Anti-Clumping Factor A Immunoglobulin Reduces the Duration of Methicillin-Resistant *Staphylococcus aureus* Bacteremia in an Experimental Model of Infective Endocarditis

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SA-IGIV is a human polyclonal immunoglobulin containing elevated levels of antibodies specific for the fibrinogen-binding MSCRAMM protein clumping factor A (ClfA). In vitro, SA-IGIV specifically recognized ClfA that was expressed on the surface of *Staphylococcus aureus* and inhibited bacterial adherence to immobilized human fibrinogen by >95%. Moreover, SA-IGIV efficiently opsonized ClfA-coated fluorescent beads and facilitated phagocytosis by human polymorphonuclear leukocytes. To determine its potential therapeutic efficacy, SA-IGIV was evaluated in combination with vancomycin in a rabbit model of catheter-induced aortic valve infective endocarditis (IE) caused by methicillin-resistant *S. aureus* (MRSA). The combination therapy was more effective than vancomycin alone in sterilizing all valvular vegetations when used therapeutically during early (12-h) IE. The combination therapy resulted in clearance of bacteremia that was significantly faster than that of vancomycin alone in animals with well-established (24-h) IE. Therefore, in both early and well-established MRSA IE, the addition of SA-IGIV to a standard antibiotic regimen (vancomycin) increased bacterial clearance from the bloodstream and/or vegetations.

The incidence of methicillin-resistant *Staphylococcus aureus* (MRSA) infections continues to rise. According to the Centers for Disease Control and Prevention, the incidence of nosocomial infections in intensive care unit patients due to MRSA increased by 40% in 1999 compared to that of the previous 4 years in the United States (4). Vancomycin, the drug of choice for such infections, is often suboptimal, and indeed, the first documented case of an infection caused by vancomycin-resistant *S. aureus* in the United States has recently been reported (5). The increased incidence of antibiotic resistance observed in *S. aureus* clinical isolates has underscored the need for alternatives to current antibiotic strategies (6). An emerging option in this regard is antibody-based immunotherapy approaches, via targeting of epitopes on critical virulence proteins expressed in vivo. Mounting evidence suggests that microbial adherence is central to the initiation and metastatic spread of *S. aureus* infections. Therefore, the MSCRAMM (microbial surface components recognizing adhesive matrix molecules) family of proteins, which play a central role in adherence to host tissues, represents a cadre of potential antigenic candidates for the development of novel immunotherapies (8, 24).

One well-characterized MSCRAMM protein that is a candidate target for immunotherapy is clumping factor A (ClfA), a fibrinogen-binding adhesin expressed on the surface of nearly all strains of *S. aureus* (3, 17, 18). It is well established that ClfA recognizes the C terminus of the γ chain of human fibrinogen (10, 19, 26) and that the interaction between ClfA and fibrinogen can be inhibited by antibodies raised against the A domain of ClfA (18). In vivo data suggest that ClfA plays a critical role in the induction and persistence of experimental endovascular infections. For example, mutant strains of *S. aureus* which lack ClfA expression were less virulent in a rat endocarditis model when the rats were challenged with low levels of bacteria (21). Furthermore, it has recently been shown that when commensal organisms, such as *Streptococcus gordonii* or *Lactococcus lactis*, are genetically engineered to express ClfA, the bacteria become more virulent in a rat infective endocarditis (IE) model (20, 25).

The present study was designed to further characterize potential antibody therapies against *S. aureus*, with a particular focus on ClfA as an antigenic target. As a first step towards the development of an anti-ClfA immunotherapy, we have developed a hyperimmunoglobulin (SA-IGIV) derived from plasma donors with naturally occurring high titers of anti-ClfA immunoglobulin G (IgG). It has been shown that human antibody preparations containing high titers of anti-ClfA-specific IgG can be successfully used as a prophylactic agent to decrease mortality in a murine model of *S. aureus* sepsis (13). Similarly, we have found that human anti-ClfA antibodies have potent prophylactic efficacy when they are tested in a rabbit model of MRSA-induced IE (data not shown). In this report, we have demonstrated that the anti-ClfA antibodies in the SA-IGIV preparation are able to recognize the staphylococcal cell surface and specifically inhibit *S. aureus* adherence to immobilized fibrinogen. Further, we have shown that the anti-ClfA antibodies present in SA-IGIV can function as an efficient opsonin in an in vitro assay of human polymorphonuclear leukocyte (PMN) opsonophagocytosis. Finally, we have shown the therapeutic efficacy of SA-IGIV, when used in combination with the glycopeptide antibiotic vancomycin, in a rabbit model of catheter-induced aortic valve IE caused by MRSA.
MATERIALS AND METHODS

Bacteria. *S. aureus* strain 67-0 is an oxacillin (methicillin)-resistant wound isolate (provided courtesy of Henry Chambers, University of California San Francisco and San Francisco General Hospital, San Francisco), previously determined to be virulent in an animal model of IE (2). Newman *spa:kan*, a protein A knockout mutant of *S. aureus* strain Newman, *L. lactis* (KS880), and *L. lactis* CIA− transfectant strains were provided by Timothy Foster (Trinity College, Dublin, Ireland).

Clf40 recombinant protein. Clf40 is a recombinant protein corresponding to the A domain (amino acids 40 to 559) of the CIA molecule. The fibrinogen-binding domain of CIA is completely encompassed by the Clf40 construct (23). The recombinant protein contains an N-terminal 6-His tag and was purified from *Escherichia coli* lysates by metal affinity chromatography on a chelating Sepharose Fast Flow resin (Amersham Biosciences, Piscataway, N.J.) followed by Q Sepharose (Amersham Biosciences) chromatography.

SA-IGIV antibody. SA-IGIV is a sterile, solvent- and detergent-treated liquid preparation of highly purified IgG. Plasma donors with elevated titers of anti-CIA antibody were selected from the general donor population for the manufacture of SA-IGIV. SA-IGIV was manufactured under good manufacturing practices by Massachusetts Public Health Biological Laboratories (Skatcon, Plain, Mass.) using cold ethanol fractionation (7). The resulting product had an anti-CIA titer that was approximately five times greater than that measured in random commercial lots of immunoglobulins for intravenous use (IGIV) prepared from unscreened donors (data not shown).

Flow cytometry. The recognition of CIA expressed on the bacterial cell surface by SA-IGIV was detected by flow cytometry. Bacteria were fluorescently stained with a fluorescein isothiocyanate (FITC)-conjugated Fab fragment of affinity-purified goat anti-human IgG (heavy and light chains) (Rockland, Inc., Gilbertsville, Pa.) and were incubated with each of the reagents for 1 h. To evaluate the bacteria, a fluorometer (FACStar; Becton-Dickinson, Mountain View, Calif.) was used. Eighty-seven percent of Newman *S. aureus* strain Newman and 75% of Newman *S. aureus* strain Newman SPA:kan were stained with 1:250 dilutions of FITC-conjugated anti-clumping factor antibodies.

RESULTS

Recognition of *S. aureus* by SA-IGIV. It was necessary to demonstrate that the antibodies present in the SA-IGIV preparation were capable of recognizing native epitopes expressed on the surface of *S. aureus*. To this end, bacteria were incubated with SA-IGIV, and bound antibodies were detected with a fluorescently labeled anti-human IgG F(ab')2 antibody. To eliminate protein A-mediated antibody binding, a protein A knockout mutant of *S. aureus* strain Newman (Newman *spa:kan*) was used. Eighty-seven percent of Newman *spa:kan* cells exhibited antibody surface binding after exposure to anti-CIA antibodies in SA-IGIV and to eliminate any activity contributed by other anti-staphylococcal antibodies that might have been present. Fluorescein isothiocyanate (FITC)-conjugated anti-clumping factor antibodies were used to stain the bacteria, and FITC-conjugated anti-clumping factor antibodies were used to stain the bacteria, and FITC-conjugated anti-clumping factor antibodies were used to stain the bacteria. The percentage of bacteria that were stained with FITC-conjugated anti-clumping factor antibodies was determined by flow cytometry.

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SA-IGIV (Fig. 1A). To demonstrate that some of this reactivity is due to the presence of ClfA-specific antibodies in SA-IGIV, we performed a similar experiment with an *L. lactis* strain transfected with a ClfA expression vector. Antibodies in SA-IGIV were able to recognize the surface of the *L. lactis* ClfA/H11001 strain but did not recognize a control *L. lactis* strain which lacks ClfA expression (Fig. 1B). These data demonstrated that SA-IGIV was capable of recognizing native epitopes expressed on the surface of *S. aureus* and that some of this recognition is attributable to ClfA-specific antibodies present in SA-IGIV.

**Inhibition of *S. aureus* binding to fibrinogen.** A binding inhibition test was performed to determine if ClfA-specific antibodies in SA-IGIV were capable of mitigating the interaction between ClfA and fibrinogen. Fluorescently tagged *S. aureus* strain Newman spa:kan was incubated with different concentrations of SA-IGIV (solid line) or with SA-IGIV which had previously been absorbed with *S. aureus* to remove specific antibody (control, dashed line) (A) and an inhibiting concentration of SA-IGIV (2 mg/ml) and *S. aureus* prior to incubation on fibrinogen-coated plates. The presence of soluble Clf40 protein completely blocked the ability of SA-IGIV to inhibit *S. aureus* binding to fibrinogen (Fig. 2B). These data support the conclusion that ClfA-specific antibodies present in SA-IGIV rec-
unconjugated beads.

SA-IGIV; No Antigen Control, Clf40 conjugated beads replaced with each reaction. No Antibody Control, all reactants with the exception of flow cytometry. The data are presented as the phagocytic product of rotation. Fluorescent bead association with PMNs was quantitated by ° incubated with human PMNs for 30 min at 37°C with end-over-end rotation. Fluorescent bead association with PMNs was quantitated by flow cytometry. Controls included the addition of all reactants with un conjugated beads replaced with un conjugated beads.

ognize the fibrinogen-binding domain of ClfA and inhibit the ability of S. aureus to adhere to human fibrinogen-coated surfaces.

Opsonizing activity of ClfA-specific antibodies in SA-IGIV. Although antibodies in SA-IGIV can recognize the surfaces of S. aureus organisms and can inhibit the bacteria from adhering to fibrinogen-coated surfaces, the ability of this antibody to act as an opsonin to facilitate OP clearance of bacteria may play a critical role in its overall therapeutic effectiveness in vivo. To specifically delineate the opsonic capacity of anti-ClfA antibodies present in SA-IGIV, fluorescent microspheres were coated with Clf40 and incubated with a saturating concentration (2.5 mg/ml) of SA-IGIV and complement. Antigen-coated beads were used in place of S. aureus organisms to eliminate the opsonizing effects of other anti-staphylococcal antibodies that may be present in SA-IGIV. The opsonized beads were incubated with freshly isolated human PMNs, and the association of PMNs with fluorescent beads was quantitated by flow cytometry. Controls included the addition of all reactants with the exception of SA-IGIV and the use of control beads lacking the Clf40 antigen. The results are shown in Fig. 3. When SA-IGIV was used, the resulting phagocytic product was 4.5-fold greater than that in control reactions. To determine if the cell-associated fluorescence was due to simple surface adherence or phagocytosis, reaction samples were analyzed by transmission electron microscopy. The results of transmission electron microscopy showed that beads were internalized by the PMNs in membrane-lined vesicles, and very few, if any, beads were surface associated (data not shown). The results clearly demonstrate that ClfA-specific antibodies present in SA-IGIV can act as opsonins to mediate the recognition and uptake of ClfA-coated particles by human PMNs.

In vivo impact of antibody on MRSA IE. To determine whether the in vitro properties of SA-IGIV would translate into in vivo efficacy, SA-IGIV was tested in two models of experimental IE. To establish parameters for the model, we performed a pilot analysis of the potential efficacy of SA-IGIV alone in established IE. Two animals received SA-IGIV (200 mg/kg) intravenously 12 h after induction of IE, while two animals were untreated controls. In the untreated controls, blood cultures obtained at 24 h postinfection yielded high-level bacteremia (mean, 3.77 log_{10} CFU/ml); both animals died by 48 h postinfection. For animals receiving SA-IGIV, serial blood cultures showed an initial decrease in the level of bacteremia compared to that of untreated controls at 24 h (mean, 2.0 log_{10} CFU/ml). However, over the ensuing 48 h, bacterial densities in these blood cultures reached levels virtually identical to those of untreated controls. Therefore, we did not include an antibody-alone group in the larger studies but rather investigated the effect of SA-IGIV in combination with vancomycin. To determine if there were differences in treating animals with early or well-established IE, SA-IGIV was administered at either 12 or 24 h after MRSA challenge. In animals with early IE (12 h postinfection), vancomycin and vancomycin plus SA-IGIV were equally efficacious at reducing the extent of bacteremia, with all blood cultures sterilized (data not shown). Both treatment regimens also provided similar levels of protection from metastatic renal seeding (Fig. 4A). However, 100% of valvular vegetations were sterilized by the antibiotic-antibody combination in comparison to only 43% of those receiving antibiotic alone (P < 0.025) (Fig. 4B). In animals with well-established MRSA IE, the clearance of bacteremia was substantially faster and more complete in the antibiotic-antibody-treated group than in the antibiotic-alone group (Fig. 5). Among animals treated with vancomycin alone, 67% remained culture positive at 72 h (Fig. 5A), whereas all blood samples were rendered culture negative by the combination therapy by this time point (P = 0.0110) (Fig. 5B). The effect of SA-IGIV was less dramatic in the target tissue samples. In vegetations, both vancomycin alone and vancomycin plus SA-IGIV produced similar reductions in bacterial densities compared to that in the untreated control group (P < 0.05) (data not shown). Likewise, bacterial densities in kidney tissues were similar for the vancomycin-treated and vancomycin-plus-SA-IGIV-treated groups, and neither treatment provided significant reductions relative to that of the control group (data not shown).

**DISCUSSION**

IGIV are approved for a wide variety of clinical indications, including replacement therapy in primary and secondary immunodeficiency, pediatric AIDS, allogeneic bone marrow transplantation, Kawasaki’s disease, and Guillain-Barré syndrome (22). Many clinical studies have also been conducted to assess the efficacy of IGIV treatment in the prevention of sepsis (27). In fact, at least 20 separate studies have reported the use of IGIV for the prophylaxis of neonatal infections (12). Despite some indications of efficacy, it is clear that the current IGIV preparations have quite different potencies against specific pathogens and that a customized approach designed to target specific pathogenic organisms would likely achieve more consistent and salutary results.

In an effort to create a more consistent and potent IGIV for the prevention and treatment of S. aureus infections, we have formulated a hyperimmunoglobulin with an increased antibody titer against a major, pathogenically important MSCRAMM protein, ClfA. The product was prepared from human plasma
donors who were screened for the presence of high-titer, anti-
Clf40 (ClfA A domain) IgG. A rabbit model of MRSA IE was
used to evaluate the potential therapeutic ef-
cacy of this prod-
uct in mitigating the course of a multisystem staphylococcal
infection. Products similar to SA-IGIV have been demon-
strated to have prophylactic ef-
cacy against
*S. aureus
infection
(at doses of 200 mg/kg) in both a rodent model of sepsis (13)
and in the rabbit IE model (data not shown). In both models,
prophylaxis with nonselected, normal human IGIV prepara-
tions had no effect on the course of disease. In the present
study, we assessed the therapeutic potential of the same dose
of SA-IGIV (200 mg/kg) in the rabbit IE model. Of note, the
SA-IGIV dose chosen was also well below typical clinical doses
of IGIV, which can range from 500 mg/kg to 1 g/kg (12, 27). A
pilot study performed with a small number of animals indi-
cated that SA-IGIV alone only transiently impacted the course
of early MRSA IE (i.e., a modest, early reduction in the extent
of bacteremia). We therefore chose to evaluate the benefit of
SA-IGIV therapy in combination with a clinically relevant
standard antibiotic therapy (vancomycin). Our choice of van-
comycin dosage stemmed from long experience in using this
antibiotic in the rabbit IE model against the MRSA 67-0 strain.
Vancomycin has been successfully used to treat strain 67-0-
mediated IE in the rabbit model at doses of 7.5 to 20 mg/kg
when administered intravenously twice daily (2, 11; Kupfer-
2003). To increase the likelihood of divulging potential salu-
tary effects of SA-IGIV–antibiotic combination treatment, the
lowest effective vancomycin dose (7.5 mg/kg) was used. In both
case of early and well-established infections, the addition of SA-IGIV
to vancomycin increased the rate of bacterial clearance from
blood and/or selected target tissues. When administered 12 h
after induction of IE, the combination of vancomycin and
SA-IGIV resulted in the complete elimination of bacteria from
the heart valve vegetations and kidneys of treated rabbits. This
result clearly demonstrated that the administration of SA-

FIG. 4. Clearance of MRSA from target organs (12 h). At 12 h
after induction of MRSA IE, rabbits received one of the following: no
therapy (Control), vancomycin alone (Vanco), or vancomycin plus
SA-IGIV (Vanco + SA-IGIV). The data are presented as log_{10} CFU
per gram measured in kidney lesions (A) and cardiac valve vegetations
(B). Group means are indicated by horizontal lines. Numbers beneath
the x axis indicate the number of infected samples per total number of
samples evaluated. The asterisk indicates a significant difference be-
tween the number of infected tissues and that of the control (P < 0.025).

FIG. 5. Bloodstream clearance of MRSA in rabbits with IE (24 h).
At 24 h after induction of MRSA IE, rabbits received either vanco-
mycin alone (A) or vancomycin plus SA-IGIV (B). The data are
presented as log_{10} CFU per milliliter of blood. Group means are
indicated by horizontal lines. Numbers beneath the x axis indicate the
number of positive blood cultures per total number of blood samples
evaluated. The asterisk indicates a significant difference between the
number of positive blood cultures and that of the control (P = 0.0110).
IGIV plus vancomycin early in the infection process may be potentially curative under conditions in which vancomycin alone is only modestly effective. Even when the infection was allowed to progress for an additional 12 h, at which time blood and target tissues contained large concentrations of MRSA, the beneficial effects of SA-IGIV treatment over that of vancomycin alone were clearly demonstrable in terms of clearance of bacteremia. The results reported here establish that passive immunotherapy against an MSCRAMM protein can be effectively used in conjunction with a standard antimicrobial regimen to amplify treatment outcomes of an established S. aureus infection.

The mechanism(s) of the salutary effect of SA-IGIV may reflect both antiadhesion and opsonic properties of this antibody. Flow cytometry showed that antibodies present in the SA-IGIV preparation were capable of recognizing antigens on the surface of S. aureus and, based on the intensity of the fluorescence, the bacterial surface appeared to be highly decorated with IgG. Some of this signal was certainly due to the anti-ClfA antibodies present in SA-IGIV, although it is likely that antibodies with specificities for other staphylococcal antigens also contributed to this signal. It is reasonable to speculate that many of these antibodies may be opsonizing and therefore may also contribute to the efficacy of SA-IGIV in vivo. Using an in vitro test for opsonization, SA-IGIV was shown to be capable of triggering recognition and uptake of ClfA-coated microspheres by human PMNs. Therefore, the ClfA-specific antibodies in SA-IGIV may act as a potent opsonin which would enable recognition and clearance of bacteria by phagocytic cells. Immune clearance of bacteria by this mechanism is likely to play an important role in the therapeutic efficacy of this antibody in the rabbit IE model. This enhanced OP property of SA-IGIV may have been reflected in the facilitated blood culture clearance in antibody-treated animals in this study.

In addition to the potential therapeutic effects of opsonizing S. aureus, SA-IGIV has the capacity to inhibit S. aureus binding to fibrinogen. Fibrinogen binding mediated by CIA has been shown to be a key virulence factor for S. aureus, particularly in endovascular infections such as IE (20, 21, 25). Inhibition of fibrinogen binding would likely interfere with the capacity of S. aureus to spread hematogenously, bind to sites of vascular damage in IE (e.g., to kidneys), and reseed the damaged heart valve. Thus, this antibody may limit the capacity of S. aureus to colonize and replicate at these sites and may reduce the bacterial burden and the overall severity of disease.

Studies to adjudicate the relative impact of SA-IGIV on S. aureus adhesion and S. aureus opsonophagocytosis in vivo are in progress. The implications for the therapeutic use of SA-IGIV in the clinical setting warrant further studies to evaluate the biological role of this antibody in invasive S. aureus infections.

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REFERENCES


