INFECTION AND IMMUNITY, Aug. 2006, p. 4418–4423 Vol. 74, No. 8
0019-9567/06/$08.00+0 doi:10.1128/IAI.01794-05

gC1qR/p33 Blockade Reduces *Staphylococcus aureus* Colonization of Target Tissues in an Animal Model of Infective Endocarditis

Ellinor I. B. Peerschke,[1]* Arnold S. Bayer,[2,3] Berhane Ghebrehiwet,[4] and Yan Q. Xiong[2,3]

Department of Pathology and Laboratory Medicine, Weill Medical College of Cornell University, New York, New York[1]; Los Angeles Biomedical Research Institute at Harbor-UCLA, Torrance, California[2]; The Geffen School of Medicine at UCLA, Los Angeles, California[3]; and Departments of Medicine and Pathology, Stony Brook University, Stony Brook, New York[4]

Received 4 November 2005/Returned for modification 30 January 2006/Accepted 9 May 2006

gC1qR/p33 (gC1qR) is a ubiquitously expressed cellular protein that is also found in plasma and the extracellular matrix. In addition to its role in modulating the activation of complement and kinin cascades, gC1qR has been identified as a putative host ligand for endovascular pathogens, including *Staphylococcus aureus*. The present study provides evidence of the ability of soluble gC1qR to enhance *S. aureus*-fibrinogen interactions via simultaneously binding fibrinogen and *S. aureus*. This interaction was inhibited in vitro by two monoclonal antibodies (MAbs 74.5.2 and 60.11) recognizing distinct structural and functional domains of gC1qR. To evaluate the in vivo role of gC1qR, MAbs 74.5.2 and 60.11 were used in an experimental rat model of *S. aureus* endocarditis. Each MAb (100 mg/kg of body weight, given intraperitoneally) reached sustained (>60 h) and high (100 to 200 μg/ml) serum levels. Prophylaxis with MAb 60.11 or 74.5.2 caused substantial reductions in *S. aureus* colonization of aortic valves, kidneys, and the spleen compared to untreated controls. However, only MAb 74.5.2 prophylaxis therapy reached statistical significance, and only sera from animals protected with MAb 74.5.2 inhibited gC1qR-mediated *S. aureus* interactions with fibrinogen. Although not statistically significant, the reductions in bacterial colonization achieved with MAb 60.11 alone and in combination with MAb 74.5.2 (versus MAb 74.5.2 alone) suggest that there are effects of gC1qR blockade on *S. aureus* infective endocarditis in addition to blocking gC1qR-mediated *S. aureus* binding to fibrinogen. Such impacts may include direct modulation of complement (MAb 60.11) and kinin cascades (MAb 74.5.2) and/or activation of immune and inflammatory responses via localized immune complex formation.

Endovascular infections with *Staphylococcus aureus*, such as infective endocarditis (IE), are associated with high morbidity and mortality (1). The molecular mechanisms underlying *S. aureus* colonization and invasion of host tissues are complex, involving a number of host and bacterial factors (12, 21, 29). Experimental data show that bacterial surface adhesins (e.g., for fibrinogen), platelets, plasma proteins, endothelial cells, and subendothelial matrix components play an important role in the pathogenesis of *S. aureus* endovascular infections (1, 21, 29).

S. aureus adhesion to host cells and tissues is an essential step during microbial pathogenesis (3, 4, 10, 14). A large number of bacterial cell wall-anchored surface adhesins have been identified. Although such adhesins were thought previously to be monospecific for a given host protein, it is becoming clear that many adhesins recognize more than one ligand. For example, *S. aureus* protein A (SpA) was originally described to bind the Fc portions of immunoglobulins, supposedly providing antiphagocytic activity by camouflage (4). More recently, SpA was shown to bind von Willebrand factor (10) and a ubiquitously expressed cellular protein, designated gC1qR/p33 (gC1qR) (22). gC1qR is highly expressed on activated platelets (24) and endothelial cells (9), circulates in soluble form in plasma (25, 30), and is present in the extracellular matrix (11). In vitro studies have demonstrated a role for gC1qR in *S. aureus* adhesion to activated platelets (22). These results raised the possibility of a key role for gC1qR in endovascular pathogenesis.

gC1qR is a versatile, multiligand binding protein involved in recognizing not only *S. aureus* but a number of viral and other bacterial pathogens (6, 22). In addition, cell surface and soluble gC1qR proteins are involved in binding the complement subcomponent C1q (5, 7) and high-molecular-weight kininogen (HK) (16), thus modulating activation of the complement and kinin cascades, respectively. gC1qR also recognizes fibrinogen and cross-linked fibrin via a carboxy-terminal sequence in the fibrinogen D domain (18). Indeed, the three-dimensional structure of gC1qR predicted from X-ray crystallography is a trimer (15), providing opportunities for multivalent homotypic and/or heterotypic ligand interactions.

Since fibrinogen/fibrin is an important component of endovascular lesions (1, 21, 29) and since gC1qR recognizes both the fibrinogen D domain (18) and *S. aureus* (22), the present study examined the effect of soluble gC1qR on *S. aureus* adhesion to fibrinogen in vitro and its impact on *S. aureus* virulence in vivo in a rat model of experimental IE.

MATERIALS AND METHODS

Bacterial strains. Clumping factor A (encoded by *clfA*) mediates *S. aureus* binding to both soluble and immobilized fibrinogen/fibrin (13, 20). A wild-type *S. aureus clfA*-positive Newman strain (ALC59) and its isogenic *clfA*-negative mutant strain (ALC620) were obtained from A. Cheung (Department of Microbiology, Dartmouth Medical School, Hanover, NH). The construction of the mutant has been described elsewhere (32). The ALC59 strain was grown to mid-logarithmic phase (4 to 6 h, 37°C) in tryptic soy broth (TSB; Difco Laboratories, Detroit, MI), to maximize *clfA* expression (2).

* Corresponding author. Mailing address: New York Presbyterian Hospital, 525 East 68th Street, Room F715, New York, NY 10021. Phone: (212) 746-2096. Fax: (212) 746-8797. E-mail: epeersch@med.cornell.edu.

ALC620 was grown in TSB supplemented with 5 μg/ml erythromycin. Bacterial growth was monitored by the optical density at 620 nm (OD_{620}). Bacteria were washed by centrifugation (10,000 × *g*, 5 min) and resuspended in appropriate buffers as indicated below for specific experiments.

For in vivo studies, a well-characterized, virulent *S. aureus* laboratory strain (RN6390) derived from NCTC 8325, which expresses both *clfA* and *spa*, was used. This strain is methicillin susceptible and is regularly used in the study of *S. aureus* pathogenesis, including experimental IE (2, 17). Moreover, it is significantly more virulent in the rat IE model than strain Newman, presumably because of the naturally truncated and low-functioning fibronectin-binding adhesin in the latter strain (8). For the current study, *S. aureus* RN6390 was cultured from −70°C storage onto 6.6% sheep blood agar plates (Clinical Standards Laboratory, Inc., Rancho Dominguez, CA). Cells were grown overnight in brain heart infusion broth (Difco Laboratories, Detroit, MI) at 37°C. Cells were then harvested by centrifugation, washed twice with phosphate-buffered saline (pH 7.2), sonicated briefly to ensure single cells, and then adjusted spectrophotometrically (optical density at 600 nm) to the final desired inoculum for animal infections. All spectrophotometric approximations were verified by quantitative culturing.

Biotinylation of *S. aureus*. *S. aureus* strains ALC59 and ALC620 were diluted in 0.2 M $NaHCO_3$, pH 8.3, to a 2% cell suspension and were biotinylated by the addition of *N*-hydroxysuccinimide–LC biotin (60 mg/ml; Pierce Chemical Co., Rockford, IL) freshly dissolved in dimethyl sulfoxide (Sigma Chemical Co., St. Louis, MO). After being mixed gently by tumbling for 60 min at ambient temperature, bacterial cells were washed extensively in 0.01 M Tris-buffered 0.15 M NaCl (TBS). Biotinylated organisms were used immediately or stored at 4°C for use within 24 to 48 h.

Recombinant gC1qR. The construction and expression of a plasmid, pGex-2T, containing an insert encoding the full-length/mature form (MF) (residues 74 to 282) and a truncated form (TF) (Δ74-95) of gC1qR, as well as the expression, purification, and characterization of the recombinant gC1qR protein, have been described in detail previously (7). The homogeneity of the full-length and truncated forms was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting (25), using monoclonal antibody (MAb) 74.5.2, which reacts with both forms of recombinant gC1qR. Previous studies have confirmed that full-length recombinant gC1qR possesses the same activities as the native molecule (7).

MAbs to gC1qR. MAbs 60.11 and 74.52, directed against specific gC1qR epitopes within amino acids 76 to 93 and 204 to 218, respectively, were described previously (5). These MAbs are associated with distinct gC1qR functional domains: the 60.11 binding domain is associated with C1q binding and modulation of complement activation (5), whereas the 74.5.2 binding site is involved in HK binding and activation of coagulation and kinin cascades (5, 16). MAb 74.5.2 has been shown in vitro to inhibit HK binding to endothelial cells and to block activation of the contact system of coagulation (16).

High-titer MAbs were produced in culture by using Integra Biosciences CELLine 1000 and serum-free medium (ADCF medium; HyClone Labs) by the Cell Culture/Hybridoma Facility, Department of Molecular Genetics and Microbiology, Stony Brook University, Stony Brook, N.Y. In vitro antibodies were harvested every 4 to 6 days. Antibody yields were in the range of 1.3 to 7.5 mg/ml.

***S. aureus* interaction with fibrinogen.** To avoid artifacts, *S. aureus* interactions with fibrinogen were investigated by several complementary methods. A traditional *S. aureus* clumping assay (28) was designed to assess the interaction between fibrinogen and the *clfA*-positive and -negative isogenic *S. aureus* strains in vitro, in the absence or presence of increasing concentrations of soluble gC1qR (10 to 300 μg/ml).

In addition, a solid-phase *S. aureus* adhesion assay was developed to evaluate biotinylated *S. aureus* interactions with immobilized fibrinogen under static conditions. Biotinylated bacteria were incubated in fibrinogen-coated microtiter wells for 60 min (22°C). The wells were washed, and bound biotinylated organisms were detected with alkaline phosphatase-conjugated streptavidin (Sigma Chemical Co.) and *p*-nitrophenyl phosphate (p-NPP) substrate.

Lastly, native *S. aureus* adhesion to immobilized fibrinogen was examined under low-shear conditions by use of a cone and plate viscometer developed by D. Varon, Hadassah-Hebrew University Medical Center, Jerusalem, Israel (27). For these studies, four-well microtiter plates (Nunc; Nalge Nunc International) were coated with 200 μl of purified fibrinogen (Kabi Vitrum, Stockholm, Sweden) (10 μg/ml) for 60 min at 37°C and blocked with 1% bovine serum albumin (BSA) (fatty acid free; Sigma Chemical Co.). Cultured *S. aureus* strains were added at a final inoculum of 10^8 CFU/ml, in the absence or presence of soluble gC1qR (50 to 100 μg/ml). The bacterial cell suspensions were sheared (500 s^{-1}) for 20 min at ambient temperature and removed from the microtiter wells. The wells were washed, and adherent microorganisms were stained with May Grunwald stain (Sigma Chemical Co.) as described previously (31). Bacterial cell adhesion was evaluated microscopically (×40), using a computer-driven software program that quantifies the percent surface coverage (27, 31). The same assay was used to evaluate the effect of gC1qR blockade in vitro by the addition of either MAb 60.11 or MAb 74.5.2 (10 to 100 μg/ml) or purified *S. aureus* protein A (100 μg/ml) (Sigma Chemical Co.). An isotype-matched antibody (MOPC-21; Sigma Chemical Co.) served as a control.

Rat endocarditis model. To evaluate the in vivo prophylactic efficacies of MAbs 74.5.2 and 60.11, we induced IE in a rat model with *S. aureus* strain RN6390. In brief, anesthetized rats (Sprague-Dawley, Indianapolis, Indiana) underwent transcarotid-transaortic-valve catheterization with a polyethylene catheter to induce sterile aortic-valve vegetations as previously described (33). The catheter was left in place throughout the study. Immediately after catheterization, animals were randomized to receive either (i) no antibody treatment (control), (ii) MAb 60.11 at 100 mg/kg of body weight, (iii) MAb 74.5.2 at 100 mg/kg, (iv) MAb 74.5.2 at 200 mg/kg, or (v) a combination of MAb 60.11 (100 mg/kg) and MAb 74.5.2 (100 mg/kg). All MAb administrations were single doses and were given intraperitoneally (i.p.). These regimens were selected based on detailed pilot pharmacokinetic analyses and in vitro antimicrobial activity studies, which showed that these doses produced prolonged serum antibody levels (≥60 h) of at least 100 μg/ml. Given the ubiquitous distribution of gC1qR in plasma (25, 30), in the extracellular matrix (11), and on peripheral blood and somatic cells (7), circulating MAb concentrations were targeted to achieve approximately fivefold higher levels than those required for functional inhibition of *S. aureus*-fibrinogen interactions in an in vitro clumping assay. Serum samples were obtained after MAb administration at selected time points for measurements of antibody levels and for use in vitro in functional assays (see below). At 48 h post-MAb administration, animals were challenged intravenously with *S. aureus* RN6390 (5×10^4 CFU). This inoculum caused IE in >95% of catheterized rats in extensive pilot studies. At 24 h postinfection, animals were euthanized with a rapid i.p. injection of 200 mg/kg sodium pentobarbital (Abbott Laboratories, Chicago, Illinois). Upon sacrifice, vegetations, kidneys, and spleens were aseptically removed to determine *S. aureus* densities in these target tissue lesions. Animals were included in the analysis only if the catheters were correctly positioned across the aortic valve and macroscopic vegetations were detected. All animals with correctly placed catheters developed vegetations. Target tissue samples were weighed and homogenized in 1.0 ml of sterile saline with a tissue homogenizer. Tissue homogenates from each sample were quantitatively cultured on TSB agar plates and incubated for 24 h at 37°C. Animals with culture-positive vegetations were considered to have IE. Bacterial densities were expressed as the $\log_{10}$ CFU/g tissue (± standard deviation [SD]). Mean tissue densities in the different treatment groups were compared statistically to evaluate the prophylactic efficacy. All animals were treated in accordance with institutional and U.S. Public Health Service guidelines for the humane care and treatment of animals.

Quantitation and functional evaluation of gC1qR antibodies in rat serum. An enzyme-linked immunosorbent assay was designed in which soluble, recombinant gC1qR was immobilized on 96-well plates. After blocking of the wells with 1% BSA, standard concentrations of anti-gC1qR antibodies (0 to 10 μg/ml) were prepared in naïve rat serum diluted 1/10 with 0.3 M NaCl and then added to the wells. Test rat sera (after i.p. MAb administration) were diluted 1/10 in 0.3 M NaCl and added to separate wells. Following incubation for 60 min at ambient temperature, bound antibody was detected spectrophotometrically (OD_{405}), using alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (IgG; Sigma Chemical Co.) and p-NPP substrate. The reactivities of test plasmas were compared to those of standard anti-gC1qR antibody solutions. The results are expressed in μg anti-gC1gR/ml serum (± SD). Each serum sample was evaluated in duplicate for data reproducibility.

The functional blocking activity of i.p.-administered antibody was assessed in vitro by examining gC1qR-enhanced *S. aureus* interactions with fibrinogen in the presence of rat sera from animals receiving MAbs. For these studies, the *clfA*-positive *S. aureus* parental strain was grown to logarithmic phase and biotinylated as described above. A 10% suspension of the biotinylated bacteria was made in rat serum diluted 1/20 in 0.01 M TBS. *S. aureus* suspensions were applied to fibrinogen-coated microtiter wells in the presence of 100 μg/ml soluble gC1qR and incubated at 37°C for 60 min. Bacterial adhesion was measured spectrophotometrically (OD_{405}), using alkaline phosphatase-conjugated streptavidin (Sigma Chemical Co.) and the p-NPP substrate. Baseline adhesion was assessed in the absence of added gC1qR.

Immunohistochemistry. Immunohistochemical staining of infected rat aortic valves was performed (23) to visualize the localization of infused anti-gC1qR antibodies on such lesions. Tissues were obtained from animals with microbiologically confirmed endocarditis at autopsy. Tissues were fixed in Zeus fixative (Zeus Scientific Inc., Raritan, NJ), embedded in paraffin, and sectioned by the Department of Surgical Pathology, Weill Medical College of Cornell University. Antibody was detected by using a biotinylated secondary antibody reagent at room temperature for 2 h (Vectastain Elite ABC kit; Vector Laboratories, Inc., Burlingame, CA) (23).

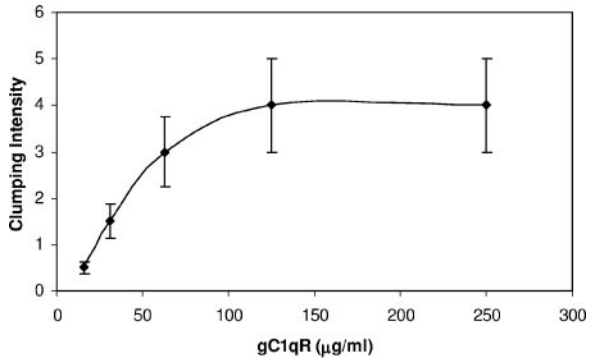

FIG. 1. Effect of increasing concentrations of soluble gC1qR on *S. aureus* interaction with fibrinogen, measured by a traditional clumping assay (28). A *clfA*-negative *S. aureus* strain was exposed to 10 μg/ml fibrinogen and increasing concentrations of gC1qR. After 30 min of shaking at 37°C, clumping was scored visually on a scale of 0 to 4. The data represent means ± SD ($n = 8$).

After being washed, the slides were treated with streptavidin-peroxidase reagent for 30 min at room temperature. The reactions were developed with a 3,3′-diaminobenzidine solution, and the slides were counterstained with hematoxylin and then mounted. Control immunostaining was performed by replacement of the secondary antibody with biotinylated polyclonal rabbit anti-ovalbumin.

Statistical analysis. For in vitro studies, quantitative data sets were compared by using the paired Student *t* test. For in vivo experiments, Kruskal-Wallis analysis of variance with the Tukey post hoc modification for multiple comparisons was utilized. *P* values of ≤0.05 were considered statistically significant.

RESULTS

gC1qR mediates interactions between fibrinogen and *S. aureus* in vitro. Since gC1qR is a multimeric protein that was shown previously to bind both fibrinogen (18) and *S. aureus* protein A (SpA) (22), we hypothesized that soluble gC1qR could mediate *S. aureus* interactions with fibrinogen. Initial studies were performed to assess the ability of gC1qR to bridge *S. aureus* and fibrinogen, using a *clfA*-negative *S. aureus* strain exhibiting a markedly diminished ability to recognize fibrinogen in a standard clumping assay. Figure 1 demonstrates the ability of soluble gC1qR to restore clumping activity to the *clfA*-negative *S. aureus* strain in the presence of fibrinogen in a dose-dependent manner. Maximum clumping activity was attained in the presence of 100 to 150 μg/ml gC1qR. Assuming a trimeric structure for gC1qR (15), this concentration represents a molar ratio of gC1qR to fibrinogen of ~5:1.

Additional studies (Fig. 2) examined the effect of soluble gC1qR (100 μg/ml) on *S. aureus* interaction with immobilized fibrinogen under low-shear conditions, using the *clfA*-positive and -negative strain pair. *S. aureus* binding to fibrinogen is shown relative to that of a buffer control, set at 100%, using the *clfA*-positive *S. aureus* strain without gC1qR. The full-length recombinant gC1qR protein (MF) significantly ($P < 0.05$) enhanced the interaction of both *S. aureus* strains with fibrinogen. Consistent with the results of the *S. aureus* clumping assay, gC1qR had a particularly pronounced effect on the adhesion of the *clfA*-negative mutant strain. gC1qR increased the adhesion of the *clfA*-positive *S. aureus* strain to fibrinogen approximately 30%, whereas the adhesion of the *clfA*-negative strain increased over 100%.

Interestingly, a truncated form of gC1qR (TF), lacking amino

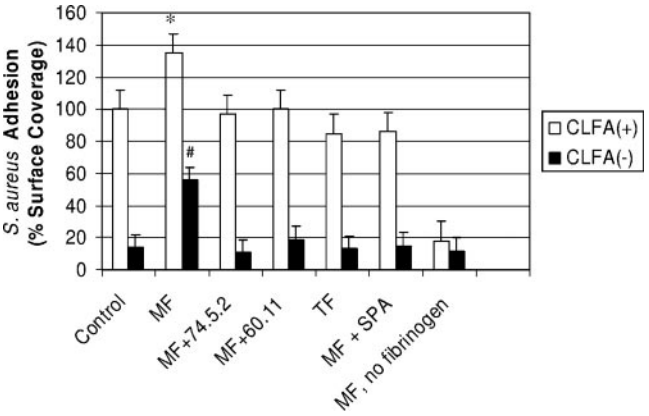

FIG. 2. Adhesion of *S. aureus* to immobilized fibrinogen. Microtiter wells were coated with fibrinogen (10 μg/ml) and blocked with 1% BSA as described in Materials and Methods. Cultured *clfA*-positive [CLFA(+)] and -negative [CLFA(−)] *S. aureus* strains were added (10^8/ml) in the absence (control) or presence of soluble full-length gC1qR (MF) or a truncated form (TF) (Δ74-95) (100 μg/ml), with or without either MAb 74.5.2 or MAb 60.11 at 10 μg/ml or purified protein A (SPA) (100 μg/ml). The bacterial cell suspensions were sheared (500 s^{-1}) for 20 min. The supernatant was removed, and the wells were washed and subsequently stained with May Grunwald stain. Bacterial cell adhesion was quantified microscopically (×40) using a computer-assisted software program that quantifies the percent surface coverage. The results reflect *S. aureus* adhesion normalized to that of *clfA*-positive *S. aureus* in the absence of gC1qR ($CLFA^+$ control). Error bars indicate SD ($n = 5$). * and #, statistically significant differences ($P < 0.05$) in bacterial cell adhesion compared to that of the relevant *clfA*-positive or -negative control, respectively. The effect of gC1qR on *S. aureus* interactions with bovine serum albumin instead of fibrinogen-coated surfaces is shown as a negative control.

acids 74 to 95 and involved in C1q binding (7), failed to enhance *S. aureus* interactions with fibrinogen (Fig. 2). In addition, MAb 60.11, directed against the same region (amino acids 76 to 93), inhibited the effect of full-length gC1qR on *S. aureus* interactions with fibrinogen. gC1qR-mediated *S. aureus* interactions with fibrinogen were also inhibited by MAb 74.5.2, directed against amino acids 204 to 218. Maximum inhibition (80 to 100%) of the observed gC1qR effect was noted at or above 10 μg/ml MAb. The inhibition of gC1qR-mediated *S. aureus* interactions with fibrinogen by both MAb 60.11 and MAb 74.5.2 suggests either the involvement of multiple functional epitopes or steric hindrance effects. In addition, excess SpA prevented gC1qR-mediated *S. aureus* interactions with fibrinogen. gC1qR did not induce *S. aureus* clumping in the absence of fibrinogen (data not shown) or *S. aureus* adhesion to albumin (Fig. 2).

TABLE 1. gC1qR MAb titers in rat sera[a]

Time (h)	gC1qR MAb titer (μg/ml)	
	74.5.2	60.11
0	0	0
17	172 ± 16	131 ± 15
24	167 ± 13	137 ± 18
41	162 ± 20	138 ± 18
65	153 ± 15	127 ± 11

[a] Rat sera were examined by enzyme-linked immunosorbent assay for the presence of gC1qR MAb 74.5.2 ($n = 5$) or 60.11 ($n = 3$) as described in Materials and Methods. Data are representative of all subsequent animal studies.

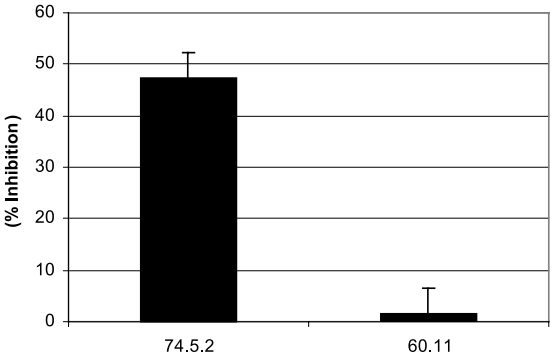

FIG. 3. Functional activities of gC1qR MAbs 74.5.2 and 60.11 in rat serum. Biotinylated, *clfA*-positive bacteria were suspended (10% cell suspension) in selected serum samples from animals treated with gC1qR MAbs. Samples were applied to fibrinogen-coated microtiter wells after dilution (1/20) in TBS and the addition of gC1qR (100 μg/ml). Following incubation for 60 min at 37°C, wells were washed, and bacterial cell adhesion was quantified using alkaline phosphatase-conjugated streptavidin and p-NPP substrate. The results reflect percentages of inhibition of gC1qR-mediated *S. aureus* adhesion over the baseline. Baseline gC1qR-induced adhesion was defined for naïve rat serum as the difference between bacterial cell adhesion in the presence and absence of added gC1qR. The data depict means ± SD for 10 representative sera from animals treated with MAb 74.5.2 and 3 representative sera from animals treated with MAb 60.11.

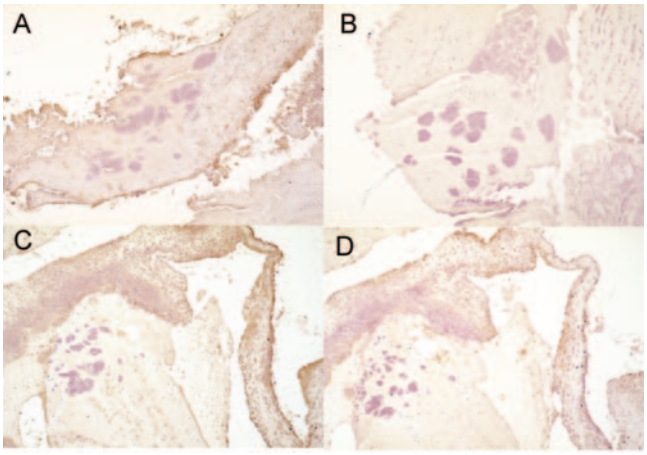

FIG. 4. Deposition of gC1qR MAbs on infected rat aortic valve vegetations after i.p. administration. Vegetations were obtained at the end of the experiment from control animals (B) or animals treated with MAb 74.5.2 (A) or MAb 60.11 (C). Specimens were fixed in Zeus fixative, embedded in paraffin, and sectioned. Immunohistochemical staining was performed using a VectaStain ABC kit. Serial sections were processed using a nonimmune secondary antibody (D). Sections were counterstained with hematoxylin. The brown-staining precipitate reflects MAb deposition. No reaction was seen in sections from control animals (B). Minimal staining occurred with nonimmune rabbit IgG in serial sections of valves from MAb-treated animals. Magnification, ×200.

In vivo studies of gC1qR blockade using a rat model of *S. aureus* IE. Since *S. aureus* binding to fibrinogen is a key event in initiating endovascular infections (26), the results from the above studies suggested that gC1qR blockade might exert prophylactic effects on IE. This hypothesis was addressed by using a rat model of *S. aureus* IE. Animals were given prophylaxis therapy with the anti-gC1qR MAbs 74.5.2 and 60.11 (6).

Sustained high levels of both anti-gC1qR MAbs (>100 μg/ml) were routinely achieved in rat sera following i.p. administration (Table 1). However, only circulating MAb 74.5.2 demonstrated inhibition of the gC1qR-induced increase in *S. aureus* (*clfA*$^+$) interactions with immobilized fibrinogen in vitro (Fig. 3). To minimize nonspecific effects of high concentrations of serum proteins on the *S. aureus* adhesion assay, rat sera were diluted 1/20 in TBS for a final MAb concentration between 5 and 8 μg/ml, slightly below the maximum inhibitory concentrations defined by in vitro studies using purified antibodies in buffer systems.

To validate that the MAbs to gC1qR bound to gC1qR expressed at sites of endovascular injury, further studies used an immunohistochemical approach to visualize antibody deposition on infected aortic valve vegetations. Figure 4A depicts the presence of MAb 74.5.2 on a representative vegetation. The presence of antibody is indicated by a brown stain precipitate along the surface of the vegetation. Similar results were noted for animals given prophylaxis therapy with 60.11 (Fig. 4C). An aortic valve vegetation from an infected animal that was not treated with MAb is shown for comparison (Fig. 4B). In addition, nonspecific staining with biotinylated nonimmune IgG (Fig. 4D) is shown in a serial section of the infected valve from the animal treated with 60.11.

Microbiologic evaluation of endocarditis model. All animals developed IE, with hematogenous dissemination to the kidneys and spleen. No differences in vegetation weight were noted between control and antibody-treated animals (data not shown). However, there were substantial differences between controls and MAb-treated animals in terms of achievable target tissue *S. aureus* densities. Table 2 summarizes the *S. aureus* densities in aortic valve vegetations, kidneys, and spleens for the different treatment groups. Monotherapy with MAb 74.5.2 at 100 mg/kg caused significant reductions in *S. aureus* densities in vegetations and kidneys ($P < 0.05$ versus control) while causing significant reductions in all target tissues at 200 mg/kg ($P < 0.005$ versus control). In contrast, MAb 60.11 yielded smaller reductions in the three target organs compared to untreated controls that trended towards but did not reach statistical significance. Notably, the combination MAb regimen caused significant reductions in *S. aureus* densities versus those in control animals ($P < 0.005$) in all target tissues and versus MAb 60.11

TABLE 2. Effect of prophylaxis with gC1qR antibodies on *S. aureus* colony counts in a rat model of infective endocarditis

Treatment group (n)[b]	Colony count (mean $\log_{10}$ CFU/g ± SD)[a]		
	Vegetations	Kidneys	Spleen
Control (7)	9.49 ± 1.1	5.04 ± 0.64	5.54 ± 0.74
MAb 74.5.2			
100 mg/kg (10)	6.45 ± 1.2*	3.53 ± 1.10*	3.8 ± 1.05
200 mg/kg (6)	5.51 ± 0.76*	2.61 ± 0.77*	3.57 ± 0.45*
MAb 60.11 (100 mg/kg) (6)	7.37 ± 0.88	3.49 ± 1.07	3.99 ± 1.09
MAbs 74.5.2 and 60.11 (100 mg/kg [each]) (5)	3.56 ± 1.25*#	0.94 ± 0.05*#	3.19 ± 0.198*

[a] *, $P < 0.05$ compared to colony counts for tissues from the control group; #, $P < 0.05$ compared to colony counts for tissues from animals treated with the 60.11 antibody alone.

[b] All MAb treatments were administered i.p.

alone ($P < 0.05$) in both vegetations and kidneys (but not spleens). Combination therapy also appeared to reduce microbial densities on vegetations and kidneys compared to monotherapy with MAb 74.5.2, although this was not statistically significant.

DISCUSSION

S. aureus is a pathogenic bacterium that causes a variety of infections, including IE, in humans (1, 21). At the cardiac valvular surface, the interaction of *S. aureus* with platelets and fibrin is critical for the induction of infective endocarditis (4, 29). Local inflammation and vasodilation likely contribute to disease progression and bacterial dissemination to distant organs.

We previously described a specific interaction between SpA and human platelets involving gC1qR (22). gC1qR is a multiligand binding protein (6) that has been identified in plasma/serum (25, 30), the extracellular matrix (11), and a variety of somatic cells, including activated platelets (24) and endothelial cells (9). gC1qR has been shown to bind fibrinogen (18), high-molecular-weight kininogen, and F XII (16) and to be involved in complement activation on platelets and endothelial cells (34). In addition, gC1qR interacts with several viral and bacterial antigens (6). Thus, gC1qR may play an important role in inflammation and infection.

The current studies were designed to further delineate the role of gC1qR in *S. aureus* pathogenesis in vitro and in vivo. Several interesting observations emerged from these investigations. Firstly, our data confirmed that soluble gC1qR enhances the *S. aureus* interaction with fibrinogen and can compensate well for any diminished fibrinogen adhesion of *S. aureus* strains. The ability of gC1qR to bridge *S. aureus* and fibrinogen was documented using a variant *S. aureus* strain deficient in one of the major fibrinogen adhesins, *clfA*. Enhanced adhesion to fibrinogen in the presence of gC1qR was demonstrated using soluble *S. aureus* clumping assays and solid-phase fibrinogen binding assays under static or low-shear conditions.

The effect of gC1qR on the *S. aureus* interaction with fibrinogen/fibrin may be influenced by physiologic shear stress. The present study did not address the impact of physiologic or pathological shear on *S. aureus* adhesion to fibrinogen. Conceivably, the bridging effect of gC1qR may stabilize bacterial interactions at endovascular lesions under high shear stress. Studies are under way to evaluate this hypothesis.

Secondly, the observed increase in bacterial cell adhesion to fibrinogen mediated by gC1qR appears to require gC1qR N-terminal amino acids 74 to 95. A truncated gC1qR variant lacking this sequence failed to demonstrate activity in solid-phase *S. aureus* adhesion assays, and MAb 60.11, which recognizes an epitope defined by this sequence, inhibited the gC1qR effect when added in vitro. In addition, gC1qR-mediated bacterial cell adhesion to fibrinogen was abrogated by excess soluble SpA, consistent with gC1qR bridging of SpA (22) and the fibrinogen D domain (18).

Interestingly, rat sera containing MAb 74.5.2 demonstrated the ability to inhibit gC1qR-mediated increases in *S. aureus* interactions with fibrinogen, but MAb 60.11-containing sera failed to do so. The reasons for this are unclear. Perhaps MAb 60.11 binds more avidly to circulating gC1qR and/or cross-reacting antigens in serum, mitigating the inhibitory effect of serum samples after in vivo MAb administration.

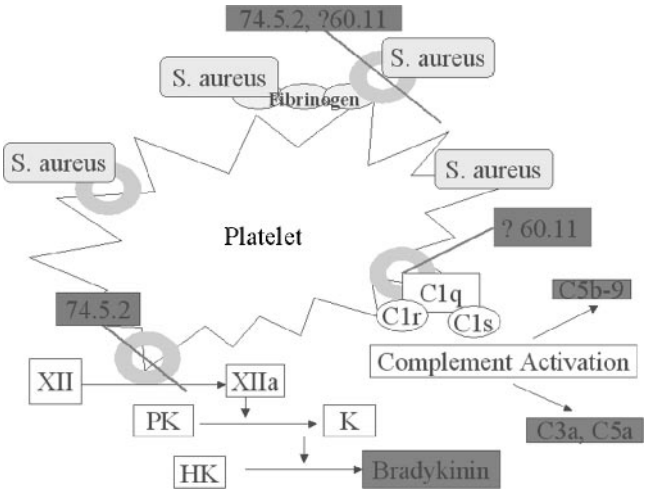

FIG. 5. Model depicting potential gC1qR effects on *S. aureus* infective endocarditis. gC1qR (circles) is shown to enhance *S. aureus* colonization of endovascular lesions by supporting bacterial interactions with activated platelets and platelet-associated fibrinogen/fibrin. In addition, gC1qR may contribute to infection by supporting local bradykinin generation and vascular permeability as well as complement activation, leading to increased inflammation and tissue damage. Abbreviations: XII, coagulation factor XII; PK, prekallikrein; K, kallikrein; HK, high-molecular-weight kininogen.

gC1qR blockade by either MAb 74.5.2 or 60.11 reduced bacterial colonization of vegetations, kidneys, and spleens. However, the reductions achieved by MAb 60.11 did not reach statistical significance, perhaps due to the smaller number of animals in the MAb 60.11 treatment group. Animals treated with MAb 60.11 and MAb 74.5.2 in combination demonstrated more profound reductions in bacterial colonization and/or proliferation in all target tissues than those in animals treated with MAb 60.11 alone. The antibody combination was also more effective than prophylaxis with MAb 74.5.2 alone with respect to reducing bacterial infections of aortic valves and kidneys.

These observations support the hypothesis that gC1qR plays an important role in *S. aureus* endovascular infections in vivo, in part by enhancing *S. aureus* interactions with fibrinogen. Soluble circulating levels of gC1qR in human plasma have been reported to range between 1 and 5 μg/ml (30). Whereas this is significantly lower than the maximum gC1qR concentrations required in the present in vitro studies, the accrual of soluble gC1qR from plasma on fibrin in evolving endovascular lesions over time in vivo may be compensatory. Furthermore, the in vitro assays allowed for only a short (20 to 30 min) exposure time for gC1qR-fibrinogen-*S. aureus* interactions.

The enhanced efficacy of combination MAb therapy may suggest additional specific MAb-mediated inhibitory effects. The postulated activities of gC1qR in *S. aureus* infective endocarditis and their inhibition are summarized in Fig. 5. Direct effects of gC1qR include recognition of *S. aureus* protein A by platelet-associated gC1qR (22) and enhanced *S. aureus* interactions with fibrinogen, contributing to the recruitment of *S. aureus* to vegetations rich in platelets and fibrin. In addition, gC1qR-mediated activation of the kinin cascade (16) at sites of infection may contribute to local vascular permeability. Indeed, focal bradykinin generation has been shown by exper-

imental infections of mice to contribute to intravascular dissemination of the infectious organism (19). Finally, complement activation, with the generation of proinflammatory peptides and C5b-9, may contribute to tissue damage. Thus, the blockade of gC1qR by use of MAbs may mitigate infective endocarditis by influencing several of these pathways.

Alternatively, or in addition, localization of MAb 74.5.2 or 60.11 to sites of endovascular damage via locally expressed gC1qR may lead to targeted immune complex formation and activation of the classical complement pathway. The activation of complement in the immediate vicinity of *S. aureus* may lead to complement deposition on bystander bacterial cells, with subsequent complement-mediated cell lysis. Furthermore, the generation of complement-derived anaphylatoxins may increase the recruitment of inflammatory cells with the potential for enhanced *S. aureus* phagocytosis. In this manner, prophylaxis with either MAb 74.5.2 or MAb 60.11 would be expected to enhance killing of the bacterial pathogen.

In summary, the results of the present study identify gC1qR as a key participant in *S. aureus* endovascular pathogenesis and as a potential prophylactic target in this context. Further studies are required to elucidate the specific mechanism(s) of action of gC1qR MAbs in this model system.

ACKNOWLEDGMENTS

We are grateful for the expert technical assistance provided by Sage E. Grigg and Yin Li.

This work was supported in part by grants from the National Institutes of Health (HL67211 to E.I.B.P. and AI-39108 to A.S.B.) and from the American Heart Association (Western affiliate) (0265054Y and 0465142Y4 to Y.Q.X.).

REFERENCES

1. **Archer, G. L.** 1998. Staphylococcus aureus: a well armed pathogen. Clin. Infect. Dis. **26:**1179–1181.
2. **Blevins, J. S., K. E. Beenken, M. O. Elasri, B. K. Hurlburt, and M. S. Smeltzer.** 2002. Strain-dependent differences in the regulatory roles of *sarA* and *agr* in *Staphylococcus aureus*. Infect. Immun. **70:**470–480.
3. **Foster, T. H., and M. Hook.** 1998. Surface protein adhesin of Staphylococcus aureus. Trends Microbiol. **6:**484–488.
4. **Foster, T. J., and D. McDevitt.** 1994. Surface-associated proteins of Staphylococcus aureus: their possible roles in virulence. FEMS Microbiol. Lett. **118:**199–205.
5. **Ghebrehiwet, B., J. Jesty, and E. I. B. Peerschke.** 2002. GC1qR/p33: structure-function predictions from the crystal structure. Immunobiology **105:**421–432.
6. **Ghebrehiwet, B., B. L. Lim, R. Kumar, C. Feng, and E. I. B. Peerschke.** 2001. GC1qR/p33, a member of a new class of multifunctional and multicompartmental cellular proteins, is involved in inflammation and infection. Immunol. Rev. **180:**65–77.
7. **Ghebrehiwet, B., B.-L. Lim, E. I. B. Peerschke, A. C. Willis, and K. B. M. Reid.** 1994. Isolation, cDNA cloning, and overexpression of a 33kDa cell surface glycoprotein that binds to the globular 'heads' of C1q. J. Exp. Med. **179:**1809–1821.
8. **Grundmeier, M., M. Hussain, P. Becker, C. Heilmann, G. Peters, and B. Sinha.** 2004. Truncation of fibronectin-binding proteins in *Staphylococcus aureus* strain Newman leads to deficient adherence and host cell invasion due to loss of the cell wall anchor function. Infect. Immun. **72:**7155–7163.
9. **Guo, W.-X., B. Ghebrehiwet, B. Weksler, K. Schweitzer, and E. I. B. Peerschke.** 1999. Upregulation of endothelial cell binding proteins/receptors for complement component C1q by inflammatory cytokines. J. Lab. Clin. Med. **133:**541–550.
10. **Hartleib, J., N. Kohler, R. B. Dickinson, G. S. Chharwas, J. J. Sixma, O. M. Hartford, T. J. Foster, G. Peters, B. E. Kehrel, and M. Herrmann.** 2000. Protein A is the von Willebrand factor binding protein on *Staphylococcus aureus*. Blood **96:**2149–2156.
11. **Hasan, A. A. K., T. Zisman, and A. H. Schmaier.** 1998. Identification of cytokeratin 1 as a binding protein and presentation receptor for kininogens on endothelial cells. Proc. Natl. Acad. Sci. USA **95:**3615–3620.
12. **Hawiger, J. S., S. Streckley, and D. Hammond.** 1979. Staphylococci-induced human platelet injury mediated by protein A and immunoglobulin G Fc receptor. J. Clin. Investig. **64:**931–937.
13. **Hawiger, J., S. Timmons, D. D. Strong, B. A. Cottrel, M. Riley, and R. F. Doolittle.** 1982. Identification of a region of human fibrinogen interacting with staphylococcal clumping factor. Biochemistry **21:**1407–1420.
14. **Herrmann, M. P., P. Vaudaux, D. Pittet, R. Auckenthaler, P. D. Lew, F. Schumacher-Perdreau, G. Peters, and F. A. Waldvogel.** 1988. Fibronectin, fibrinogen and laminin act as mediators of adherence of clinical staphylococcal isolates to foreign material. J. Infect. Dis. **158:**693–701.
15. **Jiang, J., Y. Zhang, A. R. Krainer, and R.-M. Xu.** 1999. Crystal structure of human p32, a doughnut shaped acidic mitochondrial matrix protein. Proc. Natl. Acad. Sci. USA **96:**3572–3577.
16. **Joseph, K., B. Ghebrehiwet, and A. P. Kaplan.** 1999. Cytokeratin 1 and gC1qR mediate high molecular weight kininogen and factor XII binding to endothelial cells. Clin. Immunol. **92:**246–255.
17. **Kahl, B. C., M. Goulian, W. van Wamel, M. Herrmann, S. M. Simon, G. Kaplan, G. Peters, and A. L. Cheung.** 2000. *Staphylococcus aureus* RN 6390 replicates and induces apoptosis in a pulmonary epithelial cell line. Infect. Immun. **68:**5385–5392.
18. **Lu, P. D., D. K. Galanakis, B. Ghebrehiwet, and E. I. B. Peerschke.** 1999. The receptor for the globular "heads" of C1q, gC1qR, binds to fibrinogen/fibrin and impairs its polymerization. Clin. Immunol. **90:**360–367.
19. **Maruo, K., T. Akaike, T. Ono, and H. Maeda.** 1998. Involvement of bradykinin generation in intravascular dissemination of *Vibrio vulnificus* and prevention of invasion by a bradykinin antagonist. Infect. Immun. **66:**866–869.
20. **McDevitt, D., T. Nanavaty, K. House-Pompeo, E. Bell, N. Turner, L. McIntire, R. Foster, and M. Hook.** 1997. Characterization of the interaction between the *Staphylococcus aureus* clumping factor A (*clfA*) and fibrinogen. Eur. J. Biochem. **247:**416–424.
21. **Moreillon, P., and Y. Que.** 2004. Infective endocarditis. Lancet **363:**139–149.
22. **Nguyen, T., B. Ghebrehiwet, and E. I. B. Peerschke.** 2000. *Staphylococcus aureus* protein A recognizes platelet gC1qR/p33: a novel mechanism for staphylococcal interactions with platelets. Infect. Immun. **68:**2061–2068.
23. **Peerschke, E. I. B., J. O. Minta, S. Z. Zhou, A. Bini, A. Gotlieb, R. W. Colman, and B. Ghebrehiwet.** 2004. Expression of gC1qR/p33 and its major ligands in human atherosclerotic lesions. Mol. Immunol. **41:**759–766.
24. **Peerschke, E. I. B., T. K. Murphy, and B. Ghebrehiwet.** 2003. Activation-dependent surface expression of gC1qR/p33 on human blood platelets. Thromb. Haemost. **89:**331–339.
25. **Peterson, K. L., W. Zhang, P. D. Lu, S. A. Keilbaugh, E. I. B. Peerschke, and B. Ghebrehiwet.** 1997. The C1q-binding cell membrane proteins cC1qR and gC1qR are released from activated cells: subcellular distribution and immunochemical characterization. Clin. Immunol. Immunopathol. **84:**17–26.
26. **Que, Y.-A., J.-A. Haefliger, L. Piroth, P. Francois, E. Widmer, J. M. Entenza, B. Sinha, M. Herrmann, P. Francioli, P. Vaudaux, and P. Moreillon.** 2005. Fibrinogen and fibronectin binding cooperate for valve infection and invasion in *Staphylococcus aureus* experimental endocarditis. J. Exp. Med. **201:**1627–1635.
27. **Shenkman, B., N. Savion, R. Dardik, I. Tamarin, and D. Varon.** 2000. Testing of platelet deposition on polystyrene surface under flow conditions by the cone and plate(let) analyzer: role of platelet activation, fibrinogen, and von Willebrand factor. Thromb. Res. **99:**353–361.
28. **Strong, D. D., A. P. Laudano, J. Hawiger, and R. F. Doolittle.** 1982. Isolation, characterization and synthesis of peptides from human fibrinogen that block staphylococcal clumping reactions and construction of a synthetic clumping particle. Biochemistry **21:**1414–1420.
29. **Sullam, P. M.** 1994. Host-pathogen interaction in the development of bacterial endocarditis. Curr. Opin. Infect. Dis. **7:**304–309.
30. **van den Berg, R. H., F. Prins, M. C. Faber-Krol, N. J. Lynch, W. Schwaeble, L. A. van Es, and M. R. Daha.** 1997. Intracellular localization of the human receptor for the globular domains of C1q. J. Immunol. **158:**3909–3916.
31. **Varon, D., R. Dardik, S. Shenkman, S. Kotev-Emeth, N. Farzame, I. Tamarin, and N. Savion.** 1997. A new method for quantitative analysis of whole blood platelet interaction with extracellular matrix under flow conditions. Thromb. Res. **85:**283–294.
32. **Wolz, C., D. McDevitt, T. J. Foster, and A. L. Cheung.** 1996. Influence of *agr* on fibrinogen binding in *Staphylococcus aureus* Newman. Infect. Immun. **64:**3142–3147.
33. **Xiong, Y.-Q., J. Willard, J. L. Kadurugamuwa, J. Yu, K. Francis, and A. S. Bayer.** 2005. Real-time bioluminescent imaging for evaluating the efficacy of antibiotics in a rat *Staphylococcus aureus* endocarditis model. Antimicrob. Agents Chemother. **49:**380–387.
34. **Yin, W., B. Weksler, D. Varon, N. Savion, B. Ghebrehiwet, and E. I. B. Peerschke.** 2005. Activation of the classical pathway of complement by resting and shear-stress stimulated human endothelial cells. Blood **106:**2664.

Editor: V. J. DiRita