Anti-Glucosaminidase Monoclonal Antibodies as a Passive Immunization for Methicillin-Resistant *Staphylococcus aureus* (MRSA) Orthopaedic Infections

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Abstract

Recently, methicillin-resistant *Staphylococcus aureus* (MRSA) has surpassed HIV as the most deadly pathogen in the United States, accounting for over 100,000 deaths per year. In orthopedics, MRSA osteomyelitis has become the greatest concern in patient care, despite the fact that improvements in surgical technique and aggressive antibiotic prophylaxis have decreased the infection rate for most procedures to less than 5%. This great concern is largely due to the very poor outcomes associated with MRSA osteomyelitis, which includes 30–50% failure rates for revision surgery. Thus, there is a need to develop additional therapeutic interventions such as passive immunization, particularly for immunocompromised patients and the elderly who are typically poor responders to active vaccines. Using a novel murine model of implant-associated osteomyelitis in which a stainless steel pin is coated with bioluminescent *S. aureus* and implanted transcortically through the tibial metaphysis, we discovered that mice protect themselves from this infection by mounting a specific IgG2b response against the peptidoglycan hydrolase, glucosaminidase (Gmd), an enzyme involved in cell wall digestion during binary fission. Since this subunit of *S. aureus* autolysin is essential for bacterial growth, and no genetic variation has been identified among clinical strains, we propose that monoclonal antibodies against this enzyme would have multiple mechanisms of action, including promotion of opsonophagocytosis and direct inhibition of enzyme function. Here we review the field of MRSA osteomyelitis and our research to date on the development of an anti-Gmd passive immunotherapy.

Keywords

Osteomyelitis; Methicillin-Resistant *Staphylococcus aureus* (MRSA); Autolysin; Glucosaminidase; Passive immunization

Introduction

Osteomyelitis is a bacterial infection of bone that is characterized by progressive inflammatory bone destruction (osteolysis) coupled with reactive bone formation, and can involve either a small portion or several regions of any bone. The initial infection that causes

osteomyelitis can occur either from hematogenous seeding of the pathogen from another site in the body, or from direct inoculation via a traumatic or surgical wound (1). This results in an acute infection that usually lasts several days or weeks, and may require antibiotic and/or surgical intervention. When the causative microorganism persists for more than 10 days and causes further destruction of bone, the infection is then considered chronic osteomyelitis (2). *Staphylococcus aureus* (*S. aureus*) is the single leading cause of both acute and chronic osteomyelitis in children and adults, accounting for approximately 80% of these infections (3). Other microorganisms such as coagulase-negative staphylococci, *Streptococcus* spp, *Enterococcus* spp, and *Mycobacterium tuberculosis* may also cause osteomyelitis, but *S. aureus* is by far the most prevalent bacteria found due to virulence factors that help it evade a number of host defenses (1).

Osteomyelitis is generally classified using either of two systems: the Waldvogel (4–7) and the Cierny-Mader (8;9) systems. The Waldvogel classification system is based on the extent, duration, and mechanism of bone infection, whereas the Cierny-Mader classification system also considers the immune state and risk factors of the host, thus offering comprehensive treatment options that best fit the patient's needs. Because of this, the Cierny-Mader classification system is considered more clinically relevant and is therefore more widely used (10). Regardless of the model of classification used, antibiotic and surgical treatments are designed on a patient-to-patient basis according to the distinct type of osteomyelitis presented, and not all patients will respond to either strategy (1;3;11).

The number of bone infections has increased over the last few decades, due to an increase in the number of prosthetic and fracture-fixation devices being placed by orthopedic surgeons (12). Although improvements in surgical technique and aggressive antibiotic prophylaxis have decreased the infection rate following orthopedic implant surgery to less than 5%, osteomyelitis remains a serious problem (13;14). The gravity of these infections is amplified by the fact that approximately 50% of clinical isolates are drug-resistant strains of *S. aureus*, such as methicillin-resistant *S. aureus* (MRSA), that are commonly acquired in both hospital and community settings (15). Additionally, MRSA has surpassed HIV as the most deadly pathogen in North America and continues to make the management of chronic osteomyelitis more difficult. Current estimates of two-stage revision surgery for MRSA periprosthetic infection suggest that reinfection rates are approximately 15–25% (16–18). However, these numbers may actually be higher because they do not account for the fraction of patients who cannot undergo revision surgery due to persistent infection that does not permit device reimplantation. This indicates that there is a major need for alternative interventional strategies, particularly for immunocompromised individuals (i.e., patients with diabetes or who are HIV-infected), those taking immunosuppressive medications, and the elderly who collectively comprise the majority of patients undergoing total joint replacement (TJR) surgery.

The great need for novel interventions is reinforced by the fact that approximately 112,000 orthopedic device-related infections occur each year in the United States, at an approximate cost of $15,000–70,000 per incident (3). While the infection rates for joint prosthesis and fracture-fixation devices have been only 0.3–11% and 5–15% of cases, respectively, over the last decade (1;19), these infections may lead to amputation or even death. Additionally, although unproven, the popularization of "minimally invasive surgery" for elective TJR, in which a very small incision can lead to complications from the prosthesis contacting skin during implantation, has been associated with a marked increase in the incidence of osteomyelitis (20). These infections require a very expensive two-stage revision surgery, and recent reports suggest that success rates could be as low as 63% (16–18).

At present, the only prophylactic treatments available for preventing MRSA infection in patients undergoing TJR surgery are preoperative antibiotics, such as vancomycin. However, the overuse of these "last resort" antibiotics is resulting in the emergence of strains with resistance to even our most potent antibiotics (21). Therefore, it is imperative that immunocompromised and elderly patients, who collectively account for most of the 1.5 million TJRs performed annually in the United States, have access to alternative interventional strategies. Thus, a vaccine that would decrease the MRSA incidence by 50–80% would reduce the number one complication of joint replacement and open fracture repair procedures, and it would also cut the healthcare burden by a similar amount (22).

Microbial Pathogenesis of Osteomyelitis and Targets for Immunotherapy

Based on over 150 years of research, a clear paradigm to explain microbial pathogenesis has emerged. This model also applies to osteomyelitis. The initial step of infection occurs when a single bacterium invades the body. At this point the microbe must respond to environmental changes and express virulence genes that will help it defeat innate immunity and provide it with adhesin receptors to attach to the host. The microbe is also dependent on the presence of host structures from necrotic tissue or a foreign body such as an implant. Successful completion of these steps leads to an exponential growth phase, which ceases at the point of nutrient exhaustion and/or the development of adaptive immunity. Following the exponential growth phase, the bacteria are forced to persist under dormant growth conditions within a complex extracellular matrix referred to as a biofilm. At this point the infection is now chronic and cannot be eradicated by drugs or host immunity. Because of the critical importance of initial attachment, the focus in this field has been on the cell surface adhesins that specifically interact with extracellular matrix components known as MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) (23). In fact, the majority of anti-*S. aureus* vaccines that have been developed to date have been directed against MSCRAMMs that are important to host tissue colonization and invasion (24). The goal of these vaccines was to generate antibodies that block the attachment to host tissues by binding to the adhesins. Unfortunately, *S. aureus* has many adhesins, such that inhibition of one may not be sufficient to prevent bacterial attachment.

S. aureus Autolysin as a Primary Target

In gram-positive and gram-negative bacteria, autolysins play an important role in cell separation and cell wall remodeling during normal binary fission. The 138-kDa *S. aureus* autolysin is proteolytically processed on the cell surface to produce two active enzymes, *N*-acetylmuramyl-L-alanine amidase (amidase, 62-kDa) and endo-β-*N*-acetylglucosaminidase (glucosaminidase, 51-kDa), that remain non-covalently attached to the cell surface (25–28).

There are many features that make the *S. aureus* autolysin a very attractive target for anti-*S. aureus* vaccine investigation. First, it is highly conserved among staphylococci. For example, glucosaminidase (Gmd) is greater than 95% conserved across all strains of *S. aureus* in the public database, and approximately 85% conserved among other staphylococci (29). Second, it is essential for complete separation of daughter cells following binary fission. *S. aureus* bacteria deficient in autolysin still divide, but daughter cells fail to separate, leading to the generation of large clusters that fall out of suspension (30). Third, Gmd is located on the extracellular surface of the bacterium, potentially focusing the immune response on a vulnerable part of the cell. In support of this, scanning electron micrographs of anti-Gmd immune complexes on the surface of *S. aureus* demonstrated that the antibody binds in immediate proximity to digested cell wall (28). Fourth, autolysins may have a potential role in biofilm formation (31;32). For example, previous studies demonstrated that autolysins are involved in the initial attachment of Staphylococcus

bacteria to a polymer surface (33), and that murein hydrolases are regulated by effector genes that control bacterial death and lysis in the case of biofilm formation (31;32). Finally, elevated levels of anti-Gmd antibodies were detected in serum from mice that survived a challenge with *S. aureus* (34). Collectively, this information provides a strong rationale for anti-Gmd therapy for osteomyelitis, as the antibodies have multiple potential mechanisms to achieve cytostatic and cytolytic activity, and the extremely high evolutionary conservation of the enzyme suggests that compensatory mutations to achieve antigenic variation and immune evasion may not be possible.

Glucosaminidase as a Protective Antigen

Recently, Li *et al.* identified Gmd as an important component of immune protection in mice (34;35). A time course study was performed in which a stainless steel pin coated with 1×10^6 UAMS-1 *S. aureus* was inserted transcortically through the tibia of each of five mice. At sacrifice, DNA was extracted from the infected tibia and analyzed by quantitative real time PCR to determine the number of copies of the *nuc* gene per infected tibia. Because each *S. aureus* bacterium contains a single copy of the *nuc* gene in its genome, we can directly quantify the *in vivo* bacterial load as a measure of infection (36).

In order to identify potentially protective antigens, pre-immune and convalescent sera were used as the primary antibody in Western blots of total *S. aureus* protein extract. Bands with significantly increased intensity were observed in the convalescent serum at 26-, 34-, 38-, and 56-kDa, suggesting that these antigens are somehow involved in the protective immune response.

The molecular identity of these potentially protective antigens was then determined. Convalescent serum was used to probe total *S. aureus* extract that was separated by two-dimensional polyacrylamide gel electrophoresis (35). A polypeptide with strong reactivity in the convalescent serum was detected that was not detected in the pre-immune serum. This protein was isolated from preparative Coomassie blue-stained gels, digested with trypsin, and then analyzed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), which resolved 70 individual peptide peaks. The amino acid sequence from every peptide was a 100% match with the active Gmd subunit of *S. aureus* autolysin.

Preliminary Experiments Involving Anti-glucosaminidase Antibodies

We hypothesized that inhibiting the function of Gmd would disrupt critical steps in the growth cycle of *S. aureus*. Thirty-six mouse IgG1 monoclonal antibodies were generated against the Gmd enzyme, and a series of experiments was designed to test the effects of these antibodies on enzyme activity. Five were chosen as candidate anti-Gmd antibodies for the first round of screening based on their high-affinity binding to both native and recombinant His-tagged Gmd.

We predicted that *S. aureus* cultures grown in the presence of anti-Gmd antibodies would be inhibited in their growth. Initial experiments focused on the change in light scattering detected at A_{490} as cultures of *S. aureus* grew in the presence or absence of anti-Gmd antibodies. Using Xen29 *S. aureus* as a model organism, we set up cultures and measured light scattering at various time points using a plate reader. We found that antibodies against Gmd reduced the growth-related light scattering of *S. aureus* in our *in vitro* growth assays. This led us to believe that we were indeed altering the enzymatic activity of Gmd, but we needed additional evidence to understand the mechanism by which we were altering *S. aureus* growth.

To try and understand how our anti-Gmd antibodies were working, we set up several cultures of Xen29 *S. aureus* similar to those used in the growth assays, only larger in volume. If we were indeed inhibiting Gmd enzymatic activity, we predicted that we should see an increased number of cell clusters and sedimentation since Gmd is intimately involved in cell separation. Gross observation of cultures treated with anti-Gmd antibodies showed sedimentation of cells at the bottom of the vial in comparison to Xen29 grown in the absence of these antibodies (Fig. 1), leading us to believe that failed binary fission may be causing the bacteria to fall out of suspension. This was consistent with similar observations made in two separate reports using autolysin mutant *S. aureus* strains that could not produce glucosaminidase and amidase (30;37).

Scanning electron microscopy also revealed that *S. aureus* treated with anti-Gmd antibodies grew as large clusters of twenty or more bacteria, in contrast to the mostly single-cell and doublet suspension of the control culture (Fig. 2). Additionally, *S. aureus* treated with anti-Gmd antibodies had a rough outer appearance in comparison to cultures grown in the absence of these antibodies, which is consistent with a previous report using a Gmd-deficient strain (38).

Potential Future Clinical Applications

For practical reasons that pertain to the size and scope of a potential phase 3 clinical trial designed to prove the efficacy of an anti-Gmd passive immunization, we have chosen to focus our efforts towards an adjuvant immunotherapy for MRSA-infected TJR patients who are candidates for a two-stage exchange arthroplasty. The primary goal of this intervention is to prevent the seeding of residual bacteria onto the sterile implant during revision surgery and in the post-operative period. Since these bacteria must go through a planktonic growth phase to colonize the prosthesis, anti-Gmd mAbs in combination with standard chemotherapy could inhibit the re-seeding process better than antibiotics alone and significantly increase the success rates.

Ultimately, we envision a role for our passive immunotherapy in prevention of the primary infections that cause so much subsequent trauma. Specific indications include prophylaxis for all patients undergoing TJR surgery, patients receiving artificial heart valves, and children at risk for infection in neonatal intensive care units.

Conclusion

As indicated by the increase in bone infections over the last few decades, there is a great need for alternative interventional strategies for the treatment of osteomyelitis. This demand is reinforced by three situations. First, not all patients respond to antibiotic or surgical intervention. Second, approximately 50% of clinical isolates are drug-resistant strains of *S. aureus*, most notably MRSA. Finally, with the exception of several "last resort" antibiotics, there are no prophylactic treatments that can help high-risk patients, especially immunocompromised patients and the elderly who are the primary recipients of TJR surgery. By using a novel murine model of implant-associated osteomyelitis in which a stainless steel pin is coated with bioluminescent *S. aureus* and implanted transcortically through the tibial metaphysis, we discovered that mice protect themselves from this infection by mounting a specific IgG2b response that includes antibodies against Gmd, an enzyme that is intimately involved in cell separation and cell wall remodeling during *S. aureus* growth and binary fission. These initial experiments show that anti-Gmd antibodies alter the growth habit of *S. aureus*, and suggest that Gmd may be a target for direct growth inhibition and focusing immune effectors.

Acknowledgments

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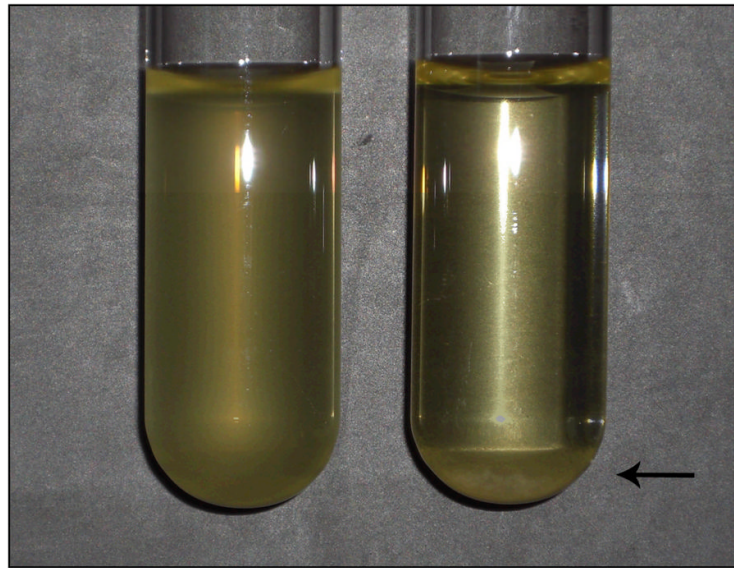
Fig. 1.
S. aureus cultures treated with an anti-glucosaminidase antibody grew as large clusters that fell out of suspension. *S. aureus* grown in Luria-Bertani broth under normal conditions (left), as compared to *S. aureus* grown in the presence of the anti-glucosaminidase monoclonal antibody 1C11 (right). Sedimentation is indicated by an arrow.
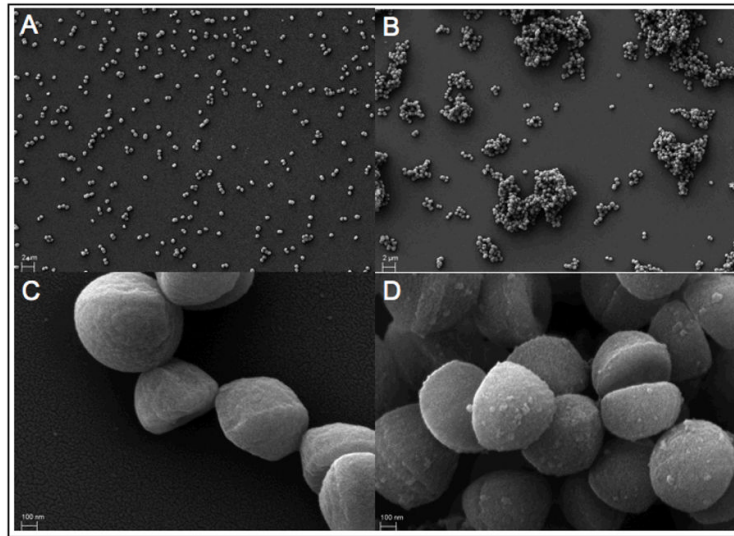
Fig. 2.
Anti-glucosaminidase antibodies alter the growth habit of *S. aureus in vitro*. *S. aureus* grew as a uniform suspension of mostly single bacteria and doublets in Luria-Bertani broth under normal growth conditions (A). In contrast, *S. aureus* grown in the presence of the anti-glucosaminidase monoclonal antibody 1C11 grew as large clusters (B), as revealed by scanning electron microscopy. Micrographs C and D are representative magnified views of A and B, respectively. Note the presence of a rough outer surface on *S. aureus* treated with the antibody, as seen in micrograph D.