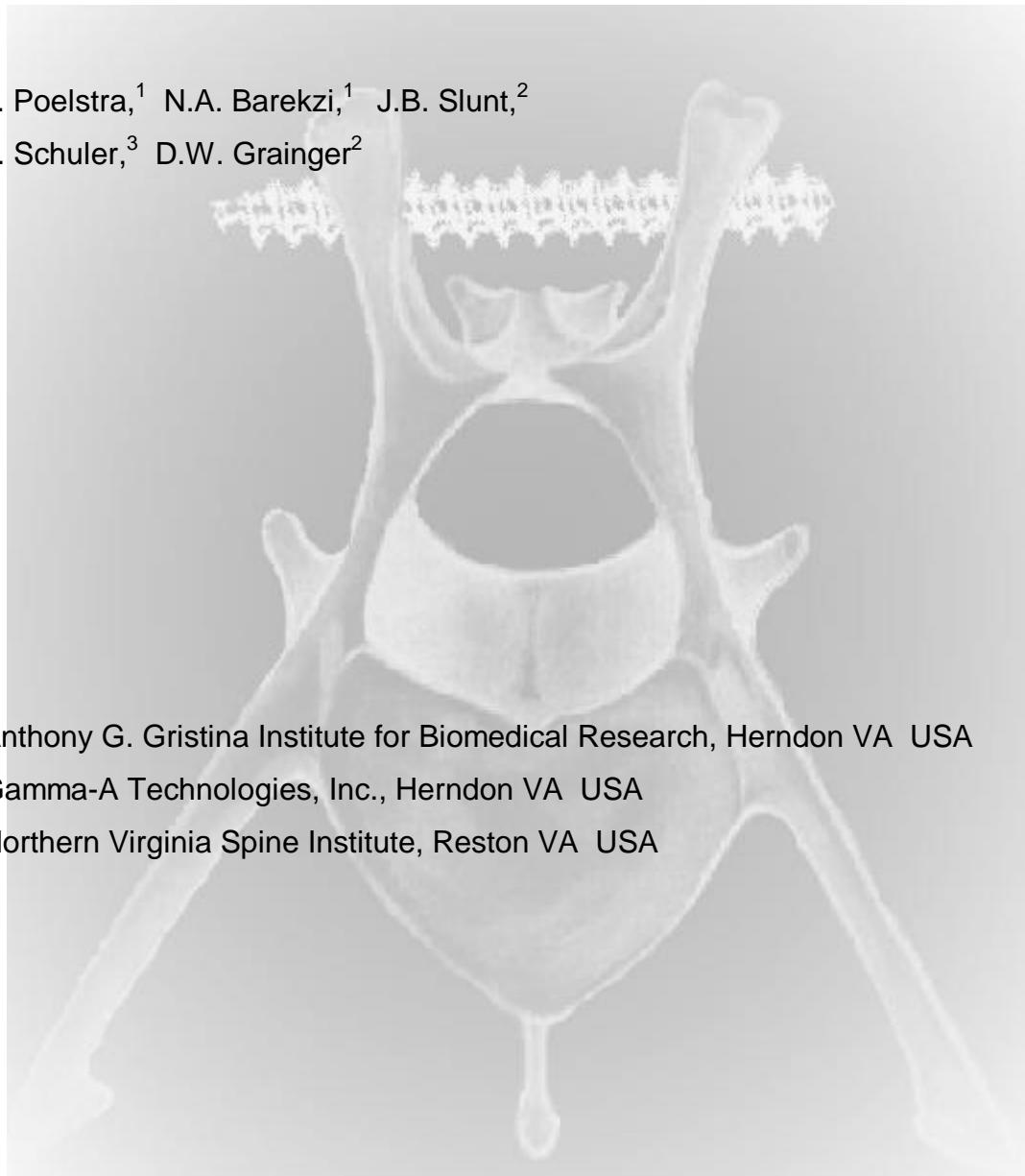
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Surgical irrigation with pooled human IgG to reduce post-operative spinal implant infection

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Abstract

A multiple site, non-lethal rabbit surgical model of spinal implant infection was used to assess the efficacy of a spinal wound lavage to reduce post-operative infection from methicillin resistant *Staphylococcus aureus* (MRSA). Multiple aqueous lavages of isotonic saline were compared to the same procedure using 1wt% pooled human immunoglobulin G (IgG) applied directly to the surgical implant sites. Visually observed clinically relevant signs of infection (e.g, swelling, erythema, pus) were supported by bacterial enumeration from multiple biopsied tissue and bone sites post-mortem, 7- and 28-days post-challenge. Clinical signs of infection were significantly reduced in IgG-lavaged infected spinal sites. Bacterial enumeration also exhibited statistically significant reductions in soft tissues, bone and on K-wire spinal implants using IgG lavage compared with saline. Complete healing of all surgical wounds was seen after 28 days, although isolated fibrosed abscesses were observed in autopsied sites treated with both IgG- and saline-lavages. Local use of IgG wound lavage is proposed as infection prophylaxis against antibiotic resistant implant-centered or surgical wound infection.

New innovations in spinal instrumentation have brought major advantages for improving treatment of a large number of spinal pathologies. Stabilization of the spine permits more rapid mobilization of the patient, facilitates more reliable and effective correction of deformities and maintenance and reconstruction of the spine after decompressive surgery. Hardware failure and neurologic injury caused by the introduction of stabilizing equipment are relatively rare, but risk of postoperative infection clearly increases after implantation of spinal instrumentation. Depending on the patient population, procedure-specific incidences of infection after application of posterior instrumentation vary from 0.1 to 12%, with *Staphylococcus aureus* as the most common wound site pathogen.¹⁻¹⁰ These infections carry a high morbidity for the patient and are extremely costly due to requirements of prolonged hospital stay, surgical re-intervention and aggressive antibiotic therapy.^{1,5,11,12} Unfortunately, many wound-site pathogens have become resistant to first-line antibiotics. According to the National Nosocomial Surveillance

Systems, methicillin resistance is present in 80% of all *S. aureus* isolates (MRSA).¹³ Most recently, vancomycin resistant MRSA emergence has been reported.^{14,15}

One routine surgical prophylaxis attempting to reduce this potentially devastating postoperative complication involves intra-operative wound irrigation with 0.85% saline together with routine systemic antibiotic infusion.¹⁶ Various other anti-infecting irrigation solutions (e.g., containing antibiotics) investigated recently in an attempt to decrease infection rates have met with inconsistent results.¹⁷⁻²¹ Topical application of antibiotics carries associated risk of promoting antibiotic resistance at the site of application as this strategy often results in sublethal antibiotic doses in the site after wound closure.²²

One possible alternative for conferring protection against infection at wound sites while avoiding antibiotic resistance involves local potentiation of host immune responses using natural immune components. Manipulation of inflammatory, immunomodulatory and healing cascades in wound sites has not yet been explored in detail. Passive local immunotherapy, involving the direct application of pooled immunoglobulins (e.g., IgG, IgA, IgM), has been recognized for decades as a strategy to locally and rapidly bolster host immune response to confer anti-infective protection.²³⁻²⁷ Application of a local bolus dose of IgG to sites of potential infection enhances opsonization of pathogens, facilitates neutralization, and promotes clearance by recruited invading neutrophils and macrophages.²⁵⁻²⁷ Local, direct immunotherapy will also be effective against antibiotic resistant bacteria, since acquisition of pathogen antibiotic resistance does not change their susceptibility to opsonization and phagocytosis.²⁸ This strategy, however, has not been investigated as a topical wound lavage to prevent postoperative spinal implant infection.

The aim of this study was to compare the effectiveness of routine surgical saline lavage with a lavage solution containing 1wt% pooled human IgG in reducing postoperative surgical wound infection in the absence of any systemic antibiotics. The approach utilized a recently reported spinal implant infection model in rabbits that has been shown to produce a reliable, localized spinal implant infection using MRSA.²⁹

Materials and Methods

Animals. After approval of all protocols by the institutional animal care and use committee, twelve New Zealand white (NZW) female rabbits were obtained, weighing 2.5 – 3.0 kg each. Rabbits were allowed one week of routine care and feeding to acclimate prior to surgery.

Bacterial inoculum. Methicillin resistant *Staphylococcus aureus* (MRSA, ATCC 33593) was prepared in TSB (BBL® Tryptic Soy Broth USP, Becton Dickinson, Cockeysville, MD 21030, USA, Lot H8DFLS) and washed by centrifugation in isotonic saline just prior to surgery. Inoculum concentrations of 5×10^2 - 1×10^4 Colony Forming Units (CFU) per 100 μ l were adjusted using a standard spectrophotometric assay and confirmed by direct bacterial enumeration on Tryptic Soy Agar (TSA, Difco - Becton Dickinson, Sparks MD 21152, USA, Lot 128882XA).

Immunoglobulin and saline lavage preparation. Pooled human immunoglobulin (Gammimune® N, 10%, Bayer Corporation, Pharmaceutical Division, Elkhart, IN 46515, USA, Lot 648R009B) was diluted in sterile glycine according to recommendations from the manufacturer to provide a 1% IgG solution (10mg IgG/ml). This solution (3ml) was stored in sterile syringes with 30G needles for use in the animal model. Control lavage of 0.85% saline was drawn into identical syringes with 30G needles. The use of saline or IgG as a lavage treatment was blinded and randomized, with two IgG- and one saline-lavaged surgical site per rabbit as described below.

In vitro IgG-MRSA titer determination. An anti-human IgG ELISA^{23,24} was used to determine polyclonal human IgG titers against MRSA strain ATCC 33593. The ELISA was modified to detect gram positive MRSA by using 3% rabbit serum as a blocking agent. Titer numbers express the inverse log dilution of IgG concentration at 50% ELISA optical absorbance (450nm) from the inflection mid-point on each IgG-MRSA binding curve. A higher titer number reflects higher IgG binding to MRSA.

Surgical model. The non-lethal rabbit spinal implant surgical infection model has been described recently in detail.^{29, this thesis} The following represents a brief description of the implantation and lavage procedure. Each rabbit was premedicated with butorphanol tartrate (0.1 mg/kg) and anaesthetized via intramuscular cocktail comprising ketamine HCl (44 mg/kg), xylazine (5 mg/kg) and acepromazine maleate (0.75 mg/kg) thirty minutes later. The positions of the desired vertebrae --thoracic 13 (*Th13*), lumbar 3 (*L3*) and lumbar 6 (*L6*) -- were marked on the back of the animal, and 0.5 - 1.0 ml of antibiotic-free Marcaine® 0.5% was injected subcutaneously at every site as local anaesthetic. After preparing the shaved animal with povidone-iodine, a 2.5 cm dorsal skin incision was made longitudinally in the midline on top of each desired vertebra, followed by an incision into the fascia to expose the spinous process. Using a small rongeur, the entire spinous process with surrounding musculature and ligaments, was excised from the base, creating a hollow self-contained defect, mimicking a partial laminectomy defect. The ligamentum flavum was not violated, nor was the dura exposed. At this time, 1ml of either IgG or saline (selected blind, randomized) was used to lavage the pocket and sponged out immediately using sterile gauze.

Subsequently, a 0.85 mm diameter stainless steel threaded Kirschner wire (ASTM F138, donated by Smith&Nephew-Richards, Memphis, TN 38116, USA) was screwed through both vertebral transverse processes, crossing the laminectomy defect, and cut adjacent to the lateral wall of the left transverse process at the appropriate length. Again, 1ml of either IgG or saline (blinded) was used to lavage the site and sponged out using sterile gauze. In implant sites, bacterial inoculum in 100 µl saline was then applied from a sterile syringe needle (30G) onto both the K-wire implant and the tissue bed inside the defect pocket and left for exactly 60 seconds. Subsequently, the last blinded 1ml lavage of either IgG or saline was performed and sponged out. The wound was closed in multiple sutured layers using Vicryl® and silk (donated by Ethicon Inc., Somerville, NJ 08876, USA).²⁹

On the same rabbit, the next two spinal sites were laminectomized and inoculated in random order, following the same protocol and using new sterile equipment. The

MRSA-infected sites were proven to be reliably locally infected, isolated, non-communicative and non-contiguous in earlier control experiments, even when local inoculations exceeded 10^5 CFU MSRA.²⁹ All three spinal surgical sites were therefore inoculated in this model and used to compare the 1wt% IgG lavage solution with the standard saline surgical lavage solution. Variability was minimized and surgical trauma was standardized by using the same surgeon (KP) to perform all operations. After the procedure, the closed wounds were left uncovered to prevent bandage irritation of the skin. The animals were housed individually in standard cages, provided with water and standard antibiotic-free rabbit chow and monitored daily with special regard to their wounds, temperature, signs of sepsis and body weight.²⁹

In another series of rabbits, the same surgical procedure was performed as described above, but no K-wire was implanted. To establish consistent infections in this case without a biomaterial implant, spinal sites were inoculated with 500, 1,000, 5,000 or 10,000 CFU MRSA and lavaged following the same blinded protocol described above.

Evaluation. According to approved animal protocols, rabbits were sacrificed by intravenous injection of pentobarbital (10mg/kg) after either 7- or 28-days post-surgery. Prior to euthanasia, blood was drawn from the ear vein and cultured to determine systemic sepsis. Still blinded for the applied lavage fluid, the surgical sites were individually visually evaluated for clinical signs of infection: swelling, erythema, pus. Different areas were rated by the same surgeon that performed the procedures (- : no signs; + : moderate signs; ++ : severe clinical signs of infection). Under sterile conditions, biopsies of the skin (suture area), the fascia and muscle (suture area), the vertebral lamina, the soft tissue that surrounded the implant in the defect (fibrous tissue), the K-wire implants and both transverse processes were removed from all sites. Additionally, a piece of the right liver lobe (approximately 5 g) was removed to assess systemic presence of MRSA infection. Harvested tissue samples were immediately homogenized (Omni-International GLH homogenizer) and implants were sonicated (NEY Ultrasonik 100) for 30 minutes in saline to detach bacteria. Serial dilutions of each sample were plated on TSA and incubated for 24 hours at 37°C to enumerate

pathogens at every site. Randomly, different samples were also plated on TSA containing 5 μ g/ml nafcillin (Sigma Chemical, St Louis MO 63178, USA, Lot 66H0044) to determine if indeed MRSA was cultured in the tissues. Data analysis was performed using a statistical package component of Microsoft® Excel97 for Windows.

Results

No animal morbidity or mortality problems were encountered with either the anesthesia or surgical procedure. All animals demonstrated a small rise in temperature one day post-surgery that renormalized quickly. After completing the entire process of harvesting and enumerating bacteria in all the sites post-mortem, the identity of the surgical lavage fluids used in the different sites per rabbit was revealed.

Table 1 lists data acquired for the 12 animals in the 7-day short-term study for MRSA burdens at various sites following each lavage treatment and clinical signs of infection observed per site per animal. The difference in visually observed clinical signs of infection at 7 days between the different lavaged sites was calculated following a (subjective) numerical representation of (-)=0, (+)=1, (++)=2. A statistical T-test analysis between saline- (avg. value = 1.50) and IgG-lavaged (avg. value = 0.83) sites resulted in a p-value of 0.005 in favor of the IgG lavage treatment providing improved visual signs of wound healing.

Figure 1 graphically compares log values (CFU/g wet tissue) of MRSA enumeration from tissue biopsies of saline- and IgG-lavaged surgical sites after seven days. Differences between bacterial enumeration after the two different lavage treatments summed over all tissues is statistically significant (IgG log CFU = 6.56, saline log CFU = 6.94, t-test, p=0.02), indicating that IgG-lavage reduces MRSA growth in surgical sites. Statistics improve for IgG-treated sites when directly quantified biopsied MRSA colony numbers are compared, although the difference from saline controls is within the same log value (IgG = 3.65×10^6 vs. saline = 8.80×10^6 , p-value 0.01, student's t-test).

Rabbit	Site	Lavage	Clinical Score
1	Th13	Saline	++
	L3	IgG	-
	L6	IgG	-
2	Th13	IgG	-
	L3	IgG	+
	L6	Saline	++
3	Th13	Saline	++
	L3	IgG	+
	L6	IgG	-
4	Th13	IgG	++
	L3	Saline	+
	L6	IgG	+
5	Th13	IgG	++
	L3	Saline	+
	L6	IgG	+
6	Th13	IgG	+
	L3	IgG	+
	L6	Saline	++
7	Th13	IgG	++
	L3	IgG	-
	L6	Saline	+
8	Th13	Saline	++
	L3	IgG	-
	L6	IgG	-
9	Th13	IgG	++
	L3	Saline	+
	L6	IgG	+
10	Th13	IgG	+
	L3	IgG	+
	L6	Saline	+
11	Th13	IgG	++
	L3	IgG	-
	L6	Saline	+
12	Th13	Saline	++
	L3	IgG	-
	L6	IgG	+

Table 1:

Directly observed clinical description of MRSA-inoculated spinal implant sites after days: 12 rabbits, 36 implant sites, lavage fluid used per site and clinically observed infection score after 7 days. No sign of infection (-), moderate infection (+) and severe infection (++).

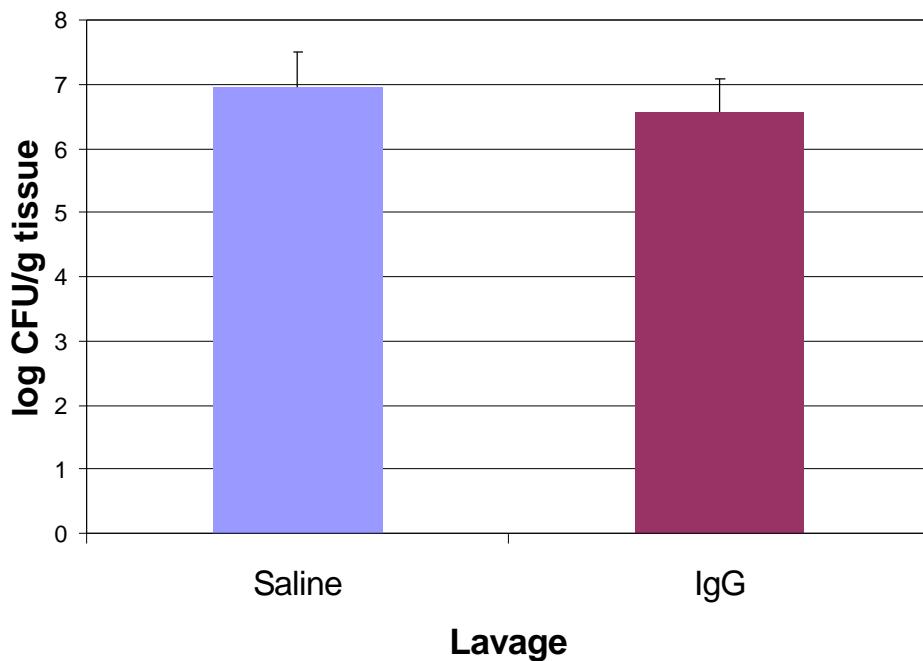
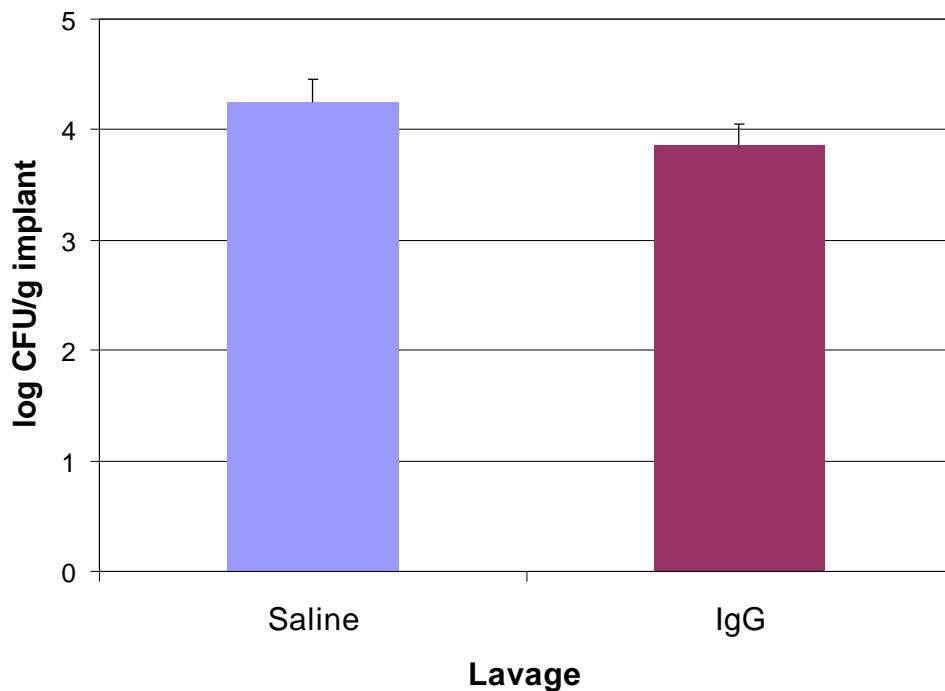


Figure 1:

Enumeration of methicillin resistant *Staphylococcus aureus* ATCC 33593 cultured and collectively summed from all tissue biopsies in the saline- versus IgG-lavaged spinal implant sites (mean CFU value per gram tissue) 7 days post-infection. P-value for the difference is 0.02 (student's t-test).

Selective bacterial enumeration from the implanted K-wires alone, calculated per gram implant, lavaged with either IgG or saline, is presented in Figure 2. Differences are close to significance (IgG log CFU = 3.86, saline log CFU = 4.25, t-test, p=0.07), with the mean MRSA count post-harvest being reduced in the IgG-lavaged sites seven days post-surgery. Statistics do not improve when enumerated cultured CFU values are compared (IgG = 7.25×10^3 vs. saline = 1.76×10^4 , p-value 0.07, student's t-test).

Significantly, eleven implants of the 24 IgG-lavaged sites had no countable bacteria (46%), while only three of the 12 saline-lavaged sites (25%) presented no culturable bacterial colonies. This correlated with the lack of clinical signs of infections described in Table 1. If non-colonized implants in these two groups are excluded from the comparison, MRSA enumeration for 13 IgG-lavaged implants and 9 saline-lavaged implants are 2.19×10^4 and 2.34×10^4 , respectively (no significant difference).

**Figure 2:**

*Enumeration of methicillin resistant *Staphylococcus aureus* ATCC 33593 detached and cultured from the stainless steel K-wire spinal implants from the saline- versus IgG-lavaged spinal implant sites after 30 minute sonication (mean CFU value per gram implant) 7 days post-infection. P-value for the difference is 0.07 (student's t-test).*

Table 2 presents the data from four rabbits in the 28-day long-term study, including the MRSA CFU values enumerated after plating both the tissue biopsies and harvested implants on TSA. In this long-term study, the center surgical site was left unchallenged with MRSA (sham site) to prevent any direct cross-over contamination from the inoculated head (*Th13*) and tail (*L3*) sites over the 28-day time period.

Comparison of bacterial enumeration demonstrated that only the superficial fascia immediately beneath the skin consistently contained countable bacterial colonies, with no evidence for deep MRSA wound infection. The fascia was nearly always associated in these sites with fibrosed, isolated self-contained abscesses containing viable MRSA. Specifically, bacterial enumeration from IgG-lavaged sites (1.12×10^6 CFU/gram tissue, 89% of biopsies exhibit no bacteria) and saline-lavaged sites (5.40×10^4 CFU/gram tissue, 85% of biopsies exhibit no bacteria) show no differences at 28 days. Importantly,

no bacteria were detected on any stainless steel K-wire at the 28-day point: the host consistently cleared the MRSA biomaterial-centered infection regardless of the type of treatment at this point, indicating the non-lethal, localized nature of the acute infection.

Rabbit	Site	Lavage	Clinical Score	Mean CFU/g
1	Th13	IgG	-	5.09E+06
	L3	sham	-	0
	L6	IgG	+	1.54E+06
2	Th13	IgG	-	0
	L3	sham	-	0
	L6	IgG	-	0
3	Th13	IgG	-	5.00E+04
	L3	sham	-	0
	L6	Saline	+	1.08E+05
4	Th13	IgG	+	5.17E+04
	L3	sham	-	0
	L6	Saline	-	0

Table 2:

Directly observed clinical description of MRSA-inoculated spinal implant sites after 28 days: 4 rabbits, 12 sites, lavage fluid used per site, clinically observed infection score and mean bacterial enumeration from the fascia immediately beneath the skin after 28 days. No sign of infection (-), moderate infection (+) and severe infection (++).

Consistent with previous studies on biomaterial-centered infections,³⁰⁻³³ surgical sites lacking any K-wire implant required substantially greater MRSA challenge (10^4 CFU MRSA) in order to establish consistent infection in the different spinal sites. Doses of 500, 1,000 and 5,000 CFU MRSA did not produce consistent infections.

This contrasts the results from implanted K-wire sites, where 500 CFU MRSA proved sufficient for reliable infection, even for the bone. Table 3 presents the data for bacterial enumeration after 7 days from four rabbits in this no implant group challenged with 10^4 CFU MRSA. Bacterial enumeration from IgG-lavaged sites was lower (3.06×10^6 CFU/gram tissue, 40% of biopsies contained no bacteria) than that from saline-lavaged sites (7.93×10^6 CFU/gram tissue, 15% of biopsies contained no bacteria) indicating IgG's ability to reduce bacterial burden over the 7-day period ($p= 0.02$, student's t-test) in the absence of implanted biomaterials.

Rabbit	Site	Lavage	Clinical Score	Mean CFU/g
1	Th13	IgG	++	5.33E+06
	L3	Saline	+	1.30E+07
	L6	IgG	+	8.60E+06
2	Th13	Saline	++	5.71E+06
	L3	IgG	++	1.45E+06
	L6	IgG	+	3.46E+06
3	Th13	IgG	++	2.23E+06
	L3	Saline	++	1.99E+06
	L6	IgG	++	1.59E+06
4	Th13	Saline	++	1.11E+07
	L3	IgG	-	4.68E+05
	L6	IgG	-	1.32E+06

Table 3:

Directly observed clinical description of MRSA-inoculated spinal implant sites after 7 days: 4 rabbits, 12 sites, lavage fluid used per site, clinically observed infection score and mean bacterial enumeration from all samples biopsied from different sites after 7 days. No sign of infection (-), moderate infection (+) and severe infection (++).

On all 5µg/ml nafcillin TSA plates selective for MRSA culture, bacterial colony presentation and quantification was no different from that observed on normal TSA plates, meaning that only MRSA was isolated from the infected sites and that no exogenous bacteria contaminated the surgical wounds during surgery. All twenty rabbits from the three experimental groups had no countable bacteria on TSA in 5ml whole blood or 5g liver tissue cultures, signifying that systemic spread of bacteria from the separate localized spinal infections does not occur in this model.²⁹

Discussion

Use of immunoglobulins against infections has a substantial history, particularly when administered systemically.^{25,26,34} Local application of immunoglobulins to various non-surgical sites against specific pathogens has also been reported.²³⁻²⁶ Use of broad spectrum pooled polyclonal IgG confers advantages of wide-ranging specificity and relatively ready commercial availability, while monoclonals tailored against specific virulence factors provide opportunities to improve titers and antimicrobial potency.

In this work, efficacy of a 1wt% IgG wound lavage in reducing bacterial burden in a rabbit spinal implant infection model²⁹ was compared to standard saline surgical lavage fluid. Rabbits received multiple non-contiguous spinal K-wire implantations challenged with 500 CFU methicillin resistant *Staphylococcus aureus* (MRSA in 100 µl saline carrier). Control procedures lacking K-wire implantations required substantial higher inocula doses (10^4 CFU) to establish MRSA infection. MRSA was chosen as a pathogen because of its frequency in postoperative spine infection and the difficulty currently encountered in successfully treating clinical, biomaterial-centered, osteomyelitic, postoperative spinal implant infections. Results indicate that IgG-lavage significantly reduces bacterial burden in spinal wound site tissues and bone adjacent to implants, as well as clinical indicators of infection in these sites. Prophylactic protection that inhibits MRSA tissue and bone colonization is therefore important in preventing MRSA infection in a surgical wound.

A pilot *ex vivo* contamination trial, applying MRSA inocula followed by the aqueous lavage procedure to freshly harvested rabbit gluteal muscle, demonstrated that either blinded lavage fluid used in this comparison (saline or 1wt% IgG) consistently removed ~65% (SD=9%) of the MRSA inoculum from challenged wound sites (results not shown), leaving ~175-200 CFU MRSA inoculum to induce infection in tissue and bone surrounding K-wire implants.

In clinical settings, local levels of endogenous host immunoglobulin typically drop in wound sites following extensive surgical procedures, compromising the host's ability to efficiently opsonize adventitious, contaminating planktonic bacteria to prevent tissue adherence and biofilm modes of pathogen growth. The initial 6 hours post-surgery, or "decisive period", are critical in this process, with the introduced pathogens still quiescent in the so-called lag-phase, or metabolically inactive state.³⁵ This is the optimal window of opportunity for the host immune system to neutralize invading microorganisms, assisted by routinely applied systemic prophylactic antibiotics. Complex spinal surgical procedures are at high risk for postoperative infection, reflected by incidences reported up to 12%¹⁻⁹ and increasingly virulent wound site pathogens are

becoming antibiotic resistant.^{14,15,36} Additionally, the host is often immunocompromised or malnourished (often after both anterior and posterior spine surgery),³⁷ increasing the risk of bacterial colonization, osteomyelitis and wound site biofilm formation that results in devastating postoperative wound infection. Local application of IgG supplements wound site immune defenses to potentially enhance pathogen killing and is indifferent to antibiotic resistant mechanisms in this susceptible phase.

Doughty reviewed topical antiseptics for use in wounds and could only identify Dakin's solution as a useful efficacious antiseptic lavage without cytotoxicity.³⁸ A study on the efficacy of various irrigation solutions for removing an important slime-producing *Staphylococcus* from stainless steel cortical bone screws showed that standard antibiotic-containing solutions for intra-operative irrigation had no significant effect on bacterial removal when compared to saline alone.^{19,22} Others have reported beneficial effects from topical antibiotic application over regular saline, either as a lavage or introduced by different carriers into wound sites.^{17,20,39}

Bacterial quantification results from this non-lethal rabbit spinal surgical model demonstrate a high number of viable MRSA present in nearly all challenged sites, regardless of the lavage treatment. The efficacy of this infection model to consistently produce such high bacterial burden is attributed to the surgical creation of a laminectomy dead-space defect that fills with blood after closure, providing an ideal medium for applied MRSA pathogens to multiply. Additional K-wire implants provide continuous biomaterial-based seeding centers for proliferating MRSA colonies to infect surrounding tissues. Biomaterial implantation is well-documented to both increase infection risk and serve as a preferred colonization site.^{30-33,40-42} In this worst-case dead-space infected implant scenario, the IgG-lavaged sites show significantly improved clinical signs of infection incidence and magnitude compared to the saline controls. Data for animals without K-wire implantations show that increased amounts of MRSA are necessary to cause an infection, and that subsequent IgG-lavage treatment significantly lowered the bacterial burden post-mortem. Data from the long-term 28-day study show that the animals successfully overcome localized infections, regardless of lavage-treatment.

We caution against direct extrapolation of these results to clinical situations due to limitations intrinsic to this model. First, these outbred New Zealand white rabbits appear to be extremely susceptible to inoculation with MRSA ATCC 33593 when compared to other *in vivo* models in which higher 10^5 - 10^7 CFU MRSA burdens establish a consistent infection.^{31,43} These rabbits fail to develop effective phagocytic responses to MRSA even in the presence of high titer sera.⁴⁴ Additionally, this susceptibility seems to be limited to MRSA: the same spinal model employing high inoculum doses ($>10^7$ CFU) of either *Staphylococcus epidermidis* (strain RP-12) or *Enterococcus faecium* (strain UMMC) do not produce any sign of local spinal infection after 7 days (unpublished data). The issue of whether such an apparently vulnerable animal-pathogen pairing accurately assesses new anti-infective therapies remains unanswered. Additionally, no comparisons of efficacy have yet been made with existing, reported antibiotic lavages.^{17,19,38} Amounts of IgG remaining inside the surgical defects over time post-lavage and its half-life are also unknown. Lastly, effectiveness of immunopotentiating agents in poorly vascularized musculoskeletal intravertebral wound sites may be hindered by slow wound site infiltration of phagocytic cells necessary to facilitate bacterial clearance.

Pooled human immunoglobulin did not exhibit overwhelming efficacy alone as a topical wound lavage prophylaxis in this rabbit model to fight postoperative biomaterial-centered surgical wound infection. This is consistent with previous *in vitro* tests of commercial human preparations against MRSA in human blood preparations that failed to demonstrate bactericidal activity.⁴⁴ ELISA-based titer data for this specific human pooled polyclonal preparation used here show substantial IgG binding activity against this MRSA strain (titer = 3912). Opsonization of MRSA in wound sites would be predicted. Opsonophagocytic data for MRSA bacterial clearance *in vitro* using freshly harvested human blood neutrophils (data not shown) indicates that presence of this pooled IgG does not significantly enhance MRSA killing efficacy. Nevertheless, significant reductions in colonization frequency, and wound site tissue and implant-adherent bacteria were noted for local IgG treatment. Additionally, no systemic bacteria were detected either 7- or 28-days post-infection.

Because clinical surgical intervention will continue to routinely employ prophylactic systemic antibiotics, use of IgG alone does not represent a relevant anti-infective measure, but serves as a useful benchmark to continue toward more clinically relevant assessment. Combination therapy of systemic antibiotics and local IgG is complimentary. Recently, this combination strategy in other animal infection models shows both additive and synergistic⁴⁵ efficacy against several different virulent pathogens. Such an approach represents a logical, more relevant extension of the current strategy, and provides the benefit of protection against antibiotic resistant infection. Other implant models and application methods (e.g., IgG delivered from biodegradable wound-filling gel or fibrin sealant) could also improve bactericidal efficacy by providing extended delivery of immunoglobulin directly to the wound bed as phagocytes infiltration, inflammatory responses, and healing cascades proceed. These strategies will be subject to comparison with other standard topical anti-infective methods in fighting antibiotic resistant infection.

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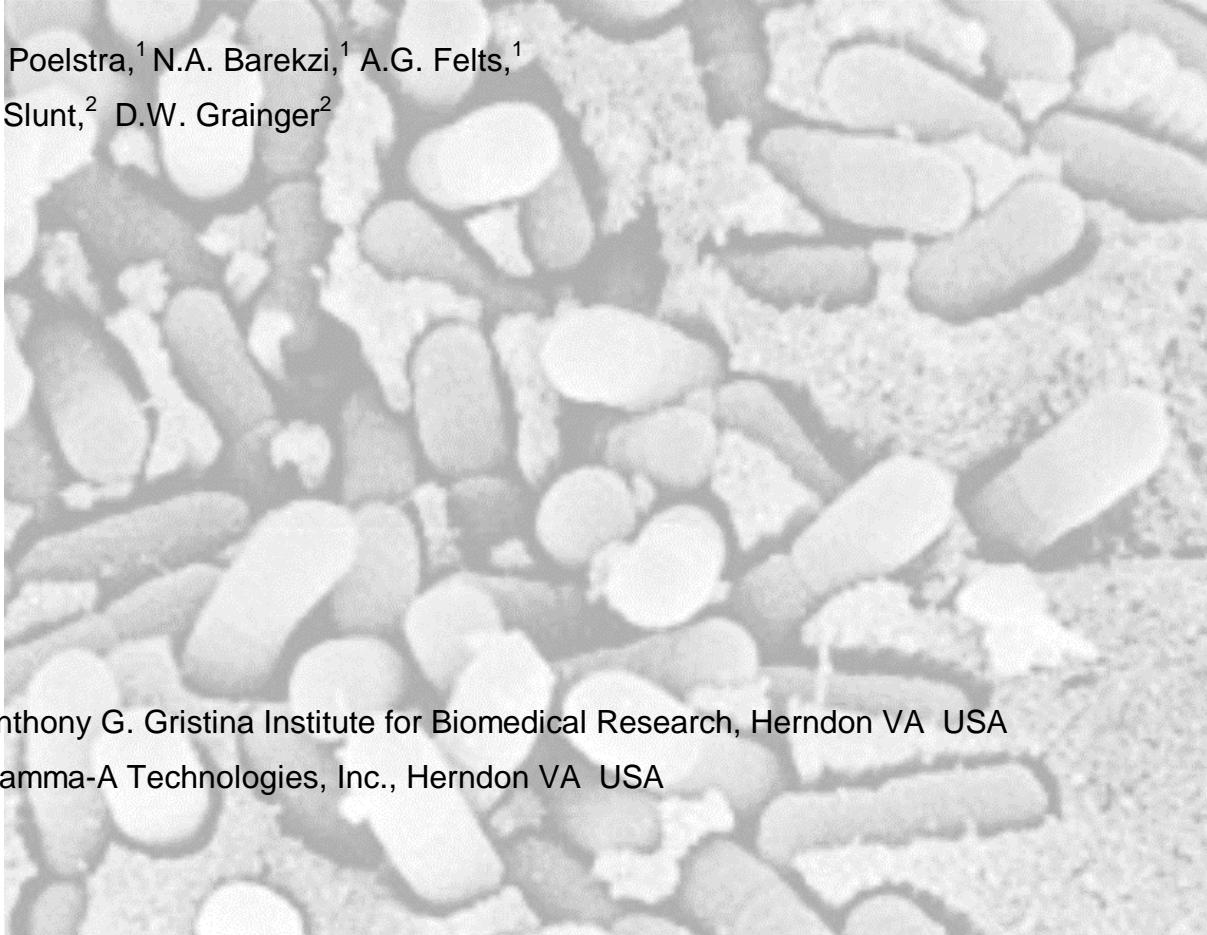
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Locally delivered polyclonal antibodies potentiate the efficacy of a systemic antibiotic against infections

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Abstract

*Antibiotic treatment of clinical infections is complicated both by the increasing emergence of antibiotic-resistant pathogens and increased patient populations intrinsically at risk for nosocomial infections. Combination therapies comprising multiple intravenous antibiotics alone, or in tandem with either intravenous immunoglobulins or local antibiotics, have all been used to improve efficacy against clinical infections. We now report that pooled human immunoglobulins applied locally to sites of infection *in vivo* substantially improve the anti-microbial benefits of a clinically important intravenous antibiotic – ceftazidime – against both *E.coli*-induced peritonitis and *Klebsiella*-induced burn wound infection. Synergistic improvements in host survival, bacterial burden, and sepsis indicators are observed with this unique treatment combination. Because immunotherapy functions independently of antibiotic resistance mechanisms, local delivery of polyclonal or monoclonal antimicrobial antibodies together with clinically routine intravenous antibiotics exploit diverse, complementary antimicrobial properties to confer improved protection against infection and extend the efficacy of front-line antibiotics.*

Current clinical standards of care often utilize, either prophylactically or therapeutically, systemic antibiotics to manage and control the threat of infection in many indications. Antibiotic resistant pathogens are an increasingly problematic cause of hospital-based infections.¹ A wide variety of pathogens now demonstrate clinical resistance to antibiotics of choice including methicillin resistant strains that account for 80% of all *Staphylococcus aureus* clinical infections,² and vancomycin resistant enterococci.³⁻⁵ The continuously increasing prevalence of antibiotic resistant bacteria has greatly elevated concern that front-line antibiotics will become ineffective in managing clinical infections.^{4,6} Recently, clinical reports of vancomycin-methicillin resistant *Staphylococcus aureus* infection provide evidence that vancomycin's clinical utility as the last antibiotic of choice is also threatened.⁷⁻¹⁰ Proof that selective pressure from increasing vancomycin use promotes even more rapid vancomycin resistance¹¹ has prompted renewed attention directed both to understanding mechanisms of antibiotic resistance, as well as to developing alternative antimicrobial methods.^{7,12-14} Although new *de novo*

antibiotic synthesis is a logical, compelling choice, few new, original synthetic antibiotics are in clinical phase trials.¹³ Furthermore, many prospective antibiotic candidates represent iterations on long-standing drug structure-function paradigms susceptible to resistance mechanisms.^{7,12}

Commercial pooled polyclonal human immunoglobulins (IgG) represent a broad-spectrum antimicrobial approach currently administered by intravenous infusion to millions of patients annually (IVIG).¹⁵⁻¹⁸ These exogenous polyclonal antibodies supplement host humoral immunity through both specific and non-specific opsonization and neutralization reactions against many ubiquitous, clinically relevant pathogens, ultimately leading to microbial phagocytic clearance. Because antibodies facilitate antimicrobial mechanisms distinct from those of antibiotics, they do not engender antibiotic resistance. Moreover, the appearance of pathogen antibiotic resistance does not alter bacterial susceptibility to opsonization and phagocytic neutralization.¹⁹ However, therapeutic benefit of IVIG alone in various scenarios has not been compelling.^{20,21} By contrast, combination therapy comprising both systemic antibiotics and systemic polyclonal IVIG has demonstrated benefit against sepsis in newborns,^{22,23} high-risk neonates,²⁴ and ventilated ICU patients.²⁵ Additionally, combination therapy has also been reported to reduce post-operative infection rates leading to sepsis after surgery for colorectal cancer²⁶ and has been applied as an adjunct therapy to systemic antibiotics against chronic sinus disease in children.²⁷ Clinical problems with antibiotic-induced release of pathogen toxins in septic patients^{28,29} have been addressed using antibodies administered systemically against these toxins.^{30,31} Most recently, systemic monoclonal antibody infusion has also been used successfully in cystic fibrosis patients infected with therapy resistant *Pseudomonas aeruginosa*,³² and systemic monoclonal antibody infusion has emerged as the treatment of choice against respiratory syncytial virus (RSV).^{33,34} Systemic antimicrobial antibodies, therefore, have shown to be complementary to antibiotics in preventing and facilitating clearance of infection. Combination therapies represent the clinical capability to exploit pathogen susceptibility to multiple antimicrobial agents that individually no longer have acceptable clinical efficacy.

Improved, often additive clinical benefits observed for simultaneously co-administered antibiotics,³⁵⁻³⁹ or antibiotic and antibody combination therapies can be attributed to several phenomena. First, pathogen antibiotic resistance is never complete. While minimum inhibitory concentrations for an antibiotic against a given antibiotic resistant pathogen can be significantly increased, they remain finite and microcidal *in vitro* at elevated doses. Clinically, however, these doses may be toxic, precluding use. Given the limited genomic and metabolic capacity of many pathogens, genetic acquisition of resistance against a specific antibiotic may compromise further broad-spectrum multiple drug resistance.⁴⁰ Secondly, antibiotic/antibody mixed infusions provide simultaneous microcidal, antimicrobial and anti-toxin efficacy important in neutralizing both pathogen growth and toxin release from viable and antibiotic-lysed pathogens.

We report here observation of enhanced efficacy for ceftazidime, a commonly used systemic antibiotic, in combination with locally applied polyclonal antibodies as a new treatment strategy against infection. Ceftazidime is a third-generation cephalosporin and first-line antibiotic against *Klebsiella pneumoniae*, *Serratia marcescens*, *Escherichia coli* and *Proteus mirabilis* infections.³⁶ However, emergence of ceftazidime-resistant *Klebsiella* and *Escherichia coli* strains^{41,42} in hospital-acquired infection scenarios warrants assessment of other strategies. Two different murine infection models (outbred CF-1 mice) detailed previously^{43,44} were used to study the efficacy of systemic antibiotic/local antibody combination therapy over each monotherapy, respectively.

In the first *in vivo* infection model,⁴⁵ full-thickness burn wounds created on the backs of anesthetized mice were immediately challenged with *Klebsiella pneumoniae* (strain 2270, 10² CFU) directly into the wound site via a subcutaneous (s.c.) injection, producing a consistently lethal infection.⁴⁴ The second *in vivo* infection model uses a direct intra-abdominal challenge with *Escherichia coli* (strain KI08ACH7, 10⁶ CFU) to consistently produce a lethal peritonitis.⁴³ Each infection model was subject to combination therapies using prophylactic, intravenous ceftazidime (tail vein infusion) and/or pooled human polyclonal antibodies either systemically infused (IVIG) or locally delivered to the site of infection.

Methods

Animals and Animal Care: Outbred female Crl-CF-1 mice (22-24 g) were obtained from Charles River Laboratories (Wilmington, MA) and housed five per cage in a biosafety level 2 facility with a 12 hour light/dark cycle. Standard mouse chow and water were provided *ad libitum*. All animals were maintained according to the *Guide for the Care and Use of Laboratory Animals*⁵² and all protocols were approved by the Gristina Institute Animal Care and Use Committee.

Bacteria: *Klebsiella pneumoniae* 2270 and *Escherichia coli* K108ACH7 (donated by Dr. Ian Holder, Shriners Burn Institute, Cincinnati, OH and Dr. J. Curtis Nickel, Queens University, Kingston Ontario, Canada, respectively) were grown for 18 hours in 20 ml trypticase soy broth at 37°C while agitated at 150 RPM using a benchtop incubator shaker. Cultured bacteria were twice sedimented by centrifugation at 7649 x g for 10 minutes, washed and diluted in saline to obtain a concentrated bacteria suspension. Serial bacterial dilutions were plated on trypticase soy agar (TSA) and colonies were counted to determine initial colony forming units (CFU) per ml after 24 hours incubation at 37°C. In parallel, optical absorbance ($\lambda=650$ nm) of these bacterial dilutions was measured (Beckman DB-GT grating spectrophotometer) and standard curves plotting optical absorbance versus CFU/ml concentrations were then constructed for each organism. Optical absorbance values of 1.16 for *K. pneumoniae* and 1.05 for *E. coli* resulted in $\sim 10^9$ CFU/ml.

Local and systemic antimicrobial therapies: Mice were treated locally (sub-eschar, s.c., intravenously, i.v., or intraperitoneally, i.p.) with commercially pooled human intravenous immunoglobulin (Lot# 2620M039A, Gammagard[®] S/D, Baxter Healthcare Corporation, Glendale, CA) and/or i.v. with sub-optimal doses of ceftazidime (Lot# 8ZP0340, Fortaz[®], Glaxo Wellcome Inc., Research Triangle Park, NC). Gammagard[®] S/D was supplied as a freeze-dried preparation, and reconstituted with supplied diluent (Sterile Water for Injection, USP) to 10wt% protein/ml (98% pure IgG) at an approximate pH of 6.8. Manufacturer specifications indicate that this reconstitution provides an iso-osmolar

solution comprising dextrose and saline. Ceftazidime, supplied as a lyophilized powder, was reconstituted to the optimal human equivalent clinical dose (200 or 22.7 mg/kg for pediatric burn wounds³⁵ and adult peritonitis,⁵¹ respectively) in sterile distilled water. Stock IgG solutions were diluted in 5% dextrose (recommended by the manufacturer) and ceftazidime was diluted in sterile water to obtain the desired working concentrations. Injections of sterile diluents (0.1 ml for s.c. and i.v. injections, 0.5 ml for i.p. injections) served as local and systemic control treatments in all experiments.

Animal Models: A previously described full thickness murine burn wound infection model was used.³⁵ Briefly, mice were shaved and then anesthetized by methoxyflurane inhalation (Metofane®, Schering Plough, Union, NJ) and a heat-resistant plastic board with a 1.0 (25 mm) by 1.5 inch (38 mm) window was pressed firmly against the shaved dorsum. Ethanol (200 proof; 0.50 ml) was spread evenly over the window opening, ignited, and allowed to burn for 10 seconds. The procedure produced a non-lethal full thickness burn wound over 10-15% of the body surface. Immediately after the burn, mice were given 0.5 ml of sterile saline intraperitoneally as fluid replacement therapy and acetaminophen (0.25 mg/ml; Children's Tylenol suspension liquid) in drinking water as a post-burn analgesic. A lethal dose of *K. pneumoniae* ($LD_{100}=10^2$ CFU/0.1 ml) and local immunoglobulin treatment (10 mg/0.1ml) were independently injected s.c. under the burn site immediately following the burn. Intravenous sub-optimal single doses of ceftazidime (44 μ g/0.1 ml; 2 mg/kg) were infused by tail vein injection either alone (monotherapy) or followed by immunoglobulin treatment (combination therapy).

In the peritonitis model,⁴³ mice were injected intraperitoneally (i.p.) with a lethal dose inoculum dose of *E. coli* ($LD_{90}=10^6$ CFU/0.5 ml) followed immediately by immunoglobulin treatment (1, 5, or 10 mg/0.5 ml i.p. or 10mg/0.1ml i.v.) and/or sub-optimal i.v. ceftazidime tail vein infusion (25 or 50 μ g/0.1 ml). Animal survival in both models was assessed for ten days thereafter.

Quantitative Microbiology: Approximately one ml of blood was collected via cardiac puncture into heparinized tubes (40 units; Sigma Chemical Co., St. Louis, MO) from each anesthetized mouse either 24 hours (burn wound model) or 12 hours (peritonitis model) post bacterial challenge. Immediately following blood collection each anesthetized mouse was euthanized by cervical dislocation. Blood was serially diluted in sterile saline and plated on trypticase soy agar (TSA) plates for enumeration of bacteria. In the peritonitis model, each peritoneal cavity was lavaged with 5 ml sterile saline. Lavage was serially diluted and plated on TSA plates for enumeration of bacteria. Resulting colony counts are expressed as log CFU/g blood or lavage, respectively. Post-euthanasia in the burn wound model, the burned eschar was surgically removed and homogenized in 10 ml sterile saline. In both models, livers were excised post-mortem and placed in 10 ml sterile saline. All tissue samples were weighed prior to homogenization (Omni-International GLH Homogenizer, Marietta, GA). Homogenate fluids were then serially diluted in sterile saline and plated on TSA plates for bacterial cultures and enumeration. Resulting colony counts are expressed as log CFU/g tissue.

Quantitation of Systemic Interleukin-6 (IL-6): Immediately following serial dilution and plating of the blood samples, serum was separated by centrifugation at 3,000 rpm for 10 minutes at room temperature, collected and assayed with an ELISA^{43,53} specifically designed to detect IL-6. Optical density was measured at 405 nm (SLT Spectra Reader, Tecan Company, Durham, NC). The detection range for the assay was 15 - 2000 pg/ml. The concentration of IL-6 present in each sample is reported as pg/ml serum.

Statistical Analysis: Data are expressed as the mean \pm standard error of the mean (SEM). Student's t tests were used to compare the control and therapy groups of the bacterial burden enumeration and IL-6 studies while ANOVA with Tukey's tests were used to compare rates of mortality. All probabilities less than 5% ($p < 0.05$) were considered significant. Data outliers, defined as any datum outside the range of the mean \pm two times the standard deviation, were excluded.

Results

Antibiotic/antibody therapy benefits in burn wound infection.

In untreated murine burn wounds, low levels of *K. pneumoniae* challenge (10^2 CFU s.c.) consistently killed 100% of burned and 80% of unburned mice (Figure 1a). Significantly, previous work has shown that other relevant burn wound pathogens required substantially higher inocula (e.g., *Pseudomonas aeruginosa*, 10^4 CFU) to produce consistent lethal infections in this model,⁴⁴ and that locally applied polyclonal IgG monotherapy protected ~90% of the animals from *P. aeruginosa* lethal infection. However, Figure 1a shows that local polyclonal antibody monotherapy, or sub-optimal i.v. ceftazidime (44 µg/animal, 2.0 mg/kg) monotherapy with *Klebsiella*-infected burn wounds each produced low survival (0% for local IgG, 50% for i.v. ceftazidime). However, the combination of local antibody treatment with systemic ceftazidime combination produced 90% survival, a significant 40% synergistic benefit ($p<0.05$).

Quantification of tissue bacterial burdens in the burn infection model at 24 hours post-infection exhibits similar trends (Figure 1b). In the harvested burned eschar, *K. pneumoniae* colony counts in dextrose-treated controls (placebo) and in cohorts treated with local s.c. antibody monotherapy increased dramatically (>6 log order increase over challenge inoculum dose) by 24 hours after bacterial challenge. Pathogen colonization of eschar tissue after systemic infusion of a sub-optimal dose of ceftazidime alone also was significantly increased (>2 log order increase over challenge inoculum dose). In contrast, combining local antibody delivery and systemic sub-optimal antibiotic treatment at the time of bacterial challenge decreases bacterial levels in this tissue by 0.5 log CFU/g tissue compared to the initial inoculum (Figure 1b).

While this strain of *K. pneumoniae* exhibited high virulence due to the presence of an extracellular polysaccharide capsule,^{46,47} a 24-hour incubation period in the host is still too brief for the infection to become fully systemized. No colonies were detected in blood samples from all groups after 24 hours (data not shown). Colonies were detected in liver homogenates, however, exhibiting similar, higher bacterial counts for placebo

and both monotherapy-treated mice cohorts after 24h. These counts will increase to lethal levels in the following 24h period, resulting in the notable increase in mortality over time (Figure 1a). By contrast, livers of all mice treated with both locally delivered antibodies and systemic sub-optimal antibiotics exhibited average pathogen levels below the 10^2 CFU/g detection limit, conferring protection consistent with survival benefits observed for this therapy group.

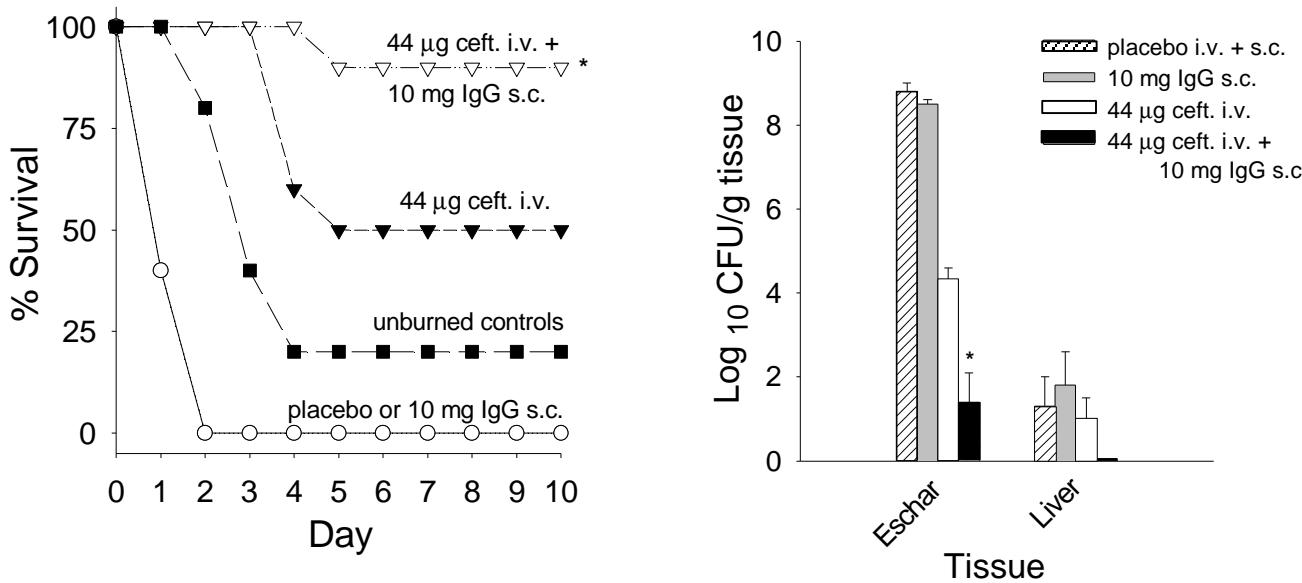


Figure 1:

(a) Host survival over time for systemic sub-optimal antibiotic dose (44 mg ceftazidime via tail vein infusion) or locally applied polyclonal antibody monotherapy (sub-eschar, s.c., injection) compared to various combination therapies in a murine burn wound infection model using a lethal dose (10^2 CFU) of *K. pneumoniae* as the pathogen inoculum. Mice were left unburned or burned and then lethally challenged following established protocols.^{35,44} Combination therapy of systemic ceftazidime and locally injected polyclonal antibodies s.c. into the wound confers synergistic survival benefit over either ceftazidime or locally applied IgG alone. (Significance: ANOVA and Tukey's test, n=10; *p<0.05 compared to all other data).

(b) Bacterial tissue burden assayed 24 hours post-burn and post-challenge with 10^2 CFU of *K. pneumoniae* following mono- or combination antimicrobial therapy. Combination therapy of systemic ceftazidime and locally injected polyclonal antibodies provides substantial reduction in eschar bacterial burden over either ceftazidime or locally applied IgG monotherapies (Student's t-test, *p<0.01 versus all harvested eschar groups).

Cytokine interleukin-6 (IL-6), a clinical indicator of systemic inflammatory acute phase reactions,⁴⁸ was detected in serum of all groups post-infection. Both systemic antibiotic monotherapy and combination therapy groups had significantly reduced IL-6 levels over that observed for placebo or local antibody monotherapy groups (Figure 1c). No significant difference in IL-6 reduction was observed for the combination treatment group compared to the antibiotic monotherapy group. Reduced systemic IL-6 is consistent with the absence of detectable systemic bacteria in blood and low liver bacterial counts observed for both of these treatments.^{43,44}

Figure 1(c)

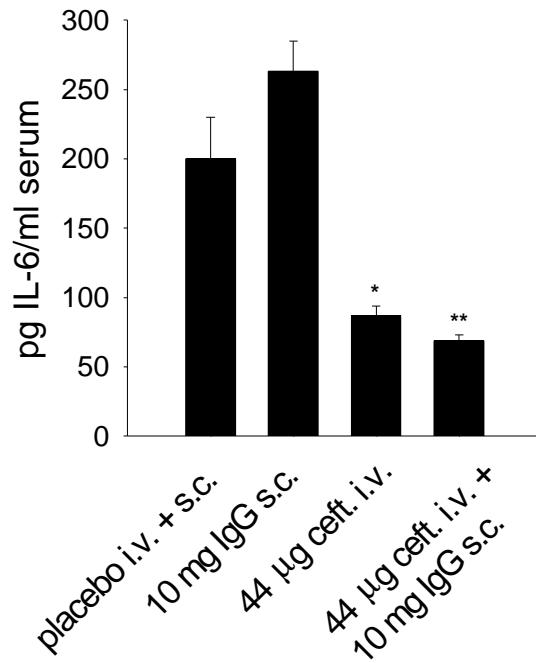


Figure 1 (continued):

(c) Levels of interleukin-6 (IL-6) in serum assayed 24 hours post-burn and post-challenge with 10^2 CFU of *K. pneumoniae* following mono- or combination antimicrobial therapy. Combination therapy of systemic ceftazidime and locally injected polyclonal antibodies provides substantial reduction in circulating IL-6 over either ceftazidime or locally applied IgG alone (Student's t-test, * $p<0.003$ versus placebo and IgG s.c. treatment, ** $p<0.05$ versus all treatments).

Antibiotic/antibody combination therapy benefits in peritonitis.

In the second model, intra-abdominal injection of a clinically isolated *E. coli* (K108ACH7, 10^6 CFU) produced consistent lethality in untreated mice (Figure 2a). This *E. coli* isolate is not fully antibiotic resistant, proving sensitive to ampicillin, cefazolin, nitrofurantoin, gentamicin and trimethoprim-sulfamethoxazole (personal communication, Dr. J. Curtis Nickel, Queens University, Kingston Ontario, Canada). Analogous to the burn infection model survival synergy shown in Figure 1a, synergistic survival benefits were also observed in this peritonitis model for combinations of sub-optimal i.v. ceftazidime (50 μ g/animal, 2.3mg/kg) with intraperitoneally administered polyclonal antibodies (Figure 2a).

Figure 2(a)

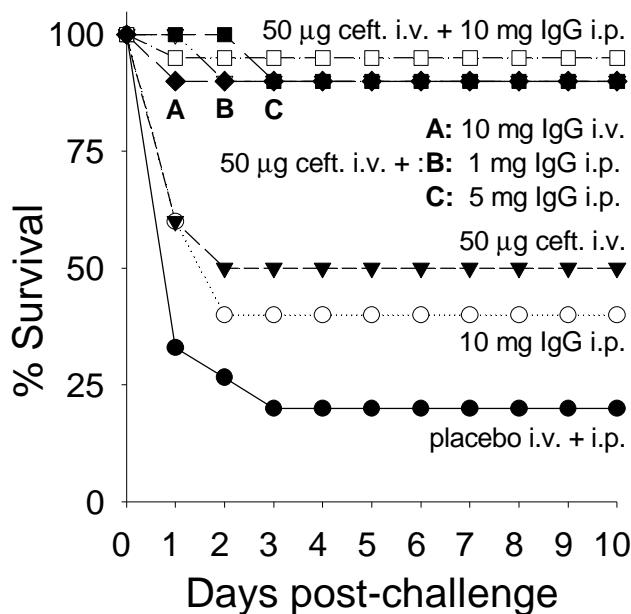


Figure 2:

(a) Murine survival over time for systemic sub-optimal antibiotic dose (50mg ceftazidime via tail vein infusion) or locally applied polyclonal antibody monotherapy (intraperitoneal injection, i.p.) compared to various combination therapies in a murine peritonitis infection model challenged with a lethal dose (1×10^6 CFU) of *E. coli*, following established protocols.⁴³ Combination therapy of systemic ceftazidime with 10mg locally (i.p.) injected polyclonal antibodies confers synergistic survival benefit over either systemic ceftazidime, locally applied IgG (i.p.) monotherapy, or the additive benefit of both monotherapies considered together.

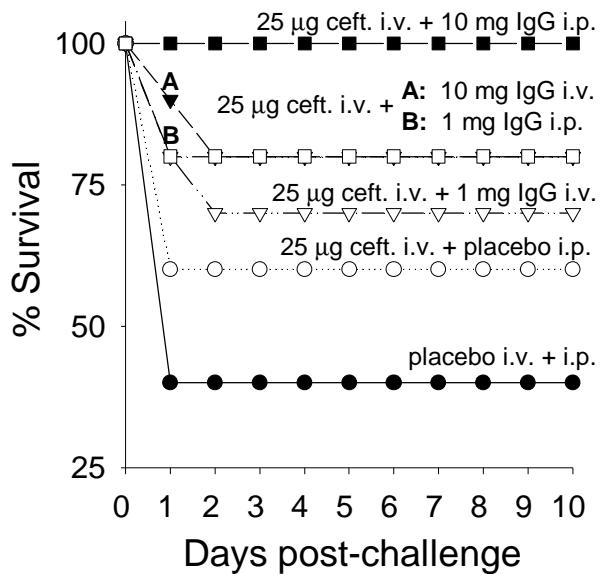
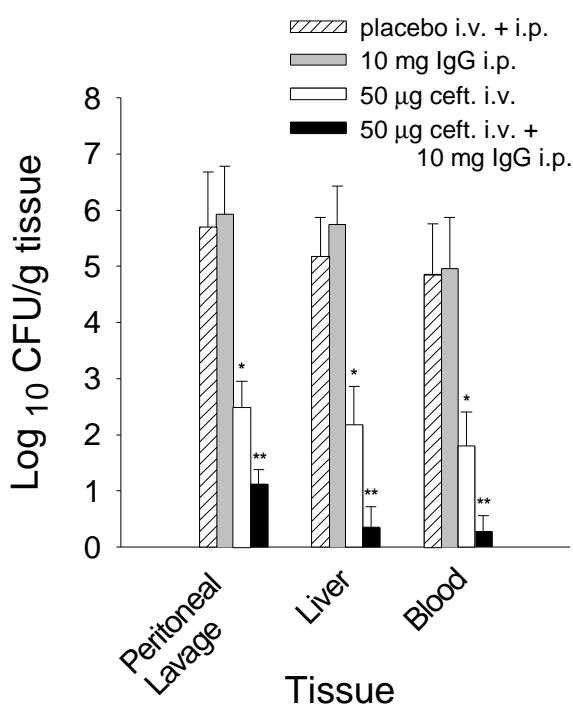
Additionally, survival benefits for combinations of systemic ceftazidime plus either 1mg or 5mg IgG i.p. were nearly identical to that for combination therapy of systemic ceftazidime and 10mg IgG intravenously.

All of these survival benefits were comparable to the additive benefit provided by systemic ceftazidime and 10mg IgG i.p. monotherapies considered together (significance: ANOVA and Tukey's test, n=5-20; p<0.001 for each combination antibiotic/IgG therapy versus each monotherapy, and p<0.001 for each monotherapy compared to placebo).

In combination with sub-optimal 50 µg i.v. ceftazidime doses, two different, reduced doses of locally applied antibodies (1 and 5mg IgG intraperitoneally (i.p.)) conferred survival equivalent to that observed for a substantially higher dose (10mg i.v.) of systemically infused IgG (conventional IVIG). Overall survival for local antibody monotherapy is 40% against this pathogen, substantially less effective than previously observed for the same local IgG monotherapy in the same model against several different virulent strains of *P. aeruginosa*.⁴³ Local antibody monotherapy proved slightly less effective than systemic antibiotic monotherapy that confers 50% survival with a single prophylactic sub-optimal dose of i.v. ceftazidime alone. Combination therapy comprising systemic antibiotic and locally delivered IgG produced a synergistic 95% survival (Figure 2a), compared to each monotherapy, a benefit significantly different than all other treatment groups (ANOVA, p<0.05).

Enhanced efficacy of locally delivered IgG i.p. over systemic IgG is more apparent when both are compared in combination with a further reduced sub-optimal 25µg ceftazidime systemic infusion and slightly reduced *E. coli* challenge (7×10^5 CFU, Figure 2b) in this peritonitis model. Addition of any antibody therapy – systemic or local – to ceftazidime i.v. treatment improves survival over ceftazidime monotherapy. Combination therapy involving local IgG applications even at this low systemic antibiotic dose produced higher survival than systemic antibiotics combined with systemic antibody infusion at two different doses (1 and 10mg IVIG). The lower local 1mg IgG dose i.p. in combination therapy conferred equivalent survival to that observed for a log higher dose (10mg) of systemic IgG with the same antibiotic infusion (Figure 2b).

Bacterial burdens found in tissues harvested from the peritonitis model at 12 hours post-infection support these trends in survival (Figure 2c). Enumeration of pathogens from peritoneal lavage, liver and blood post-treatment indicates that systemic antibiotic/local antibody therapy significantly and synergistically reduces viable bacteria in the host compared to either monotherapy. Local IgG monotherapy i.p. has little efficacy in reducing pathogen burden in all sites examined, while sub-optimal i.v. ceftazidime monotherapy reduced bacterial burdens substantially and consistently at all sites.

Figure 2(b)

Figure 2(c)

Figure 2 (continued):

(b) Murine survival over time for a lower systemic sub-optimal antibiotic dose (25 mg i.v. ceftazidime) in combination with various locally or systemically applied polyclonal antibody doses (1 and 10mg IgG i.p. or i.v.) compared to placebo and combination therapies in a murine peritonitis infection model using a lethal dose (7×10^5 CFU) of *E. coli* as the pathogen inoculum. Combination therapy of sub-optimal systemic ceftazidime and locally (i.p.) injected polyclonal antibodies (1 or 10mg IgG) confers improved survival benefit over either systemic ceftazidime monotherapy or combination of systemic ceftazidime with corresponding 1 or 10 mg systemic IgG dose i.v., respectively. Additionally, survival benefit for combination therapy comprising sub-optimal systemic ceftazidime plus 1mg IgG i.p. was equivalent to that for combination therapy of systemic ceftazidime and 10mg IgG i.v. (significance: ANOVA and Tukey's test, n=10; p<0.001 for each combination antibiotic/IgG therapy versus each monotherapy, p<0.001 for ceftazidime monotherapy compared to placebo, and p<0.001 for all combination therapies using local IgG dosing (i.p.) versus combination therapy using systemic IgG (i.v.));

(c) Tissue and blood bacterial burden assayed 12 hours post-challenge with 5×10^7 CFU of *E. coli* and mono- or combination antimicrobial therapy. Combination therapy comprising a sub-optimal systemic ceftazidime dose (25 : g i.v.) with locally injected polyclonal antibodies i.p. provides substantial reduction in bacterial burden in all sites assayed over either ceftazidime or locally applied IgG monotherapy (significance: Student's t-test, *p<0.05 comparing ceftazidime versus placebo and IgG monotherapy; **p<0.05 comparing combination therapy versus all treatments);

Together, the combined therapy would be predicted to show a benefit similar to ceftazidime alone. However, data shown in Figure 2c indicate that a bacterial reduction benefit substantially greater than simple additivity is produced by the combination of ceftazidime i.v. and IgG i.p., consistent with the survival synergy noted in Figure 2a.

Analogous to treatment effects seen in Figure 1c for the burn infection, levels of circulating IL-6 detected in serum of both antibody monotherapy and placebo treatment groups for peritonitis are dramatically increased over IL-6 quantified for antibiotic monotherapy or combination antibiotic/antibody treatment. (Figure 2d). Combination therapy reduces IL-6 to near-baseline detection limits and is significantly reduced compared to i.v. ceftazidime monotherapy. Serum IL-6 levels after combination therapy show a significant and synergistic reduction compared to placebo or either monotherapy, consistent with low bacterial burden (Figure 2c) and survival synergy (Figure 2a and b).

Figure 2(d)

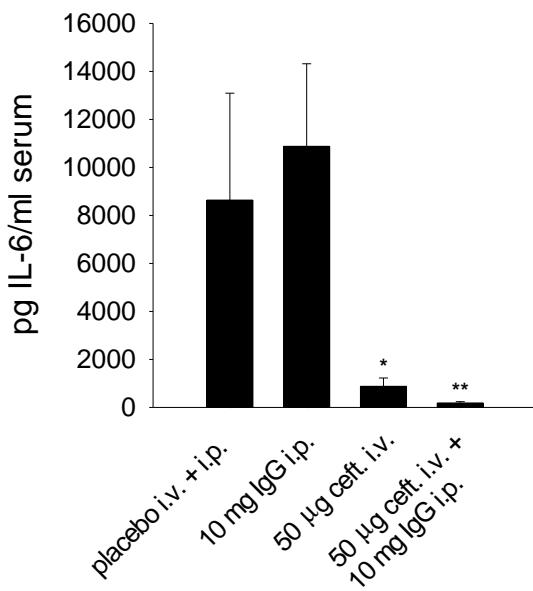


Figure 2 (continued):

(d) Levels of interleukin-6 (IL-6) in serum assayed 12 hours post-challenge with 1×10^6 CFU of *E. coli* following mono- or combination antimicrobial therapy. Combination therapy comprising systemic ceftazidime with locally injected polyclonal antibodies provides substantial reduction in circulating IL-6 over placebo, ceftazidime or locally applied IgG monotherapies (Student's t-test, * $p<0.05$ comparing ceftazidime versus placebo and IgG monotherapy, ** $p<0.05$ comparing combination therapy versus all treatments).

Discussion

Combining locally applied polyclonal antibodies with sub-optimal systemic i.v. antibiotic prophylaxis produces synergistic survival benefits over that observed for each separate monotherapy. Significantly, mortality resulting from administration of sub-optimal i.v. antibiotic doses was consistently improved by low level, local co-administration of IgG. Survival benefits from combination therapy in both infection models using two different pathogens directly correlate with observed synergistic reductions in both tissue bacterial burdens at several sites, and systemic levels for a cytokine indicator of sepsis (IL-6). The data suggest that prevention of systemization of the infection generally enhances survival, and that such prevention is significantly enhanced using combination therapy over monotherapies or double systemic infusion therapy of both antibiotic and IgG. The interesting question surrounds the mechanism for this effect. Systemic clearance of intraperitoneally administered IgG is known to be rapid (~3 hrs)^{43,49} while human immunoglobulins injected s.c. into murine full thickness burn wounds are first detectable by ELISA in mouse serum after 3 hours (data not shown). Once present in murine systemic circulation, however, human IgG is detectable beyond seven days.⁴³ Hence, the synergy observed in reducing infection mortality and morbidity in these models could be attributable to the same, unelucidated yet therapeutically beneficial systemic interactions reported between systemic antibiotics and IVIG.⁵⁰ Improved, synergistic benefit observed for the combination of systemic antibiotic/local antibody therapy over the efficacy of combination systemic IgG therapy also supports a distinct, locally enhanced contribution from doses of exogenous polyclonal IgG at the sites of infection. Local pathogen opsonization by IgG in the peritoneal cavity can occur immediately, reducing clearance of IgG to systemic circulation. Abundant, endogenous peritoneal macrophages could conceivably become rapidly activated by IgG opsonization of the bacteria as well as by pathogen proliferation, clearing bacteria in early stages of contamination locally prior to systemic spread of infection. Opsonization would not only serve to promote local phagocytic clearance but also hinder or delay rapid pathogen proliferation kinetics necessary to achieve systemization of the infection. Because the burn wound is severely immunocompromised, avascular and often necrotic, clearance of

local infection would rely on local, extravascular transport of fresh IgG, other immunocomponents (e.g., complement), and cellular elements including macrophages, monocytes and neutrophils from the unburned surrounding tissue. Direct injection of IgG into the immune-depleted site overcomes transport limitations for this important component. Lastly, increased capability for IgG binding and neutralization of toxins locally limits septicemia and systemic infection, as supported by IL-6 results.

Sub-optimal systemic antibiotic monotherapy has shown reduced efficacy against pathogens, but in conjunction or in combination with other antibiotics, synergistic effects have been previously observed.³⁸ Sub-optimal antibiotic doses were chosen in this study to permit discrimination of therapeutic benefits attributable to IgG in each infection model. Specifically, sub-optimal prophylactic antibiotic doses for each infection model were calculated from published human equivalent full clinical i.v. ceftazidime doses relevant to treating pediatric burn wounds³⁵ or adult peritonitis.⁵¹ The full equivalent antibiotic monotherapy doses in mice (440 or 500 µg/animal i.v., respectively) produced 75% survival in the burn wound model and 100% survival in the peritonitis model, regardless of initial bacterial challenge between 10^2 and 10^6 CFU (data not shown). Ceftazidime reduction to 10% of the full dose constitutes a legitimate regimen to determine antimicrobial synergy¹ and produced consistent infection mortality while permitting significant influence of local IgG administration.

Observed anti-infective benefits using combinations of antibiotics and antibodies might be general to many other infections and could have important clinical implications for both reducing selective pressure involved with producing antibiotic resistance, improving the performance and extending the clinical lifetime of current front-line antibiotics facing resistance, as well as in treating antibiotic resistant infection. Combination antibiotic/antibody therapies might improve patient health, provide better clinical control of antibiotic resistant pathogens and reduce costs by reducing dose and simplifying delivery requirements for antimicrobial antibodies administered locally and directly to the site of infection in tandem with routine systemic antibiotics. Importantly, reduction of systemic antibiotic levels by sub-optimal dosing mimics a possible future clinical

scenario in which selective pressures encouraging ceftazidime resistance are decreased while optimizing therapeutic benefit against infection using exogenous, locally delivered antibodies¹¹.

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Treatment of gram-negative and gram-positive abdominal implant infections using locally delivered polyclonal antibodies

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Abstract

*Virulent methicillin resistant Staphylococcus aureus (MRSA) and Pseudomonas aeruginosa strains produced lethal implant-associated infections in a mouse open abdominal implant surgery model. These infections were treated with prophylactic combinations of systemic antibiotics and pooled polyclonal human antibodies (IgG) locally delivered from a controlled-release hydrogel carrier applied directly onto a polymer mesh abdominal implant prior to surgical closing. In vitro antibody release from the hydrogel matrix was rapid, nearing completion after 48 hours. Human antibodies released intraperitoneally from this matrix in vivo were detected in mouse serum after 3 hours and persisted beyond 7 days. Released antibodies showed little to no benefit against MRSA infections either alone (100% animal mortality after 3 days) or in combination with systemic cefazolin or vancomycin antibiotic infusions. Controlled release IgG monotherapy produced significant survival benefit in gram-negative infections from two different strains of *P. aeruginosa*. A role for locally delivered antibodies against implant-associated gram-negative infection is suggested, utilizing humoral protection mechanisms that both circumvent increasingly antibiotic resistant pathogens and potentiate local host immune responses.*

Infection is a common complication associated with the surgical implantation of foreign materials.¹⁻⁵ Several types of biomaterials that are commonly used to gain therapeutic access to the peritoneal cavity (e.g., peritoneal dialysis catheters) are associated with high infection rates.⁶⁻⁸ Other biomaterials used to ensure proper tissue repair or wound closure after abdominal surgery also have significant infection statistics.⁹ Despite routine clinical use of prophylactic antibiotics, a major complication of abdominal implant surgery is infection caused by normal flora or ubiquitous gram-positive and gram-negative bacteria and anaerobic organisms of enteric origin seeding from the surgical site into surrounding tissues.^{1,4,10,11} Postoperative infections lead to costly, prolonged hospital stays, additional intravenous antibiotic administration, surgical interventions and patient discomfort.¹² Geriatric, immunocompromised, diabetic and obese patients all have greater risks of infections^{13,14} that contribute to increasing patient mortality and rising healthcare costs.¹²

The intrinsic ability of the host to eliminate contaminating bacteria inside the surgical wound is significantly reduced once pathogens colonize biomaterials. This is reflected in the increased incidence of infections associated with implanted foreign bodies.^{2,5,15-17} Many pathogenic bacteria require adhesion-dependent colonization to effectively establish infection in surrounding tissues and the formation of a surface-adherent and protective ‘biofilm’ that characterizes mature device-related infections. Once established, biofilms are difficult to eradicate, despite use of antibiotics with proven efficacy for killing pathogens in standard *in vitro* susceptibility tests.^{5,18-20} In addition, the emergence of various methicillin- and vancomycin-resistant bacteria together with a large and increasing immunocompromised patient population both add significantly to the surgical infection problem.²⁰⁻²² Failure of most common clinical treatments to overcome implant-centered infection often requires removal of the device in order to eradicate the therapy-resistant infection.^{1, 2, 5, 11}

In addition to clinically standard systemic prophylactic antibiotics, other treatment regimes have been used to prevent postoperative, implant-associated infection. Correction of malnutrition and normalizing albumin levels in blood assist the patient’s immune system to battle contaminating bacteria after abdominal surgery. Other therapeutic approaches as alternatives to antibiotics that promote bacterial-resistance include systemic supplementation with intravenous, broad-spectrum immunoglobulins (IVIG). The addition of exogenous opsonic antibodies has been used clinically for decades to fight infection from viruses and pathogenic bacteria.²³⁻²⁶ In animal experiments, IVIG has also been reported to synergize with β -lactam antibiotics since IVIG contains anti-lactamase antibodies against such antibiotic resistant pathogens.²⁷ Due to promising but inconsistent clinical results with IVIG in preventing bacterial infection,²⁸⁻³⁶ systemic antibody therapy is currently indicated in relatively few clinical infection situations, whereas antibiotic monotherapy remains the clinical treatment of choice.

Nevertheless, the combination of increasingly widespread antibiotic resistance, emergence of new pathogens and increases in immunocompromised patient populations leave many antimicrobial drugs less effective than ever before. The increased reliance on biomaterial implants and their propensity to promote biofilm-forming infections^{1,5} also call for the development of new anti-infective strategies. Reducing bacterial adhesion to biomaterial implant surfaces immediately during implantation³⁷⁻⁴⁰ decreases the risk for biofilm formation while improving host tissue integration of the implant. Improved tissue integration and wound healing, uncomplicated by postoperative wound infection, could also be important in preventing latent infections caused by hematogenous spread of bacteria from distant infectious foci, (e.g., after dental restoration procedures, total joint or heart valve replacement, or after penetrating traumata) that colonize biomaterials even years after initial implantation. Neutralization of contaminating organisms intra-operatively during the initial 4-6h “decisive period” prior to bacterial colonization^{41,42} could reduce postoperative, biomaterial-centered infection from virulent, antibiotic-resistant bacteria.

While currently not indicated or approved as a systemic, prophylactic antimicrobial, use of systemic IVIG may be appropriate in some indications to achieve this goal.^{23,26} However, human IgG is currently scarce and continues to be costly. Additionally, systemic administration of large quantities of IgG is associated with adverse effects, including finite risks of blood-borne pathogen infection, and renal insufficiency and failure.⁴³ Since infectious complications leading to septic shock frequently systemize initially from local device-centered infections (e.g., i.v. catheters, urinary tract (urological catheter) infections (UTI), orthopaedic implants), local anti-infective therapy applied directly to sites of infection as an alternative or supplement to systemic antibiotic prophylaxis could prove very useful. Infection incidence might be reduced by locally potentiating the body’s own intrinsic ability to neutralize contaminating pathogens prior to colonization, prevent biofilm formation and the systemic spread of pathogens leading to sepsis, ultimately allowing a reduction in systemic antibiotic use and thereby slowing the growth of antibiotic resistance.⁴⁴⁻⁴⁶ In particular, local humoral immunopotentiation side-steps antibiotic resistance mechanisms because humoral immunity functions by a

distinctly different mechanism. Since bacterial antibiotic resistance does not alter bacterial susceptibility for opsonization and phagocytosis,⁴⁷ local immunotherapy using IgG should function just as effectively against resistant pathogens. While IVIG has demonstrated limited clinical appeal, shifting the use of immunotherapy from systemic treatment to local application provides several advantages for humoral immunopotentiation at sites of high infection risk. This local approach could be effective using lower, highly selective antibody doses while exploiting sophisticated delivery strategies to reduce infection incidence, severity, and antibiotic resistance.

This research aimed primarily to investigate the efficacy of pooled human polyclonal antibodies delivered locally from a controlled-release hydrogel carrier to prevent biomaterial-centered infection in a murine open abdominal surgery model. Different therapeutic scenarios after challenge with either gram-positive (methicillin resistant *Staphylococcus aureus*) or gram-negative (*Pseudomonas aeruginosa*) bacteria utilized polyclonal human antibodies in both the absence and presence of clinically relevant systemic antibiotic prophylaxis.

Materials and Methods

Animals: After approval of all experiments by the Institutional Animal Care and Use Committee (IACUC), female CF-1 mice were purchased (30-33g) from Charles River Laboratories (Raleigh, NC). All animals were housed for at least 7 days in a 12 hour light-dark cycle, and given food and water *ad libitum* before use.

Bacterial species and preparation of inocula: Methicillin resistant *Staphylococcus aureus* (MRSA, ATCC33593),⁴⁸ *Pseudomononas aeruginosa* strains IFO 3455 and M2⁴⁹ were grown in Tryptic soy broth (TSB, BBL® USP, Becton Dickinson, Cockeysville, MD, Lot H8DFLS) for 18h at 37°C while agitated at 150 rpm. Cultured bacteria were twice sedimented by centrifugation at 7649 x g for 10min, washed, and diluted into sterile saline to obtain a concentrated bacterial suspension. Inoculum concentrations

producing reliable infections were determined from LD₉₀₋₁₀₀ values from preliminary dose-response experiments using each pathogen in the presence of the implant. Inoculum doses of 1x10⁸ Colony Forming Units (CFU) for MRSA, 1x10⁵ CFU for IFO3455 and 1x10⁴ CFU for M2 per 500 µl application were adjusted using a standard spectrophotometric assay⁴⁹ and confirmed by direct bacterial enumeration on Tryptic soy agar (TSA, Difco - Becton Dickinson, Sparks, MD, Lot 128882XA).

Hydrogel delivery vehicles and saline lavage preparation. Carboxymethylcellulose (CMC, Sigma Chemical product C-4888, St.Louis, MO, lot 125H0899) was mixed with sterile, de-ionized water and boiled to create a clear, transparent, homogeneous 5wt% gel. This vehicle was cooled and used as the controlled-release carrier for antibody delivery to the peritoneal cavity. Lyophilized, solid pooled polyclonal human immunoglobulin G (IgG, Baxter Gammagard S/D, Deerfield IL, Lot 98F03AB11) was weighed and mixed gently into the CMC gel using a syringe-syringe mixing/exchange method. This provided a homogenous 2wt% IgG formulation (20mg IgG/ml CMC-gel) after solid antibody dissolution into the CMC gel-carrier. This IgG-gel was stored a maximum of several hours in sterile syringes (3ml) with 18 gauze (G) needles for application in the animal implant model. As a control, empty CMC gel was drawn into identical syringes with 18G needles. Sterile isotonic saline was drawn into sterile syringes with 26G needles to serve as a surgical lavage solution.

In vitro IgG binding titer determination: An anti-human IgG ELISA^{22,49,51} was used to determine polyclonal human IgG binding titers against MRSA strain ATCC33593 and *P. aeruginosa* strains IFO3455 and M2. For the gram positive MRSA binding assay only, the ELISA was modified by using 3% rabbit serum as a blocking agent.⁵¹ Titer numbers express the inverse log dilution of IgG concentration at 50% ELISA optical absorbance (450nm) from the inflection mid-point on each IgG-bacterial binding curve.⁴⁹ A higher titer number reflects higher IgG binding to the bacterium strain.

In vitro IgG hydrogel release: Samples of the CMC-IgG-gel (weight ~1.0g, 10wt% IgG in CMC-gel) were loaded into sterile gauze pouches and suspended in 200ml of PBS (pH 7.0) release medium supplemented with 0.05% sodium azide at 37°C on a rotary shaker at 100rpm. Aliquots (1ml) of PBS were assayed for IgG at regular intervals up to 48h using both UV spectroscopy ($\lambda_{280\text{nm}}$) and a BCA protein assay (BCA-200 kit, Pierce, Rockford IL).⁴⁹

Murine abdominal implant surgical procedure: Mice were anesthetized with an intramuscular cocktail of ketamine (1.9mg = 60mg/kg) and xylazine (0.6mg = 20mg/kg) in a total injection volume of 50 μ l. Mice were then temporarily strapped on their backs to a surgery table, the exposed abdominal area was prepared with povidone-iodine and sterile cloth was used to cover each mouse, leaving an open oval area to perform surgery (~2.5cm max. diam.). The abdominal skin was cut longitudinally along the midline (~1.5cm), after which the exposed peritoneum was lifted using forceps and cut identically. Small stainless steel retractors were used to keep the wound open. The exposed intestines were lavaged twice with 0.5ml sterile saline and dried separately using sterile gauze. A square polypropylene mesh implant (1x1cm, 105 μ m pores, Spectrum Medical Industries, Inc. Los Angeles, CA), previously sterilized in ethanol (70%) for 15 minutes and dried in ambient air, was subsequently placed inside the abdominal cavity and challenged with the inoculum dose of one of the three bacterial strains in 500 μ l of saline immediately delivered directly onto the implant. After 60 seconds, either blank- (empty) or IgG-containing CMC-gel (500 μ l containing 10mg of IgG⁴⁹) was applied by syringe directly onto the implant and the wound was then closed in layers by suturing. The animals were kept in a cage heated by warm water under a warm light until fully recovered from anaesthesia (after ~3h) and Tylenol® was added to their drinking water as a post-surgical analgesic. Aseptic precautions were rigorously observed during the entire procedure. To standardize the surgical trauma, the same surgeons performed all operations.

A second ELISA,⁴⁹ using a capture mouse anti-human IgG and detection peroxidase-conjugated anti-human IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) was used to detect human IgG (optical absorbance at 450nm) in sampled mouse serum and peritoneal lavages. For these analyses, serum was separated from murine blood obtained via cardiac puncture after anaesthesia with Metofane® (Mallinkrodt Veterinary, Inc., Mundelein, IL) at different time points post-surgery, using a benchtop HN-SII centrifuge (10 min. at 3000rpm, IEC, Needham Heights, MA). Following euthanization by cervical dislocation, the peritoneal cavity was immediately exposed and lavaged using 5ml of sterile saline. Lavage fluid (2-4ml) was then collected and the ELISA described above was used to determine human IgG levels in both samples (detection range between 5 - 5000 ng/ml). Human IgG levels in mice were determined by comparing the experimental absorbance values from serum or peritoneal lavage to an IgG standard curve constructed from known amounts of IgG from the same lot of IgG in buffer.

Combination therapies using systemic antibiotics: Clinically relevant antibiotics were systemically infused in combination therapy with locally applied IgG. Cefazolin, (Sigma Chemical, St.Louis, MO, product C-5020, lot 115H1078) a first generation cephalosporin and common prophylactic antibiotic in abdominal surgery,⁵² or vancomycin (Sigma Chemical, product V-2002, lot 38H14001), the only current clinical choice against methicillin resistant pathogens, were administered 30min prior to surgery in volumes of 100µl via tail-vein infusion using a 30G needle. Single doses were increased step-wise in successive dose-response infection experiments from 0µg to 400µg of each antibiotic in sterile H₂O per 30-33g mouse, eventually reaching the clinical equivalent dose of systemically infused antibiotics in a human patient (1g/70kg patient). Sterile H₂O was systemically infused via the tail vein in placebo treated mice as a negative control.

Results

Polyclonal IgG binding titer determination: *In vitro* binding titers of polyclonal IgG determined using a published ELISA method⁵¹ were 1778, 423 and 489 for this IgG lot against MRSA, *P. aeruginosa* IFO3455 and M2, respectively. These values represent significant IgG binding activity against these different pathogens, representative of opsonization.

In vitro IgG hydrogel release: Figure 1 shows kinetics for 10wt% IgG release from CMC gel into buffer at 37°C over two time formats. Nearly 90% of the loaded IgG is released by 9 hours. The inset release graph plots the square root of time release and shows that this early portion of release is linear, consistent with IgG diffusion-limited release kinetics in the water-swollen gel matrix.

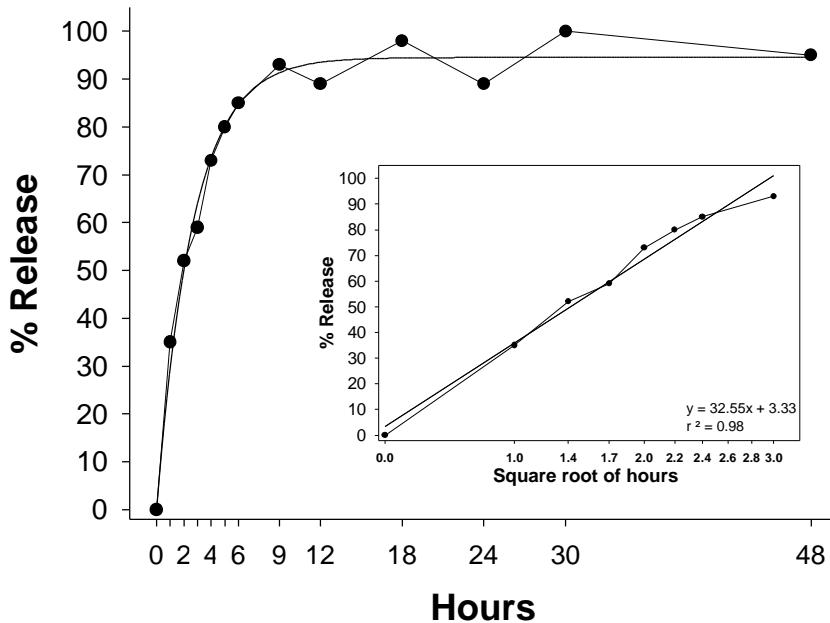


Figure 1:

Kinetics for 10wt% IgG release from the CMC controlled-release carrier into PBS *in vitro* at 37 °C. Complete release was observed after ~30h.

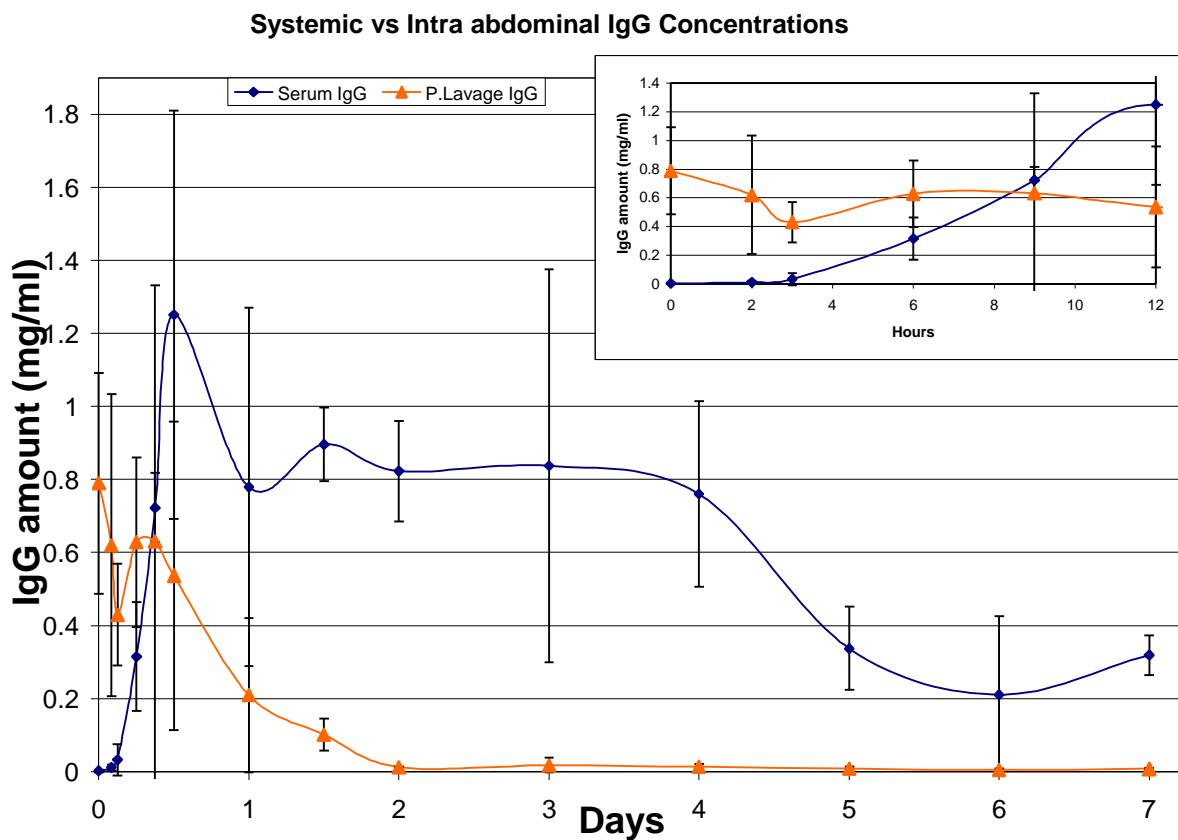
INSET: Square root of time release for IgG from the gel carrier, showing linear, diffusion-limited release kinetics from the water-swollen gel.

Complete antibody release is observed after ~30h and comparable IgG levels maintain detectable up to day 16 (data not shown). These release properties indicate that CMC-gel delivery of antibody to the implant site can be rapid, limited only by outward diffusive transport of IgG in the high water-content gel.

In vivo IgG biodistribution over time: Serum and peritoneal lavage fluids collected from groups of mice treated with 10mg of IgG in the CMC-gel carrier and euthanized at various time points up to day 7 were used to compare systemic and local biodistributions of human IgG. Placebo (empty CMC-gel)-treated mice were analyzed only at 0h and no human IgG was detectable in any sample using this assay (data not shown). Figure 2 shows both the amounts of human IgG detected intraperitoneally and systemically over time in IgG-CMC-gel treated mice. Levels of human IgG detected from peritoneal lavage begin at 0.8mg/ml at 0h and decline thereafter until 3h post-administration. Consistent with in vitro release kinetics in Figure 1, CMC-gel rapidly releases most of its IgG in vivo with a spike observed 6-8h post-gel placement.

After day 2, only small quantities of human IgG are recovered from the abdominal cavity. Simultaneously, human IgG levels in serum (Figure 2) are first detectable at the first 3h post-gel placement time point (when the animals have completely recovered). A rapid increase in serum human IgG follows, spiking to almost 1.25mg/ml after 12h, and remaining constant at 0.85mg/ml for up to 4 days post-gel placement. Human IgG remains detectable by ELISA methods thereafter in both serum and peritoneal lavage for up to 7 days, reflecting prolonged bioavailability after gel release.

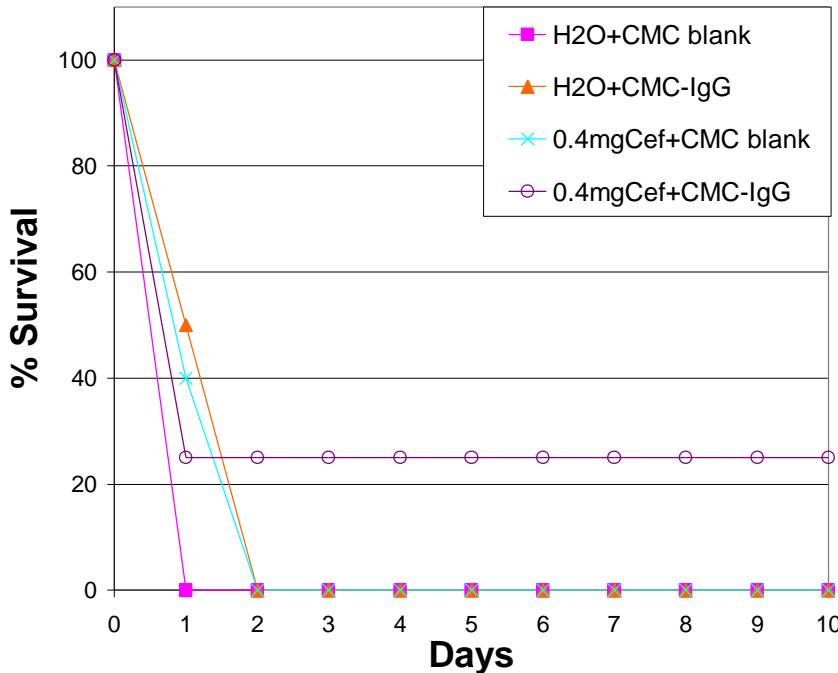
Local IgG administration against MRSA peritonitis: CMC-gel released IgG (10mg) was delivered against lethal challenges of MRSA (10^8 CFU) in 4 different abdominal implant infection experimental cohorts. Mouse survival with 10mg of IgG released from CMC-gel was not enhanced when compared to control mice treated with placebo (blank CMC-gel). Complete mortality was observed after day 4 in all cohorts with IgG monotherapy (data not shown).

**Figure 2:**

ELISA detection of human IgG levels in mouse serum (●) and peritoneal lavage fluid (▲) post bacterial challenge with *P. aeruginosa* (IFO3455, 10^5 CFU, $n=3-4$ mice per time point) and 10mg IgG treatment in CMC hydrogel carrier over 7 days. Human IgG was detectable in mouse serum after 3h, and remained present over the duration of the experiment.

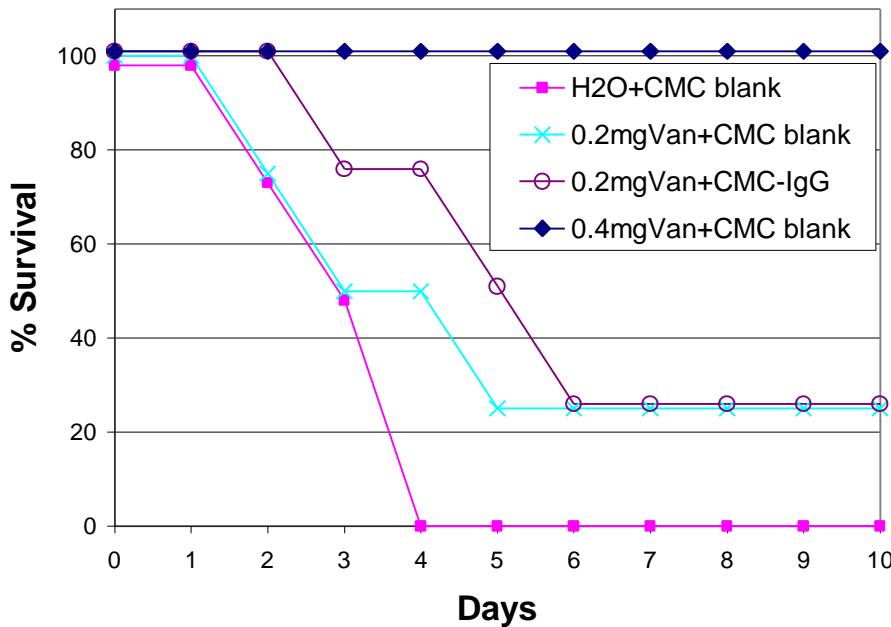
INSET: Human IgG levels in mouse serum and peritoneal lavage fluid in the first 12 hours post-surgery.

In a second set of experiments, mice were administered single systemic i.v. doses of cefazolin (increasing from 0.4 μ g to 400 μ g/mouse), ultimately approximating the clinical prophylactic dose in humans. No dose improved infection survival (data not shown), consistent with *in vitro* evidence indicating cefazolin resistance for this bacterial strain. Addition of 10mg of IgG in CMC-gel to 400 μ g systemic cefazolin prophylaxis i.v. resulted in improved survival to 25% for the combination therapy (shown in Figure 3), compared to 100% mortality in the three control groups (blank CMC-gel/systemic H₂O, CMC-IgG gel/systemic H₂O, and blank CMC-gel/systemic antibiotic). Although low, this survival enhancement was statistically significant against all other therapeutic scenarios using an ANOVA over 10 days ($p<0.05$).

**Figure 3:**

Mouse survival after MRSA challenge assessed with 400mg cefazolin i.v. tail vein injection both with and without IgG-CMC gel, delivered locally to the peritoneal cavity onto the implant surface at the time of surgery. The combination of cefazolin+IgG-CMC gel significantly enhanced survival of MRSA infection over therapeutic controls and cefazolin treatment alone (ANOVA $p<0.05$).

Prophylactic vancomycin was also infused systemically in a third set of MRSA-infected abdominal surgical experiments. Mice were administered increasing systemic i.v. vancomycin doses up to 400 μ g/mouse 30min prior to surgery, approximating the relevant clinical prophylactic dose in humans. The highest dose (400 μ g/mouse) of vancomycin conferred 100% survival (Figure 4), whereas the lower i.v. doses did not enhance survival as compared to control untreated mice (data not shown). An intermediate, sub-optimal dose of vancomycin (200 μ g) was then chosen for a further experiment to assess combination effects of additional, locally delivered human IgG on mouse survival in this model. Combination therapy (200 μ g vancomycin i.v. plus 10 mg of IgG delivered locally from the CMC-gel) only moderately improved survival to 25% after Day 6, a result no better than the survival percentage already observed in the 200 μ g vancomycin monotherapy group after day 5 (Figure 4). Additionally, lower doses of vancomycin (40 μ g) in combination with 10 mg of IgG delivered intraperitoneally from the CMC-gel did not enhance survival compared to 40 μ g of vancomycin i.v. alone (data not shown).

**Figure 4:**

Mouse survival after MRSA challenge and treatment with increasing prophylactic doses of vancomycin administered via tail vein injection, and blank CMC-gel delivered to the peritoneal cavity onto the implant surface. Survival experiments using a sub-optimal dose of vancomycin (200 mg/mouse) and local delivery of IgG via the CMC-gel carrier to implant surfaces showed no survival improvements over 200 mg vancomycin + blank CMC-gel (both 25% survival).

Survival studies were conducted analogously in this surgical implant infection model using lethal challenges of two virulent strains of gram-negative *P. aeruginosa*: IFO3455 (10^5 CFU in 500 μ l) or M2 (10^4 CFU in 500 μ l) administered directly onto the implant. Results in Figure 5 show the 10-day survival curves for mice treated with 10mg of IgG in 500 μ l CMC-gel versus placebo-treated mice (blank CMC-gel) as monotherapy (i.e., no antibiotics).

Significantly improved survival was achieved against IFO3455 infection using local IgG delivery without use of any antibiotics, producing 70% survival benefit while all control mice died by day 3 (ANOVA, $p<0.05$). Improved survival benefits were achieved using the same treatment and the more virulent *P. aeruginosa* strain M2.⁴⁹ The M2 bacterial challenge produced 80% mortality in the placebo-treated mice while 100% survival was observed in mice cohorts treated with a single prophylactic dose of 10mg IgG delivered to the implant from the CMC controlled-release hydrogel (Figure 5). These results are

statistically significant over the 10-day study period (ANOVA, $p<0.05$). Based on the significant efficacy of locally delivered IgG in enhancing survival in these gram-negative implant-centered infections, further studies with systemic antibiotics were discontinued.

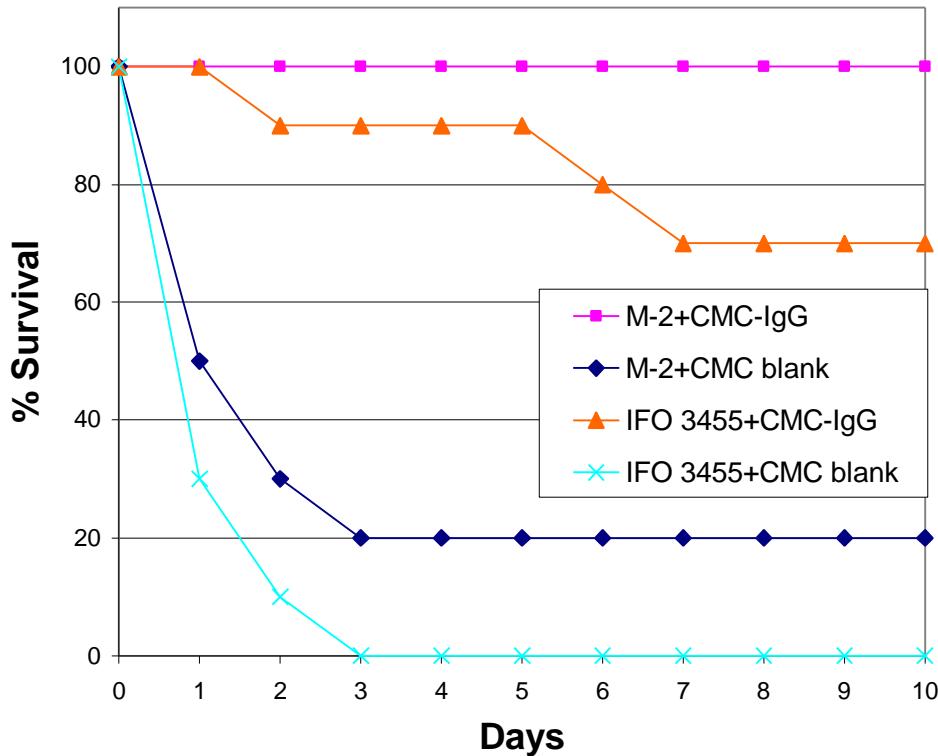


Figure 5:

Challenges of *P. aeruginosa* M2 (10^4 CFU) or IFO3455 (10^5 CFU) caused lethal infections in 80 or 100 percent of the control-mice, respectively. Local delivery of human IgG from the CMC-gel conferred significantly improved survival benefits against both pathogens, up to 100% survival after 10 days.

Discussion

A 2wt% pooled polyclonal human IgG formulation released from a hydrogel controlled release carrier was used to improve the survival of mice after abdominal implant surgery and subsequent challenge with either gram-positive and gram-negative pathogens. Mice received a polypropylene mesh implant and were challenged with lethal doses of MRSA or *P. aeruginosa* during surgery. Both pathogens were chosen because of their

frequency in postoperative and nosocomial (i.e., CAPD) abdominal implant infections and the difficulty encountered in successfully treating clinical, biomaterial-centered infections.^{48,49} Human polyclonal IgG in a CMC controlled-release gel carrier was released directly to the surgical wound and implant site in the peritoneal cavity.

Previous work has shown that human polyclonal IgG i.p. injection was effective in protecting mice against lethal closed abscess peritonitis induced by several *Pseudomonads*. In this study, however, similar mice and IgG doses as described before were applied and the resulting survival benefits were shown to be independent of either mouse strain or *P. aeruginosa* strain.⁴⁹ Timing of the local application of polyclonal IgG was important to optimize survival benefit, with prophylactic antibody applications producing the highest survival.^{22,49} Several significant differences distinguish the current study and this recently reported peritonitis model: (1) the murine infection models differ significantly, with earlier work using a closed abscess infection,⁴⁹ and the current study focusing on open surgical implant infection, and (2) the distinctly different delivery modes of antibodies. Previous work used i.p. injection,⁴⁹ known to provide a convenient route to rapidly systemize antibodies, while this current delivery method utilizes a controlled release matrix that delays systemization of IgG and sustains local delivery to the implant-centered infection. The impact of these differences lies in the kinetics and resulting bioavailability of the IgG doses to fight bolus lethal bacterial inocula, and in the increased trauma and host compromise suffered through the surgical procedure and the presence of the implant.

Comparable surgical implantation and bacterial challenge experiments were performed by Ward and coworkers¹¹ using rabbits which, prior to surgery, were primed with killed *P. aeruginosa* to induce high systemic circulating levels of specific IgG in serum to combat inocula of the same species. Their results demonstrated the failure of the peritoneal defense mechanisms to clear the infection in the presence of a biomaterial device even with these primed animals and high *systemic* levels of IgG. Our results, however, show remarkable improvement in animal survival using a *local* presence of pooled polyclonal IgG in the abdominal implant site in gram-negative *P. aeruginosa*

infections. This difference could be due to three possible factors: 1) immediate IgG-mediated reduction of biomaterial colonization by *P. aeruginosa* in the initial period after surgery,³⁷ 2) lack of distribution and diffusion barriers for IgG between the highly perfused peritoneum and systemic circulation, providing rapid biodistribution of protective antibodies, and 3) substantial non-specific opsonization benefit for gram-negative bacteria due to the wide variety of antibodies naturally occurring in pooled polyclonal human IgG.^{53,54}

Human polyclonal antibodies demonstrate sufficient binding titers *in vitro* against infective pathogens. Since antibody release from the gel *in vitro* was rapid and diffusion limited, nearing 100% release within 30h (Figure 1), and human antibodies released *in vivo* from the gel in the peritoneal cavity were detected in mouse serum after 3h (Figure 2), exogenous human IgG is presumed to become rapidly available to opsonize bacteria at the implant site. Nevertheless, initial amounts of IgG assayed in the peritoneal cavity (0, 2, and 3h time points) were lower than expected (0.8mg/ml in ~4ml of lavage fluid). This is attributed to the lavage technique, which cannot account for IgG remaining within the gel carrier at the implant site and does not sufficiently affect dissolution of residual gel with associated IgG. Complete recovery of the animals and dissolution of the CMC-gel carrier allowed for the sustained release of the remaining human IgG to mice systemic circulation, shown as the serum spike after 12h (~1.25mg/ml serum).

While the addition of high dose systemic antibiotics in both pathogenic infections significantly improved survival, only the human equivalent dose of vancomycin provided complete protection against gram-positive infection. The pronounced differences observed between the efficacy of polyclonal IgG against the gram-positive and gram-negative challenges may be attributed to the differences in antibody opsonization efficiency and reactivity due to presence of protein A on the surface of the gram-positive MRSA. Protein A can non-specifically “reverse bind” IgG thereby effectively preventing it from opsonizing the bacteria for phagocytic clearance.^{37,51} Another factor may be the dramatic changes in surface characteristics of the gram-negative *P. aeruginosa* in the presence of IgG. These surface changes have been attributed to substantial adsorptive

interactions of non-specific human IgG with these bacteria that can lead to non-specific IgG mediated killing.^{37,53,54} This effect is additive to the specific opsonization occurring against *P. aeruginosa* and has not been reported for gram-positive organisms such as *S. aureus*, in which only pathogen-specific antibodies are capable of enhancing humoral immune-mediated clearance.^{53,54}

Comparisons of the *P. aeruginosa* inoculum doses used to produce 80-100% mortality in CF-1 mice in the presence of a surgically implanted biomaterial with earlier experiments involving the same bacteria and mouse strain but without biomaterial implantation or open abdominal surgery⁴⁹ are interesting. The LD₉₀₋₁₀₀ doses used previously in a closed abscess peritonitis infection model⁴⁹ (10^7 CFU for both *P. aeruginosa* strains injected directly into the peritoneal cavity) were a log-order higher than inoculum needed to cause 100% mortality after open abdominal surgery without the polypropylene mesh implant (i.e. 10^6 CFU of *P. aeruginosa* IFO 3455, data not shown). Additionally, only 10^5 CFU (IFO 3455) and 10^4 CFU (M-2) killed 80-100% of mice in the surgical infection model if the biomaterial was implanted. This comparison provides further evidence of the compromising influence of both open surgical procedures and biomaterial implants on the host's immune response to effectively fight contaminating organisms inside a surgical wound.^{1,4,11}

Results for this abdominal surgical infection model confirm the increased propensity for infectious complications associated with surgically implanted biomaterial devices. Biomaterial-centered infection with both gram-positive MRSA and gram-negative *P. aeruginosa* strains with abdominal polymer implants consistently produced host mortality if no therapeutic was prophylactically applied. Moreover, methicillin resistant *Staphylococcus aureus*-induced biomaterial-centered infection was demonstrated to be more refractory to (1) sub-optimal monotherapy using systemic clinically relevant antibiotics (2) local polyclonal antibody monotherapy, and (3) combinations of these two treatments.

The local, controlled delivery of antibodies from a matrix or carrier directly to implant sites prone to colonization shows therapeutic potential. Pooled human IgG could be delivered locally to the peritoneal cavity or other sites of bacterial contamination or infection (e.g., appendicitis, diverticulitis, primary anastomosis surgery) using a biocompatible hydrogel vehicle capable of rapid release of bioactive antibodies to enhance host survival after contamination with relevant gram-negative organisms. We suggest, therefore, a role for locally applied IgG as a supplement to systemic antibiotic prophylaxis to potentiate local host immune responses. Antibodies function to clear pathogens via mechanisms that circumvent further antibiotic resistance, a role that complements the strengths and eliminates some of the weaknesses of current antibiotic prophylaxis. Ultimate success against infection depends on antibody-mediated neutralization of pathogens, the pharmacodynamics (e.g., release rates, clearance, bioavailability, biodistribution) of delivered antibody, required doses against various infections, and the virulence of clinically relevant pathogens.

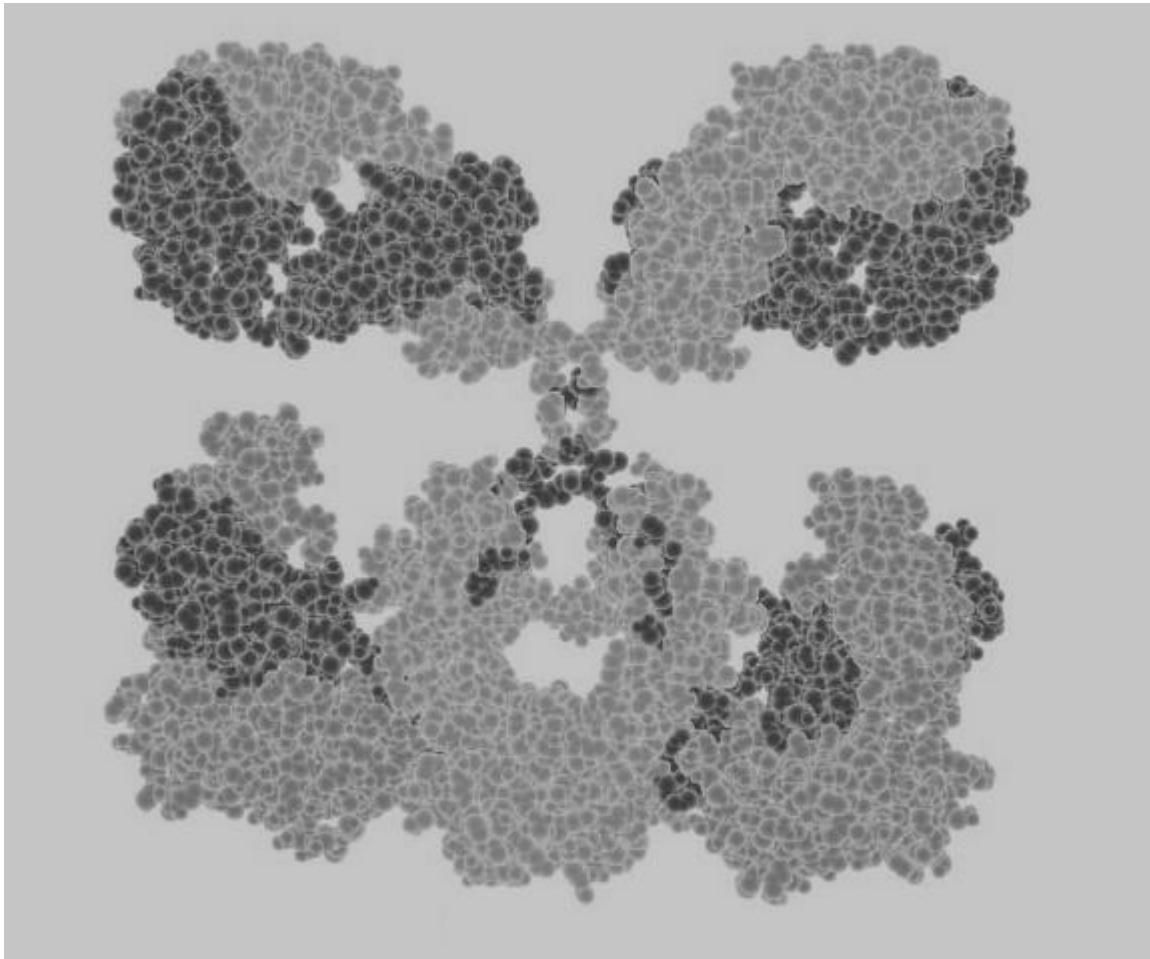
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General discussion



“The antibiotic using community requested, and the drug makers spun out new, or modified versions of old antibiotics to treat the dozen major types of disease-causing bacteria. The consequence: bacteria were trained to find new ways to penetrate the defences.”

(Elyse Tanouye, The Wall Street Journal, June 25, 1996)

“Too few new drugs are being developed to replace those that have lost their effectiveness. In the race for supremacy, microbes are sprinting ahead.”

(World Health Report 1996, WHO)

Over the last two decades, the rapid evolution of antibiotic resistance has become a major problem in managing infectious diseases. With 80% of all *Staphylococcus aureus* isolates exhibiting resistance to methicillin in the United States¹ and showing an increased resistance to vancomycin,²⁻⁶ the era of antibiotics is unavoidably coming to an end.^{7,8} Fortunately, bacteria that acquire antibiotic resistance genetically do not change their surface characteristics or surface epitopes targeted by human IgG.⁹ Thus antibody opsonization remains effective in facilitating clearance by the host immune system. This thesis describes the investigation of an alternative method to prevent (biomaterial-centered) infection using locally applied, pooled polyclonal human IgG.

Results of the described research indicate that the behavior of *Pseudomonas aeruginosa* to a glass surface, resembling a biomaterial device, could be significantly influenced (Chapter 2) by the presence of IgG. Both the pre-adsorption of IgG to the glass surface, as well as the pre-opsonization of bacteria with IgG significantly reduced the initial deposition rates and increased the generation times of adherent bacteria. Visual observations of individual bacteria suggested that bacteria were physically inhibited from ‘stretching’ or cell body expansion prior to separation after their division in the presence of adsorbed immunoglobulins.

Subsequently, mouse-survival was significantly improved with prophylactic use of local IgG against lethal peritonitis challenges with the same bacterium as used on the glass surfaces (Chapter 3). The data indicated that locally delivered human IgG lowered the incidence and severity of infection by reducing locally the acute bacterial burden and cytokine production and systemically, by inhibiting sepsis.

However good results were achieved against different *Pseudomonads*, the efficacy of local human IgG to prevent biomaterial-centered infection in surgical wounds contaminated with methicillin-resistant *Staphylococcus aureus*, a clinically relevant organism in spinal implant infection (Chapter 4), was less convincing (Chapter 5). IgG-lavaged, stainless-steel K-wire implant sites showed significantly improved clinical signs of infection incidence and magnitude compared to saline-lavaged controls, corresponding with significant biofilm reduction on the implanted K-wires. Surrounding soft-tissues, however, still contained clinically significant numbers of viable bacteria in all the tested sites.

In the next stage, immune systems of mice potentiated by locally applied human IgG alone, could not overcome lethal bacterial challenges with *Escherichia coli* and *Klebsiella pneumoniae* (Chapter 6). Bacterial killing and therapeutic efficacies of a clinically routine prophylactic antibiotic (ceftazidime, 3rd generation cephalosporin) was nonetheless significantly improved when administered *in tandem* with locally applied IgG, leading to significantly improved survival results. In some cases, the combination therapy of local IgG and systemic antibiotics exhibited even synergistical benefits, that could be general to many other infections and could have important clinical implications for extending the clinical lifetime of front-line antibiotics facing resistance, as well as in treating antibiotic resistant infections.

In the end, (Chapter 7) the local, controlled delivery of human IgG from a gel-carrier directly to polypropylene implant sites prone to colonization and bacterial infection showed therapeutic potential and is advocated as a supplement to systemic antibiotic prophylaxis to potentiate the local host immune responses. Rapid release of bioactive antibodies in areas of bacterial contamination (e.g., appendicitis, open fractures) could function to clear pathogens via mechanisms that circumvent further antibiotic resistance, a role that complements the strengths and eliminates some of the weaknesses of current antibiotic prophylaxis, and one of our necessary future tools in the battle against (antibiotic-resistant) biomaterial-centered infection.

Future prospects

The blood-derived therapeutic IVIG is currently prepared in millions of doses annually, using Cohn fractionation and solvent/detergent treatment to eradicate possible bacterial and viral contamination. However, because of the concern of spreading blood-borne pathogens, IVIG products have been bypassed by various antibody technologies providing the means to generate unlimited amounts of monoclonal antibodies (Mabs) without *any* risk of viral- or prion-mediated disease transmission. Additionally, reduction in IVIG titers, resulting from careful blood donor screening and a general crisis in the short supply of IVIG worldwide, have both prompted new efforts to use Mabs to control antibody titers and supply. In recent years, major advances have been made beyond the original monoclonal hybridoma technology using mice¹⁰ to mass produce specific therapeutic Mabs, humanize murine Mabs¹¹ and even generate human antibodies in plants.¹²⁻¹⁴ Currently, production costs for novel human or mouse Mab technology are significantly higher than the cost of pooled human IgG products. Nonetheless, the first monoclonal IgG products have entered clinical testing markets¹⁵⁻¹⁷ and can be effective in much lower doses than IVIG due to high specific antiviral or antibacterial titers.

For example, the human polyclonal hyperimmune anti-RSV globulin, RespiGam®, (MedImmune, Gaithersburg MD) exhibits at least a five-fold greater viral neutralization titer against RSV than standard IVIG.¹⁵ Recently, however, RespiGam® was bypassed on the market by MedImmune's monoclonal antibody product, Synagis™, against RSV infection. This Mab demonstrates 50-100 times increased potency in the cotton rat model of RSV prophylaxis.^{16,17} Although seemingly expensive (\$4,500 mean cost per five month anti-RSV-treatment session),¹⁸ the prophylaxis using RespiGam® was still 15 times less expensive than one hospitalization for serious RSV infection, while RSV-related hospitalizations in prophylactically treated patients could be prevented by 41-57%.¹⁹ Synagis™ is now the treatment of choice against RSV.

Global increase in Mab use will also allow companies to upscale Mab production, leading to a reduction in overall manufacturing cost and reduced wholesale prices of the final products. Production cost forecasts for "plantibodies"¹²⁻¹⁴ claim unit Mab costs of \$10-100/g, bringing this raw material cost in line with other competitive drugs.

A major disadvantage of monoclonal antibody therapy, compared to the pooled polyclonal immunooglobulins studied in this thesis, lies in the limited spectrum protection exhibited by Mab products due to the presence of highly specific antibacterial or antiviral antibodies targeting one epitope. Polyclonal antibodies exhibit broad specificities against families of epitopes or different epitopes on a single antigenic structure and, therefore, exhibit a wide range of binding activities and affinities against targets in different environments. Such a range results directly from various combinations of light and heavy chain primary sequences and their combinations (discussed in Chapter 1) to produce binding sites (Fab). However, biosynthetic design and productions of Mabs that specifically target antigenic epitopes common to entire strains or families of viruses or bacteria significantly reduces this disadvantage of monoclonal antibody prophylaxis or therapy. In the example of Synagis™, the antibody targets the so-called F protein that shows a high degree of cross-reactivity between subtypes of the RS-virus, rendering the therapeutic extremely effective against all current RSV strains. Likewise, antiherpes antibodies produced in plants are currently under development to prevent the spread of this sexually transmitted disease by local release of this therapeutic in the vagina.¹³

Current prophylactic, generalized use of broad-spectrum antibiotics has a significant disadvantage over the application of pooled human IgG and future highly specific monoclonal antibodies in fighting infections. Antibiotics are not specifically directed against pathogenic microbes; Their microcidal action also eliminates commensal flora and viable cellular elements, essential components of the body's armor against infection, therewith compromising the efficacy of the human immune response. These include strains of intraintestinal *E. coli* responsible for digestion and other cellular immune elements. Reducing the antibiotic "overkill" will render immunotherapy-receiving patients more immunocompetent to prevent pathogenic colonization and infection by maintaining and exploiting their own flora.

Many companies are currently investigating the development of other anti-microbial Mabs, including anti-menigococcal, anti-pneumococcal, and anti-Borrelia Burgdorferi (Lyme disease) antibodies (e.g., Smithkline-Beecham, Genentech, MedImmune,

Centacor, IDEC, NABI) with Mab designs selected carefully to target surface molecules conserved for centuries across species. Future Mab improvements and mixed Mab cocktail products will likely further broaden the current narrow spectrum of protection that characterize typical monoclonal antibodies.^{20,21} This will allow scientists to design and manufacture new anti-infective products that are badly needed to effectively address infectious disease in the antibiotic resistance era. However, current progress in new antibody and antibiotic synthesis may not be able to keep-up with the rapid increase in antibiotic resistant mechanisms and organisms. During this interim period where antibiotics are rendered less and less effective and potent monoclonal antibody therapies have not yet matured, hyperimmune pooled polyclonal immunoglobulins should prove very useful as a bridge therapy. By immediately opsonizing and paralyzing invading bacteria during surgical intervention within the “decisive period” resulting in possible infection,^{22,23} high titer, broad-spectrum IgG could assist the immune system to rapidly neutralize bacteria and significantly reduce biofilm formation and biomaterial-centered infection with pathogenic bacteria.

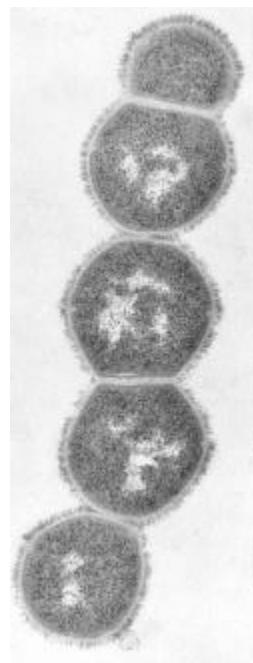
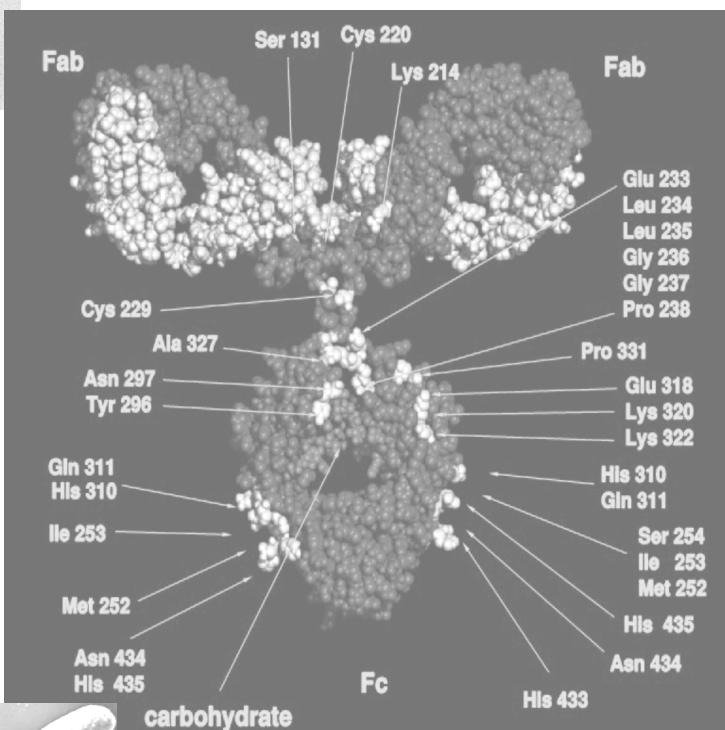
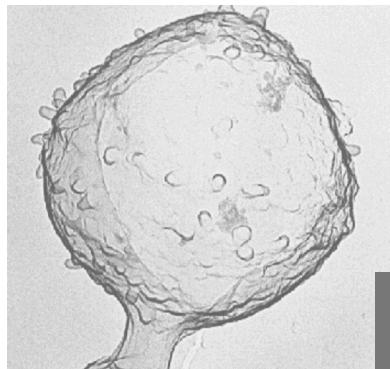
Subsequent combinations of systemic and local applications of monoclonal antibody cocktails, tailored towards specific bacterial and viral organisms and patient risk-groups, represent a logical evolution in this immunotherapy already identified in both scientific and laypersons literature. Experience gained using systemic and local applications of human IgG from many sources, by several application routes, and over many years will provide clinicians with the necessary knowledge, confidence and resources to develop non-blood derived monoclonal antimicrobials to effectively combat infections caused by antibiotic-resistant bacteria in this millenium.

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Summary



More than a century ago, medical doctors and researchers began isolating and applying sera from healthy donors to treat patients suffering from various infectious diseases. Early in this century, antibody infusion was the standard treatment for pneumococcal pneumonia and meningitis until antibiotics replaced them in the 1940's. The interest in antibody therapy then faded away, but with increases in immunocompromised individuals (e.g., HIV, elderly and obese patients and low birth-weight neonates) and antibiotic resistant pathogens, renewed attention and research efforts are focussed on antibody therapy. After approval of intravenous immunoglobulin products (IVIG) for human use by the FDA in 1981 clinical trials demonstrated large benefits of IgG therapy for neonates and immunocompromised patients to overcome lethal bacterial and viral infections. The blood-derived IgG product became safer after solvent/detergent treatment in addition to Cohn's original cold-ethanol fractionation process and highly purified hyperimmune globulins are now routinely manufactured, directed specifically against virulent pathogens. Pooled human polyclonal IgG could become a significant therapeutic in the approaching post-antibiotic era, because IVIG is an 'up-to-date' pooled human immunoglobulin product comprising thousands of specific opsonic antibodies that can improve clearance of bacteria, viruses and toxins by the host immune system. This could provide clinicians with an important therapeutic in the battle against antibiotic resistant organisms.

However, systemic, prophylactic IgG treatment is expensive, requiring large quantities of IVIG to be administered over time to achieve sufficient tissue levels to potentiate the immune response against invading pathogens. Direct, local application of IgG could overcome distribution and diffusion barriers from systemic application, reduce the required dosages per treatment and risks of adverse side effects and decrease the costs associated with IgG treatment. These were the main reasons, as described in **Chapter 1**, to investigate the efficacy of locally applied, pooled human IgG to reduce or prevent bacterial infection.

Chapter 2 describes the direct effect of pooled human IgG on the *in vitro* adherence of *Pseudomonas aeruginosa* IFO3455 in a parallel plate flow chamber. Adherence of IgG to this bacterium and pre-adsorption of IgG to the glass surface both effectively reduced initial pathogen deposition rates and subsequent surface colonization and growth. Bacterial surface characteristics changed in the presence of opsonized IgG as measured by zeta-potentials, water contact angles and X-ray photoelectron spectroscopy. Water contact angles dropped from 120 degrees to 64 degrees after IgG opsonization, while XPS elemental ratio changes for nitrogen, oxygen and phosphorus each compared to carbon were consistent with the adsorption of antibodies (e.g., amide groups) to the bacterial cell wall surfaces.

Based on results from three *P. aeruginosa* strains (IFO3455, M2 and MSRI7072), **Chapter 3** describes the prophylactic protection of local IgG against peritonitis in a murine model. Host survival was significantly increased in a dose-dependent manner following local intraperitoneal application of IgG and lethal bacterial challenges: 100% survival up to 6 hours prophylactically or at the time of bacterial challenge with 10mg of IgG. Therapeutic 10mg IgG treatment administered up to 12 hours post-challenge also significantly increased host survival. Tissue bacterial burden was significantly reduced in the liver, peritoneal lavage and the blood after IgG treatment, correlating with reduced serum IL-6 levels as a marker for sepsis.

To study the efficacy of local IgG to prevent biomaterial-centered infection, a new spinal implant infection model in rabbits was initially developed and described in **Chapter 4**. The incidence of postoperative spinal implant infection in adult spinal surgery is up to 8% due to the length of the procedure, the amount of tissue damage and the creation of dead space necessary for the implantation of spinal stabilizing systems -- the highest rates in postoperative wound infection. In the rabbit spinal implant infection model, a virulent methicillin resistant *Staphylococcus aureus* strain (ATCC33593) was used to inoculate three non-contiguous spinal implant sites with stainless steel, threaded Kirschner-wires ('K-wire', orthopaedic osteosynthesis material) after partial laminectomy to create the dangerous 'dead-space' defects. Inoculation of the sites with 5×10^2 CFU

appeared to cause a consistent, biomaterial-centered infection in all challenged sites, while cross-contamination and systemic spread of the infections were prevented. Control procedures lacking K-wire implantation required substantial higher inocula (10^4 CFU MRSA) to establish an infection, proving the local immune compromising effect of the biomaterial implant.

The spinal implant infection model in rabbits described in **Chapter 5** was then used to study the efficacy of IgG, locally applied to the site of spinal implantation and bacterial challenge to prevent postoperative biomaterial-centered infection. Multiple aqueous lavages of isotonic saline were compared to the same procedure using 1wt% pooled human IgG applied directly to the surgical implantation sites. Since three sites could be used in each rabbit, the total number of animals necessary for this study could be reduced by two-third, while IgG lavaged sites and saline-lavaged controls were present in one animal. Visually observed clinical signs of infection were supported by bacterial enumeration from multiple biopsied tissue and bone sites post-mortem, 7- and 28 days post-surgery. Clinical signs of postoperative infection were significantly reduced in the IgG-lavaged sites, while bacterial enumeration also exhibited statistically significant reductions in the soft tissues and bone, and on the stainless steel implants using IgG lavage compared with saline. After 28 days, complete healing of the surgical wounds was seen in all sites.

In the battle against infection, combination therapies comprising multiple intravenous antibiotics alone or in tandem with either intravenous immunoglobulins or local antibiotics have all been investigated and used in clinical scenarios. In **Chapter 6**, the potentiation of systemic antibiotics by locally applied IgG is described, using two different *in vivo* murine models. The anti-microbial efficacy of ceftazidime (third generation cephalosporin) against both *E. coli* induced peritonitis and *K. pneumoniae* induced burn-wound infection was significantly improved in combination with locally applied IgG. Synergistic improvements in animal survival by reduced sepsis indicators and bacterial burden post-mortem were observed with this treatment combination. Because the additional immunotherapy functions independently of antibiotic resistance

mechanisms, local delivery of polyclonal or monoclonal antibodies together with routine intravenous antibiotics could confer, also in the clinical situation, improved protection against infection and enhance the efficacy of front-line antibiotics.

Chapter 7 describes the experiments with a newly developed abdominal implant infection model in mice to study the efficacy of local IgG in a hydrogel carrier to prevent polymer biomaterial-centered infection with MRSA, and *P. aeruginosa* strains IFO3455 and M2. During open abdominal surgery, a polypropylene mesh implant (1x1cm.) was introduced into the abdominal cavity of the mice and challenged with different amounts of bacteria. Subsequently, 0.5ml of hydrogel with or without 10mg IgG was used to cover the implant. *In vivo* IgG antibody release from the hydrogel was rapid to surrounding tissues, nearing a 100% *in vitro* after 48 hours in PBS. Although released IgG alone or in combination with the systemic antibiotics cefazolin or vancomycin did not enhance survival in mice challenged with MSRA (100% mortality after 72 hours), significantly improved survival was observed in cohorts of mice against both gram-negative *P. aeruginosa* strains with IgG monotherapy released from the hydrogel.

The results of these studies are discussed in the perspective of current antibiotic-resistance problems and poly- and monoclonal antibody developments in **Chapter 8**, the general discussion of this thesis.

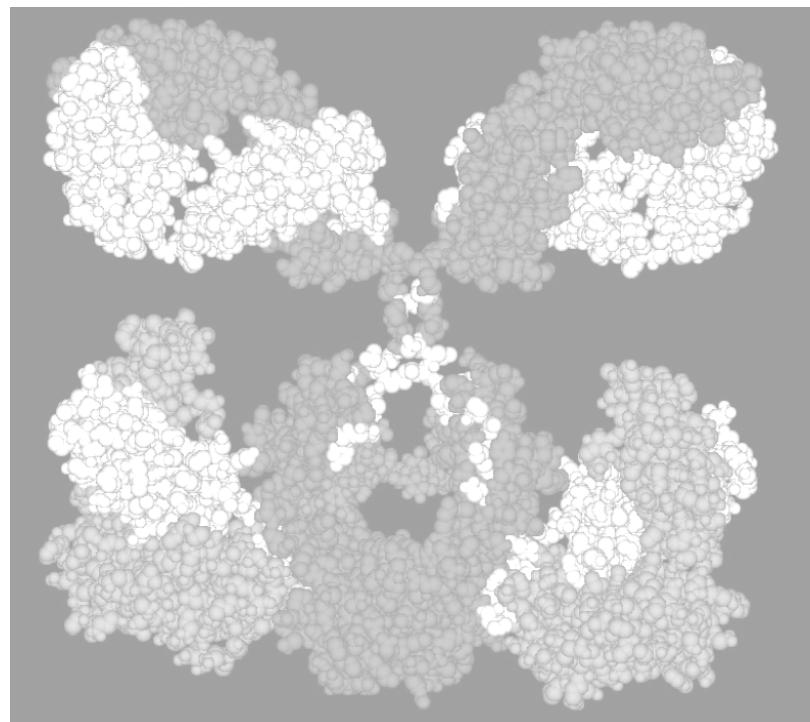
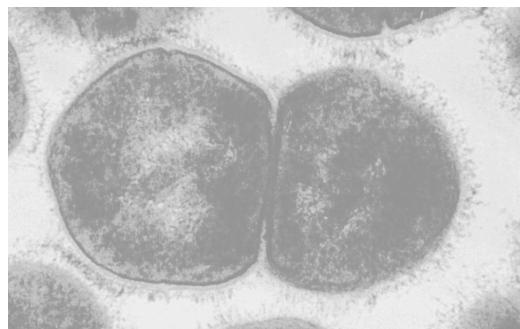
In summary, this thesis describes different studies investigating the efficacy of local application of pooled human IgG against bacterial, biomaterial-centered infection. Although local application of IgG appeared to be more efficacious than systemic IVIG administration in *in vivo* studies and could be administered in lower dosages to achieve those improved benefits, complete prevention of bacterial infection with single prophylactic applications of local IgG was not possible. Clearance of encapsulated microorganisms requires large amounts of specific antibodies for successful opsonization. These quantities are only available in highly specific hyper-immune globulins or monoclonal antibody cocktails, but unfortunately not in pooled human immunoglobulin products. However, once combined with standard intravenous antibiotic

therapy, even synergistically improved anti-infection results were achieved in some cases with combinations of antibiotics and the local application of pooled human IgG.

The combination of systemic antibiotics with local IgG could provide clinicians with an alternative prophylactic therapeutic treatment against antibiotic-resistant organisms. By combining these antibiotics additionally with locally applied hyperimmune sera or monoclonal antibody cocktails against specific bacteria for high-risk individuals, the required dosages can be reduced while achieving maximum protection of the host against disease-causing organisms at the site of surgical implantation. Immediate postoperative infection and probably also latent biomaterial-centered infections can be reduced if contaminating bacteria can be prevented from compromising initial tissue integration. This will hopefully result in the achievement of the ultimate goal of this research: an increased quality of life for many patients receiving artificial joints and other implanted biomaterials that restore both form and function as well as newly designed *tissue engineered* scaffold implants.

Because the use of implanted devices will only increase, the magnitude of the associated infection problem must be addressed with solutions that overcome antibiotic resistance. Exploitation of natural immune mechanisms is an important alternative to conventional therapies with the direct application of antibodies as an attractive addition to this mechanism.

Samenvatting



Meer dan een eeuw geleden werd het serum van gezonde donoren al gebruikt voor het behandelen van patiënten met verschillende infectieuze aandoeningen. De standaard behandeling aan het begin van deze eeuw voor pneumococcen pneumonie (longontsteking) en meningitis (hersenvlies ontsteking) was infusie met antilichamen uit dat serum, totdat antibiotica werden ontdekt in de veertiger jaren. De interesse in antilichaam-therapie verdween langzaam maar zeker, maar met een toename in patiënten met een verminderde afweer (HIV-geïnfecteerden, ouderen, patiënten met zwaar overgewicht en premature kinderen) en antibiotica-resistente bacteriën in de laatste jaren is de interesse voor antilichaam therapie weer toegenomen. Goedkeuring van immunglobulinen (IgG) voor intraveneuze toediening (IVIG) aan mensen door de *Food and Drug Administration (FDA)* in Amerika volgde in 1981 en in verschillende klinische studies werd aangetoond dat ernstige infecties makkelijker konden worden overwonnen door premature kinderen en patiënten met een slechte afweer na systemische behandeling met IgG.

Het uit bloed gezuiverde IgG bleek een ‘up-to-date’ middel tegen zelfs de nieuwste pathogene microorganismen, omdat het menselijk lichaam steeds nieuwe IgG kan produceren tegen nieuwe organismen die dagelijks het lichaam aanvallen. De IgG produkten werden bovendien veiliger door verbeterde donor-screening, en *solvent/detergent* behandeling werd toegevoegd aan de zogenaamde “Cohn” koude-ethanol fractieatie produktie methode om de risico’s voor het overbrengen van hepatitis en HIV verder te reduceren. Tegen hoog virulente pathogenen worden momenteel bovendien uiterst specifieke en gezuiverde hyper-immunglobuline produkten geproduceerd. Het leek er dan ook op begin jaren negentig dat humaan gepoolde polyclonale IgG een belangrijk wapen kon worden in de strijd tegen antibiotica-resistente bacteriën in het naderende post-antibioticum tijdperk.

De intraveneuze behandeling met IgG tegen infecties is echter bijzonder duur want grote hoeveelheden (tot 30 gram/dag/patiënt) schaarse IgG zijn nodig voor de behandeling van één persoon om een anti-infectieus effect te krijgen. Directe, lokale behandeling van gecontamineerde of geïnfecteerde weefsels is goedkoper, zou distributie en diffusie

barrieres vanuit de circulatie kunnen wegnemen, de hoeveelheid benodigde IgG per patiënt en het risico voor mogelijke bijwerkingen kunnen reduceren. Dit waren de meest voor de hand liggende redenen zoals beschreven in **Hoofdstuk 1**, voor het onderzoeken van de effectiviteit van direct, lokaal gebruik van IgG ter preventie van biomateriaal geassocieerde, bacteriële infecties.

In **Hoofdstuk 2** wordt het effect van IgG beschreven op de aanhechting van de bacterie *Pseudomonas aeruginosa* IFO3455 in een doorstroom kamer. Hechting van IgG aan het oppervlak van de bacterie en IgG pre-adsorptie aan de glas plaatjes van de doorstroom kamer leidden beide tot een significante reductie van initiële bacteriële hechting en de daaropvolgende oppervlakte colonizatie en groei. De oppervlakte eigenschappen van de bacterie, gemeten met behulp van randhoeken, zeta-potentialen en gamma-fotoelectron-spectroscopie (XPS), lieten een duidelijke verandering zien na de hechting van IgG aan de bacterie. Randhoeken gemeten met water namen af van 120 graden tot 64 graden na IgG hechting. Het voorkomen van de elementen stikstof, zuurstof en fosfor ten opzichte van koolstof veranderden consistent met de aanhechting van antilichamen (eiwitten met amide-groepen) aan de celwand van *P. aeruginosa*. IgG bleek de oppervlakte eigenschappen en daarmee het hechting gedrag van deze bacterie te veranderen.

Hoofdstuk 3 beschrijft vervolgens de profylactische bescherming van lokale IgG behandeling in een muis-model voor peritonitis. Drie verschillende *P. aeruginosa* stammen (IFO3455, M2 en MSRI7072) werden gebruikt om een buikvlies ontsteking te veroorzaken in de intra-abdominale ruimte van de muizen. IgG werd op verschillende momenten vóór, tijdens en na die contaminatie eveneens geïnjecteerd in de buikholte. Significant langere, dosis afhankelijke overleving werd gevonden in de behandelde muizen in vergelijking met de controle groepen. Tot 100% overleving werd gezien in de groepen waar 10mg IgG profylactische werd toegediend (6- en 3-uur vóór en tijdens de letale bacteriële inoculatie) en toegediend tot 12 uur volgend op de bacteriële inoculatie, gaf 10mg IgG een significante verbeterde overleving vergeleken met de controlegroepen. Bacterie aantallen post-mortem in lever-, intra-abdominale lavage- en

bloed-monsters was in alle IgG behandelde groepen significant lager, hetgeen overeen kwam met serum interleukine-6 (IL-6), als een determinant voor sepsis.

Teneinde de effectiviteit van IgG ter voorkoming van biomateriaal-geassocieerde infecties te kunnen bestuderen hebben we een diermodel ontwikkeld om postoperative wondinfectie te kunnen bestuderen na wervelkolom fusie chirurgie. De incidentie van postoperatieve wondinfectie na dergelijke chirurgie in volwassenen kan oplopen tot 8 procent als gevolg van de lengte van de ingreep, de weefselschade die wordt aangebracht, de implantatie van schroeven en staven en het creeëren van ‘dode-ruimte’ waarin bacteriën kunnen groeien in achtergebleven bloed. **Hoofdstuk 4** beschrijft de ontwikkeling van dit model waarin de hoog virulente methicilline resistente *Staphylococcus aureus* ATCC33593 (MRSA) werd gebruikt als klinische relevante bacterie voor het veroorzaken van postoperative, biomateriaal-geassocieerde infecties na wervelkolom fusie chirurgie. Drie dezelfde, onafhankelijke operaties -partiële laminectomie voor het maken van ‘dode-ruimte’- en implantaties van roestvrij stalen Kirschner-draden met schroefdraad ('K-draden', bot-fixatie materiaal) werden uitgevoerd in de wervelkolom van één konijn, waarna slechts 500 kolonie vormende units (CFU's) MRSA genoeg bleken om een consistente, biomateriaal-geassocieerde infectie te veroorzaken. De drie onafhankelijke locaties in iedere wervelkolom veroorzaakten geen onderlinge contaminatie, terwijl de infecties niet systemisch werden. Controle operaties zonder K-draad implantatie bleken met significant grotere aantallen bacteriën (10.000 CFU's) gecontamineerd te moeten worden teneinde een infectie te veroorzaken. Dit geeft aan hoe de lokale afweer verzwakt wordt in dit model na de implantatie van het biomateriaal. Doordat de drie onafhankelijke operaties in één konijn volledig op zichzelf stonden, was het model uiterst geschikt om IgG therapiën te testen en te vergelijken met een positieve en negatieve controle in één en hetzelfde konijn. Hierdoor kon het aantal benodigde dieren voor deze studies met twee-derde worden terug gebracht.

In **Hoofdstuk 5** is het wervelkolom chirurgie model in konijnen toegepast voor het bestuderen van lokale behandeling met IgG in een chirurgische lavage. Isotone 0.9% NaCl oplossingen (standaard lavage vloeistof) werden vergeleken met 1 gewichts

procent (1wt%) IgG bevattende lavages wat betreft het vermogen om infecties te voorkomen nadat de chirurgische wonden tijdens implantatie van K-draden waren gecontamineerd met 500 CFU MRSA. Klinische tekenen van infectie werden samengevoegd met de resultaten van bacteriële kwantificering van meerdere biopsieën van spier- en bot-weefsels post-mortem, 7- en 28-dagen na de initiële chirurgie.

Klinische tekenen van postoperatieve infectie waren significant gereduceerd in wonden die behandeld waren met IgG, en kleinere aantallen bacteriën werden post-mortem gekweekt in alle monsters (huid-, spier- en bot-biopsiën) en van de K-draden uit de IgG-behandelde wonden. Hoewel klinisch duidelijk verbeterd ten opzichte van placebo behandelde controles, bleek het niet mogelijk de geïnitieerde infecties na 7 dagen volledig te genezen met uitsluitend lokale IgG behandeling. Er werden nogsteeds grote hoeveelheden bacteriën gekweekt uit weefsel biopsieën en macroscopische wondgenezing ten opzichte van ongeïnfecteerde controles was trager. Na 28 dagen waren alle wonden wel volledig geheeld, onafhankelijk van de behandeling.

In de strijd tegen ernstige infecties zijn combinatie therapiën van i.v. antibiotica met locale antibiotica, andere i.v. antibiotica of systemische IgG lang bekend en onderzocht op hun effectiviteit. **Hoofdstuk 6** beschrijft de combinatie therapie van i.v. antibiotica met lokale IgG behandeling in twee verschillende diermodellen. Deze resultaten tonen aan dat de anti-microbiële effectiviteit van ceftazidime (een derde generatie cefalosporine) tegen *Escherichia coli* peritonitis en *Klebsiella pneumoniae* brand-wond infecties significant kon worden verbeterd in combinatie met lokale IgG behandeling. Synergistische verbeteringen in overleving door een reductie in de mate van sepsis en bacterie aantallen in de weefsels post-mortem werden gezien in beide *in-vivo* situaties. Omdat immunotherapie onafhankelijk werkt van antibiotica-resistantie mechanismen zou de combinatie van systemische antibiotica met lokale behandeling van polyclonale en/of monoclonale antilichamen, ook in een klinische situatie, de weerstand tegen infecties kunnen verhogen, en de effectiviteit van eerste-keus antibiotica kunnen verbeteren.

Hoofdstuk 7 beschrijft de studies gedaan in een nieuw muis-model voor intra-abdominale polymeer ge-associeerde infecties. We bestudeerden de biomateriaal-infectie preventie door lokale IgG in een *hydrogel carrier* tegen MRSA en *P. aeruginosa* IFO3455 en M2. Tijdens open abdominale chirurgie werden 1x1cm polypropyleen mesh implantaten ingebracht in de abdominale ruimte van de muizen en gecontamineerd met verschillende hoeveelheden van de gekozen bacteriën. Vervolgens werd 0.5ml *hydrogel* mét en zónder 10mg IgG op de gecontamineerde mesh aangebracht. Antilichamen werden snel vanuit de gel afgegeven aan de omliggende weefsels *in vivo*, met een 100% release na 48-uur *in vitro* in buffer. Alhoewel deze IgG antilichamen alleen of in combinatie met de systemische antibiotica cefazoline of vancomycine de overleving van muizen na MRSA infectie niet verbeterden (100% mortaliteit na 72 uur) ten opzichte van de controles, werd wel significant verbeterde overleving aangetoond tegen beide gram-negatieve *P. aeruginosa* stammen in muizen behandeld met alleen IgG antilichamen.

In **Hoofdstuk 8** worden de resultaten van alle studies bediscussieerd met het oog op de toekomstige problemen van antibiotica-resistantie en de huidige, snelle ontwikkelingen in poly- en monoclonale antilichaam producten en technologieën.

Samenvattend geeft dit proefschrift een beschrijving van verschillende studies naar de effectiviteit van lokale behandeling met humaan, gepoolde polyclonale IgG in de strijd tegen bacteriële, biomateriaal-geassocieerde infecties. Hoewel lokale IgG behandeling in de gebruikte *in vivo* modellen beter werkte dan systemische IgG en zelfs in lagere doseringen, is het helaas niet mogelijk gebleken infecties volledig te voorkomen met alleen lokale IgG. Vooral tegen kapsel vormende bacteriën zijn specifieke antilichamen nodig om succesvolle opsonizatie en phagocytosis te initiëren door neutrofielen en macrofagen in geïnfecteerde weefsels. Die antilichamen zijn alleen in hyperimmunoglobuline produkten aanwezig of kunnen gecombineerd worden in cocktails van monoklonale antilichamen en niet in polyclonale IgG produkten. In combinatie met systemische antibiotica was echter in sommige gevallen zelfs een synergistische verbetering aantoonbaar in overleving die niet door IgG of door het antibioticum alleen kon worden bereikt.

Dergelijke combinatie therapiën van i.v. antibiotica met lokale polyclonale IgG zouden een therapeutisch alternatief kunnen zijn tegen antibiotica-resistente bacteriën. Door daarnaast bestaande i.v. antibiotica te combineren met direct, locaal gebruikte hyperimmun sera en specifieke, gehumanizeerde monoclonale cocktails, zouden benodigde doses kunnen worden gereduceerd, terwijl maximaal prophylactische bescherming tegen infecties kan worden geboden op de plaats van chirurgisch ingebrachte biomaterialen. Daardoor zouden direct postoperative infecties en wellicht ook latente infecties van implantaten kunnen worden verminderd door voorkoming van de verstoring van initiële weefsel integratie door contaminerende bacteriën. Dit kan hopelijk resulteren in het uiteindelijke doel van deze studies: een verbeterde *quality of life* voor de patiënten die langdurig kunstgewrichten, andere biomaterialen voor kortere duur en zelfs moderne *tissue engineered* materialen geïmplanteerd krijgen om verloren vorm en functie te herstellen.

Omdat het gebruik van implanteerbare materialen alleen maar zal toenemen over de komende decennia, zal er intensief aandacht moeten worden besteed aan “*biomaterial-centered*” infecties die gemakkelijk antibiotica-resistant kunnen worden. Exploratie van natuurlijke immuun-mechanismen is een aantrekkelijke optie voor de versterking van conventionele antibiotica therapiën. Behandeling met poly- of monoclonale antilichamen levert in ieder geval een bijdrage aan het patiënt-eigen immuun-systeem om effectief hardnekkige infecties te kunnen bestrijden.

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Kornelis A. Poelstra (Kees) was born on March 6, 1973 in Zierikzee, The Netherlands. After primary and secondary school he studied Medicine at the University of Groningen and became involved with research. As a student, he was co-founder and chief executive/organizing officer together with Eric J.W. Maarsingh of the first students' research conference. He graduated from Medical School in August 1997 and received his American Medical Degree that same year.

He started his graduate program at the Department of Biomedical Engineering at the University of Groningen, studying the effect of vacuum mixing on antibiotic release from bone cement, microbial growth and biofilm formation. After receiving an Ambassadorial Fellowship award from the Nuffic in Den Haag (Talentenfonds), Kees was invited to join the research-team of Dr. Anthony G. Gristina in Herndon, Virginia, USA. While in the US, in collaboration with his advisors from Groningen, he finished his graduate research regarding the use of IgG to prevent biomaterial-centered infection, the subject of this thesis.



At present, he is a resident at the Department of Orthopaedic Surgery of the University of Virginia in Charlottesville, USA. Currently, his research encompasses cell-based gene delivery to improve the healing of fractures and non-unions.

In his private time, he enjoys an active social and athletic life with his wife Angelique and loves to work on his classic automobiles.

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