New prophylactic approaches are needed to control infection with the Gram-positive bacterium Staphylococcus aureus, which is a major cause of nosocomial and community-acquired infections. To develop these, greater understanding of protective immunity against S. aureus infection is needed. Human immunity to extracellular Gram-positive bacterial pathogens is primarily mediated by opsonic killing (OPK) via antibodies specific for surface polysaccharides. S. aureus expresses two such antigens, capsular polysaccharide (CP) and poly-N-acetyl glucosamine (PNAG). Here, we have shown that immunization-induced polyclonal animal antisera and monoclonal antibodies specific for either CP or PNAG antigens have excellent in vitro OPK activity in human blood but that when mixed together they show potent interference in OPK activity. In addition, reductions in antibody binding to the bacterial surface, complement deposition, and passive protection were seen in two mouse models of S. aureus infection. Electron microscopy, isothermal calorimetry, and surface plasmon resonance indicated that antibodies to CP and PNAG bound together via an apparent idiotype–anti-idiotype interaction. This interaction was also found in sera from humans with S. aureus bacteremia. These findings suggest that the lack of effective immunity to S. aureus infections in humans could be due, in part, to interference in OPK when antibodies to CP and PNAG antigens are both present. This information could be used to better design S. aureus vaccine components.

Conflict of interest: Tomas Maira-Litran and Gerald B. Pier have received research support and licensing income related to the development of active and passive immunotherapies targeting PNAG. David Skurnik, Gerald B. Pier, and Jean C. Lee have received consulting income related to development of vaccines for Staphylococcus aureus.

Citation for this article: J Clin Invest. 2010;120(9):3220–3233. doi:10.1172/JCI42748.
the OPKA in the adsorbed sera was lost. Finding that antibodies to PNAG and CP antigens have neither additive nor synergistic OPKA against *S. aureus*, and may, in fact, interfere with protective efficacy mediated by the antibodies specific to the individual antigens, has implications for vaccine development and might explain, in part, the lack of development of effective human immunity to *S. aureus* following infection.

**Results**

Opsonophagocytic activity of polyclonal animal antisera to CP and PNAG antigens. We raised polyclonal rabbit, mouse, and goat antisera to tetanus-toxoid– or diphtheria toxoid–conjugated (TT- or DT-conjugated) CP5, CP8, or the deacetylated glycoform of PNAG (dPNAG) (15) to induce opsonic antibodies, then combined the different antisera in various proportions to determine whether addi-
Mouse mAbs to CP8 or PNAG. We used two different mouse Pseudomonas aeruginosa μ that was lost when combined with rab CP8 strain PS80 had signifi (Supplemental Table 1). Combining the mAbs to PNAG or CP8 with a rabbit antisera to dPNAG or CP8, respectively, decreased OPKA to 20%–25% (Supplemental Table 1). Mixing the mouse mAb to CP8 with the mouse mAb to PNAG also decreased OPKA to 23% (Supplemental Table 1).

**Opsonophagocytic activity of mAbs.** Mouse mAbs to CP8 or PNAG and a human mAb to PNAG (21) at a concentration of 6.25 μg/ml had OPKA of 62% or greater against S. aureus PS80 (Supplemental Table 1). Combining the mAbs to PNAG or CP8 with a rabbit antisera to CP8 or PNAG, respectively, decreased OPKA to 20%–25% (Supplemental Table 1). Mixing the mouse mAb to CP8 with the mouse mAb to PNAG also decreased OPKA to 23% (Supplemental Table 1).

**In vivo animal protection studies.** We used two different mouse models of S. aureus infection — bacteremia (22) and a model of skin infection produced by combining bacteria with 131- to 220-μm-diameter Cytodex beads (23) — to determine whether mixing antibodies to CP antigens and PNAG also interfered with in vivo protection. Mouse passively given antibody to either CP8 or PNAG and challenged i.v. with S. aureus CP8 strain PS80 had significantly (P < 0.001, ANOVA and Tukey’s multiple comparisons test [TMCT]) lower S. aureus cells in their blood when compared with controls given NRS (Figure 3A). Combining antisera to PNAG and CP resulted in no reduction in blood bacterial levels compared with mice given NRS (P > 0.05, TMCT). The higher levels of S. aureus in the blood of mice given antibody to both PNAG and CP compared to animals receiving monospecific antibodies to these antigens indicated interference in the activity of the monospecific antibodies when both were present.

A similar outcome was obtained in a murine skin abscess model (Figure 3B), in that antibody to either PNAG or CP8 significantly (P < 0.001, ANOVA and TMCT) reduced the levels of S. aureus PS80 in the abscesses 72 hours after infection, but when the antibodies were mixed together, the protective efficacy was lost (P > 0.05 vs. NRS, TMCT). When the CP-negative, PNAG-positive USA 300 MRSA strain LAC was used in the skin abscess model (Figure 3C), antibody to PNAG significantly (P < 0.001, ANOVA and TMCT) reduced the mean CFU/abscess compared with that in mice given antibody to CP8, antibody to both PNAG and CP8, or NRS. When antibody to CP5 was used in the skin abscess model with strain LAC (Figure 3D), antibody to PNAG, but not antibody to CP5, again sig-
nificantly (P < 0.001, ANOVA and TMCT) reduced the mean CFU/abscess compared with that in animals given NRS, and combining antibodies to PNAG and CP5 abrogated the ability of the antibody to PNAG to reduce infection levels, even if the two antibodies were injected into the mice using two different syringes (Figure 3D).

The experiments coadministering antibodies to both CP and PNAG antigens were repeated, but each serum was adsorbed with a strain of \textit{S. aureus} to remove or leave behind antibodies to one of these antigens. When the antiserum to CP8 was adsorbed with the CP8 PNAG+ \textit{S. aureus} strain MN8Δcap8, to leave behind the antibodies to CP8, then added to antiserum to PNAG, there was still an interference with the protective activity of the antiserum to PNAG (Figure 4A, red P values and lines). When the antibodies to CP8 antigen were adsorbed from this antiserum using the CP8–PNAG− \textit{S. aureus} MN8Δica strain, the antiserum to PNAG no longer interfered with the protective activity of the antibody to CP8 (Figure 4C, red and green).

Mechanism of interference between antibodies to \textit{S. aureus} PNAG and CP antigens. We determined whether mixing together rabbit antibody raised to PNAG and CP antigens interfered with the deposition of the key complement opsonin, C3, onto the surface of \textit{S. aureus}. Both antibody to PNAG and antibody to CP8 deposited C3 onto the bacterial surface in a dose-dependent manner, and this was inhibited when the sera were mixed together (Figure 5A). We further evaluated whether the concurrent presence of antibodies to both PNAG and CP8 inhibited antibody binding to the bacterial surface using \textit{S. aureus} cells treated with trypsin to remove protein A. When used alone, each antibody bound well to the bacterial surface, but when combined, the antibodies in the two antisera no longer bound to the bacterial surface (Figure 5, B–G).

Electron microscopy. We used electron microscopy to visualize the interactions of mouse mAbs to PNAG and CP8. When viewed as individual preparations, mostly monomeric IgG molecules were observed with only rare, electron-dense aggregates (Figure 6, A–C). When these two mAbs were mixed together, large amounts of elec-
To confirm that the antibody interactions occurred through their antigen-binding variable regions, we added the PNAG antigen into the isothermal titration calorimetry cell along with the mAb to PNAG and found that this prevented generation of any heat of interaction when antibody to either CP8 or CP5 was added, indicating that the PNAG antigen blocked the binding of antibody to PNAG to antibody to either of the CP antigens (Supplemental Figure 2, D and E). When we used monovalent Fab fragments generated from the human mAb to PNAG and added them to polyclonal rabbit IgG to CP8 (Figure 7G) or CP5 (Figure 7H), binding was also observed, whereas the Fabs did not bind to NRS (Figure 7I).

Further confirmation that antibody to PNAG and antibodies to S. aureus CP antigens bound to each other was obtained by SPR analysis using, as a ligand, IgG purified from a rabbit antiserum to dPNAG coupled to a CM-5 sensor chip and, as a titrant, rabbit antiserum to either CP8 (Supplemental Figure 3, A and C) or CP5 (Supplemental Figure 3, B and D). These results showed a clear binding of the anti-Cap antibodies to CP8 (compared with NRS, black lines) is lost when this antibody is combined with antibody to PNAG (blue). Adsorption of the anti-Cap antibody to PNAG with S. aureus MN8 Δica does not restore protective efficacy (red), but adsorption with S. aureus MN8 Δcp8 to remove antibody to PNAG relieves interference (green). Each point represents an individual mouse; lines represent medians. P = 0.01 for overall Kruskal-Wallis ANOVA. P values for pairwise comparisons shown in the graphs were determined by the Dunn procedure.

![Image](http://www.jci.org)
body, we tested the effect of adding in increasing concentrations of NaCl during an SPR analysis to measure the effect on the antibody interactions. A clear, dose-dependent inhibition in binding of antibody to either CP5 (Supplemental Figure 3E) or CP8 (Supplemental Figure 3F) to immobilized IgG to PNAG occurred when NaCl was added, with 0.3 M NaCl reducing the binding to background levels. Antibody to the negatively charged alginate antigen of *P. aeruginosa* (20) did not bind to the antibody to PNAG (Supplemental Figure 3G), indicating that both electrostatic charge and an additional specific factor were involved in the binding of antibody to PNAG to antibody to staphylococcal CP.

**OPKA specific to CP or PNAG surface polysaccharides in human sera.** We next evaluated the OPKA against *S. aureus* of antibodies to CP and PNAG in human sera made specific for PNAG or CP by adsorption with either a CP-PNAG− *S. aureus* strain or CP-PNAG+ *S. aureus* strain, respectively. We initially tested 98 sera from 22 French patients with bacteremia (Supplemental Table 2). Figure 8, A–G, shows specific examples of the 7 different patterns of OPKA found among these samples. Supplemental Table 2 summarizes the results of the OPKA in the 98 serum samples from the 22 patients. Table 1, group 1, and Supplemental Figure 4 summarize the OPKA results by patient. Among the 98 individual serum samples, results with 7 sera were inconclusive (6 samples from 1 patient) due to PMN-independent killing. Four (4%) serum samples showed increased OPKA when monospecific anti-CP8 and anti-PNAG were combined, 28 of the 98 serum samples (28%) had no OPKA, 9 serum samples (9%) had OPKA to PNAG only, and 11 serum samples (11%) had CP8-specific OPKA only. Sixteen serum samples (16%) had OPKA to both PNAG and CP8 that was present in both monospecific samples and when these were combined. Overall, only 40% of the samples had detectable OPKA to *S. aureus* surface polysaccharides, and the mean level of bacterial killing was only 49% ± 2% (SEM) in a 1:10 serum dilution.

Notably, there were 23 additional serum samples (23%) from 14 of 22 patients with bacteremia that had OPKA to both CP and PNAG antigens in adsorbed, monospecific samples that was reduced to less than 30% bacterial cells killed when the monospecific samples were recombined (Figure 8, F and G). Animal sera with OPKA of 30% or less do not protect against experimental *S. aureus* infection (15). We could totally relieve the interference in 17 of the 23 individual samples by adding in either purified CP or purified PNAG antigen (Figure 8H), and in the other 6 samples with interference, we relieved interference when PNAG antigen was added (Figure 8I). When the 23 serum samples were adsorbed with the double Δcap8/Δica *S. aureus* MN8 strain to leave antibody to both CP and PNAG in the sample (example in Figure 8H), no OPK greater than 30% was observed. Of the 22 total patients in the group of French patients with *S. aureus* bacteremia, 14 (64%) had at

Figure 5
Effect of mixing antibodies to PNAG and CP8 on binding of complement and antibody to *S. aureus*. (A) Complement deposition onto *S. aureus* PS80 (CP8) in the presence of monospecific antibody to PNAG or CP8 or when these antibodies were mixed together. Antibody to CP8 or to PNAG was held constant at a 1:20 dilution and the heterologous antisera added in at dilutions indicated on the x axis. Bars represent mean OD at 405 nm of quadruplicate samples with SEM less than 10% (not shown). (B–E) Binding to *S. aureus* PS80 of rabbit antibody to CP8 (detected by a FITC-conjugated donkey antibody to rabbit IgG) but not in FL2 channel (C) which detects phycoerythrin-conjugated donkey antibody to goat IgG; and binding of goat antibody to PNAG (not detected in FL1 channel [D] but detected in FL2 channel [E]). (F) Binding of antibody to CP8 (filled purple curve, detected in FL1 channel) is reduced 92% when antibody to PNAG is added (green curve, FL1 channel). (G) Similarly, binding of antibody to PNAG (filled purple curve, FL2 channel) is reduced 78% when antibody to CP8 is added (green curve, FL2 channel).
least one serum sample with interference of OPKA (Supplemental Figure 4 and Table 1, group 1). Four (18%) patients had no OPKA, and only 4 (18%) other patients had OPKA toward either CP or PNAG or both without interference.

We next analyzed 10 serum samples obtained from 1 day to 2 weeks prior to the documented onset of MRSA bacteremia in 10 ICU patients in Boston, Massachusetts, USA, for OPKA specific to CP or PNAG antigens (group 2, Table 1). Analysis of monospecific OPKA showed that 3 (30%) serum samples had no OPKA; 7 had anti-PNAG OPKA; 6 had anti-CP OPKA; 5 of the 7 had OPKA to both CP and PNAG antigens; and only 1 showed interference when the monospecific samples were combined. As with the sera from the French bacteremic patients, the overall OPKA in the ICU patients' sera prior to bacteremic infection was low, with only 36% ± 3% (mean ± SEM) of the bacterial cells killed in a 1:10 dilution of the monospecific samples. Analysis of 16 sera obtained from 1 to 35 days after the first positive blood culture for MRSA from the same 10 patients (Table 1, group 3, 1–2 samples/patient) showed 2 samples from 2 patients still had no OPKA (both samples obtained more than 14 days after onset of bacteremia). Among the 14 other serum samples from the remaining 8 patients, 12 had anti-PNAG OPKA greater than 30% and all 14 had anti-CP OPKA greater than 30% when tested for monospecific OPKA, again with an overall low level of killing (mean killing, 44% ± 2% [SEM]). Recombining an aliquot of an adsorbed serum sample from a patient with anti-PNAG OPKA with an adsorbed serum aliquot from the same patient with anti-CP OPKA showed that the group average OPKA was reduced to 12% ± 3% (SEM) CFU killed ($P < 0.0001$, paired $t$ test compared with OPKA in monospecific antisera). At least one serum sample obtained after the onset of MRSA bacteremia from 8 of the 10 patients had interference of OPKA, compared with 1 of 10 prior to the bacteremia ($P = 0.005$, Fisher’s exact test). In all, 21 (66%) of 32 patients from the 2 groups with $S. aureus$ bacteremia had at least 1 serum sample with evidence of interference between antibodies to CP and PNAG antigens.

Among sera from patients with $S. aureus$ pneumonia ($n = 15$) or skin infections ($n = 15$), and 15 serum samples from 5 hospitalized patients lacking evidence of $S. aureus$ infection, 39 of 45 (86%) had no measurable CP- or PNAG-specific OPKA to $S. aureus$. Similarly, 15 (94%) of 16 samples from healthy individuals also lacked detectable CP- or PNAG-specific OPKA against $S. aureus$ (Table 1, groups 4–7). Of the 6 (10%) serum samples (of 61 total samples from 51 individuals) where we could detect monospecific OPKA to CP or PNAG antigens and greater than 30% killing, 5 of these monospecific preparations had killing to both of these antigens that was reduced when they were combined. Thus, surface polysaccharide–specific OPKA against $S. aureus$ was only detected in 1 of 61 serum samples from humans with $S. aureus$ infections, excluding the patients with bloodstream infections or no documented infection.

**Discussion**

Due to our lack of knowledge as to what constitutes protective human immunity to $S. aureus$, it has been difficult to use a rational approach to develop a vaccine. Given the high recurrence rates of $S. aureus$ infections, particularly in humans colonized with MRSA (4–7), the usual means to identify potential effectors of humoral immunity — analysis of antibodies in sera from those convalescing from infection — may not be informative. Using the surface polysaccharides CP5, CP8, and PNAG as vaccines to induce OPK antibody would be a logical choice based on analogy to successful vaccines for other bacterial pathogens (24, 25).

Because there is no reliable source of human sera with OPKA to $S. aureus$ CP or PNAG antigens, we turned to vaccine-induce, antigen-specific animal antisera to study the potential for a synergistic effect between antibodies to $S. aureus$ CP and PNAG antigens to enhance protective immunity. Individual antisera from mice, rabbits, and goats had potent CP- and/or PNAG-specific OPKA, and the rabbit and goat antibodies had protective efficacy in mouse models of bacteremia and skin infections. Quite unexpectedly, these efficacies were lost when the antibodies to the CP and PNAG antigens were combined or coadministered. Mouse mAbs to CP antigens and a human mAb to PNAG also interfered with each other's OPKA. The interference between antibodies to CP and PNAG antigens could be attributable to a specific binding interaction of these antibodies, with the most likely explanation being an idiotype–anti-idiotype binding. A similar type of antibody reactivity has been found in HIV-infected patients and suggested to contribute to AIDS-related pathogenesis and autoimmunity due to immune complexes (26). The binding of antibody to CP and PNAG antigens appears to be mediated, in part, by electrostatic
The charges between these antibodies, which are raised to oppositely charged antigens, inasmuch as binding could be negated in the presence of 0.3 M NaCl. However, there is an additional specificity to the interaction of antibody to PNAG and staphylococcal CP since antibody raised to the negatively charged alginate antigen of *P. aeruginosa* did not bind to the antibody to PNAG.

Expression of both CP and PNAG antigens on the *S. aureus* surface was not needed for interference, as the concurrent presence of antibodies to both of these antigens was sufficient to inhibit killing of CP-negative *S. aureus* or PNAG-producing *E. coli*. Combining antibodies to PNAG and CP antigens also resulted in decreased antibody binding and complement deposition onto the *S. aureus* surface. Addition of high levels (25–50 μg/ml) of either CP or PNAG antigens or adsorption with high levels of bacterial cells (approximately 10¹⁸ CFU) could relieve interference by inhibiting or adsorbing out the competitor antibody. When lower levels of *S. aureus* cells were used in opsonic assays (2 × 10⁶ CFU) or challenge experiments (1 × 10⁶ to 2 × 10⁶ CFU), this relief did not occur. This is explained by the relative levels of antigens involved in these different settings. In the critical biologic assays — OPK and protection — there is likely insufficient CP or PNAG antigen present to neutralize enough interfering antibody, allowing effective OPK and protection to proceed. Overall, it appears that when antibodies to both CP and PNAG

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**Figure 7**

Isothermal calorimetry analysis of the binding of mAb to CP8 or PNAG with polyclonal rabbit antisera to PNAG, CP8, or CP5. Background heat generated from the addition to MEM of rabbit antibody instead of the addition of mAbs to CP8 or PNAG (example in F) was subtracted. (A) Binding curve obtained when solutions containing 5 μM mouse mAb to CP8 was placed in the calorimetry sample cell (Cell) and 250 μg rabbit IgG from antisera raised to PNAG (anti-PNAG) was injected into the cell (Titrant). (B and C) Binding curves obtained when solutions containing 250 μg rabbit IgG raised to CP8 or CP5 (Titrant) were injected into the cell containing 5 μM mAb to PNAG. (D) No binding was observed when a solution containing 5 μM of mouse mAb to CP8 was placed in the cell and NRS (Titrant) was injected into the cell. (E) No binding was observed when a solution containing 5 μM of human mAb to PNAG was placed in the cell and NRS was injected into the cell. (F) Example of background heat generated by addition of rabbit antisera to CP8 or CP5 or PNAG into MEM. (G–I) Addition of rabbit antisera to CP8, CP5, or NRS as a control (Titrant) to the cell containing 5 μM of Fabs prepared from mAb F598 specific to PNAG.
antigens are present at specific levels, there is little monospecific antibody free to bind to the bacterial surface and mediate bacterial killing, the main correlate of protective immunity.

When looking at interference in OPKA in human sera, we found that OPK antibody to \emph{S. aureus} surface polysaccharides was virtually absent from sera of normal humans or humans hospitalized with \emph{S. aureus} infections other than bacteremia. Even in this latter group of patients, the majority of serum samples from 22 patients with ongoing \emph{S. aureus} bacteremia (98 serum samples) plus 10 patients recovering from \emph{S. aureus} bacteremia (16 samples) had no OPKA greater than 30% (71 of 114 total samples) due to lack of antibody activity (30 samples) or interference between antibodies to CP and PNAG antigens (41 samples). Notably, the highest level of interference was found in sera from patients recovering from \emph{S. aureus} bacteremia, with 11 of 16 (69%) sera from 10 patients having this property. The lower percentage of sera from patients with ongoing bacteremia showing interference may reflect lack of sufficient time after infection for this activity to fully develop. Nine of the 10 patients recovering from \emph{S. aureus} bacteremia had at least one sample with either no OPKA or with interference, indicating that following \emph{S. aureus} bacteremia when patients actually produce OPK antibodies to surface polysaccharides, virtually no patient made a potentially protective OPK antibody response to these antigens. This is quite distinct from the infected human response to CPs of \emph{S. pneumoniae}, \emph{Haemophilus influenzae}, and \emph{Neisseria meningitidis}, which is the basis for development of highly successful conjugate vaccines targeting the surface polysaccharides (24, 27–29). Twenty-one of the 32 (66%) betaremic or convalescing \emph{S. aureus}–infected patients had at least one serum sample lacking OPKA due to interference based on the presence of antibodies to both CP and PNAG antigens. Overall, in both normal and \emph{S. aureus}–infected human sera, the vast majority of samples studied had surface polysaccharide–specific OPKA of 30% or less due to either lack of CP- or PNAG-specific antibody or interference between these two antibodies when both were present.

Notably, there were some human sera that did not exhibit interference, and even a handful showed augmentation of OPKA when antibodies to both CP and PNAG antigens were present. This indicates it may be feasible to use either active or passive immunization to the CP and PNAG antigens to elicit high serum levels of antigen-specific antibody that would not be subject to interference from responses to the heterologous antigen. However, two clinical trials for preventing bacteremia in hemodialysis patients using CP antigens conjugated to a carrier protein as a vaccine both failed to meet their primary end points (30, 31), although in one trial (30) there was efficacy after 40 weeks, as determined by a post hoc analysis, but not at the predetermined 54-week endpoint. The loss of protection over the final 14 weeks was associated with a drop in antibody level to the CP antigens, which may have possibly resulted from development of interfering antibody to PNAG.

Overall, our finding of an interference in opsonic and protective activity between antibodies to PNAG and staphylococcal CP antigens have implications for immunity to \emph{S. aureus}, especially as related to vaccine development and insights into the high rates of recurrence of infection due to this pathogen. Clearly, vaccine components must be chosen carefully to avoid interference, which may be engendered not only by vaccine antigens but also by normal bacterial flora, many of which express PNAG antigens (32). Additionally, the apparent lack of development of effective human immunity to \emph{S. aureus} in many situations might partly be explained by our finding both of a general lack of production of polysaccharide antigen–specific OPK antibody in most human sera and of interference between antibodies to CP and PNAG antigens that were present most commonly in sera of patients convalescing from \emph{S. aureus} bacteremia. Both the lack of effective opsonic antibody responses to \emph{S. aureus} surface polysaccharides and interference between antibodies to PNAG and CP antigens might contribute to the high recurrence rate of infections associated with this pathogen (4, 7, 8, 33).

### Table 1

Summary of the OPKA by patient as determined in sera from humans with the indicated type of \emph{S. aureus} infection

<table>
<thead>
<tr>
<th>Group no. and source of serum sample</th>
<th>No. of subjects</th>
<th>No. of samples</th>
<th>No OPKA</th>
<th>Anti-PNAG OPKA</th>
<th>Anti-CP OPKA</th>
<th>Interference when combined</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Subjects with \emph{S. aureus} bacteremia</td>
<td>22</td>
<td>70</td>
<td>9 (41%)</td>
<td>14 (64%)</td>
<td>17 (77%)</td>
<td>14 (64%)</td>
</tr>
<tr>
<td>2. Subjects before \emph{S. aureus} bacteremia</td>
<td>10</td>
<td>30</td>
<td>3 (30%)</td>
<td>7 (70%)</td>
<td>6 (60%)</td>
<td>1 (10%)</td>
</tr>
<tr>
<td>3. Subjects after \emph{S. aureus} bacteremia</td>
<td>10</td>
<td>30</td>
<td>2 (20%)</td>
<td>8 (80%)</td>
<td>8 (80%)</td>
<td>7 (70%)</td>
</tr>
<tr>
<td>4. Subjects with \emph{S. aureus} pneumonia</td>
<td>15</td>
<td>45</td>
<td>13 (87%)</td>
<td>2 (13%)</td>
<td>2 (13%)</td>
<td>2 (13%)</td>
</tr>
<tr>
<td>5. Subjects with \emph{S. aureus} skin infection</td>
<td>15</td>
<td>45</td>
<td>14 (93%)</td>
<td>1 (6.5%)</td>
<td>1 (6.5%)</td>
<td>1 (6.5%)</td>
</tr>
<tr>
<td>6. Subjects without \emph{S. aureus} infection</td>
<td>5</td>
<td>15</td>
<td>3 (60%)</td>
<td>1 (20%)</td>
<td>1 (20%)</td>
<td>1 (20%)</td>
</tr>
<tr>
<td>7. Healthy subjects</td>
<td>16</td>
<td>48</td>
<td>15 (95%)</td>
<td>1 (6%)</td>
<td>1 (6%)</td>
<td>1 (6%)</td>
</tr>
<tr>
<td><strong>Totals</strong></td>
<td>103</td>
<td>310</td>
<td>59 (57%)</td>
<td>35 (34%)</td>
<td>39 (38%)</td>
<td>28 (27%)</td>
</tr>
</tbody>
</table>

\(n\), no. of subjects; \(s\), no. of samples. *Samples with inconclusive, augmented, and no interference have been omitted from the table; data are given in Results. Total percent does not add up to 100% as some samples had both anti-PNAG OPKA and anti-CP OPKA.
Figure 8
Examples of the patterns of OPKA against *S. aureus* PS80 (CP8) or Newman (CP5) in sera from patients with *S. aureus* bacteremia. (A) Serum without OPKA (activity, <30%). (B) CP8-specific OPKA inhibited by addition of CP8 antigen (25 μg/ml). (C) PNAG-specific OPKA inhibited by addition of PNAG antigen (50 μg/ml). (D) PNAG- and CP8-specific OPKA in monospecific and combined antisera, showing no effect from combining monospecific samples. (E) PNAG- and CP8-specific OPKA with augmentation by combining monospecific samples. (F) PNAG- and CP8-specific OPKA, with loss of OPKA when combined together. (G) PNAG- and CP5-specific OPKA, with loss of OPKA when combined together. (H) PNAG- and CP8-specific OPKA in adsorbed, monospecific samples, with loss of OPKA when recombined together, an effect that is unchanged after adsorption by the *S. aureus* MN8Δcap + Δica strain that leaves both antibodies in the serum. OPKA is restored when specific antigen to either PNAG or CP8 is added to the recombined, previously monospecific, adsorbed samples. (I) PNAG- and CP8-specific OPKA in adsorbed, monospecific samples, with loss of OPKA when combined together and with no OPKA in the serum after adsorption by the *S. aureus* MN8Δcap + Δica strain and with OPKA restored when specific antigen to PNAG but not CP8 is added to the recombined, previously monospecific, adsorbed samples. Bars represent means of quadruplicate counts with SEM less than 10% (not shown). Controls lacking PMNs all showed less than 10% OPKA (not shown).

Methods

**Bacterial strains.** *S. aureus* CP8 strains were: PS80, MN8, Sanger 252, and Reynolds (CP8), the latter produced by replacing the indigenous cap5 locus with the cap8 locus (34). *S. aureus* CP5 strains were: Newman and Reynolds. Genetic manipulations to delete either the cap or ica locus were as described previously (22, 34–36). Non-CP-producing MRSA USA 300 strain LAC (14) was also used. *E. coli* strains H, J, and P have been described (18).

**Human sera.** Clinical information, as well as discarded routine clinical blood specimens, were obtained under an approved human subjects protocol (Hôpital Bichat-Claude Bernard, Paris, France) from patients hospitalized between October 2002 and June 2003. Patients were identified from microbiology laboratory reports as having *S. aureus* infections of the blood, skin, or lung. Clinical information, as well as discarded clinical blood specimens collected prospectively from September 1, 2003, to May 31, 2004, were also obtained for all adult intensive care unit patients at Brigham and Women’s Hospital under a protocol approved by the Partner’s Health Care System Institutional Review Board (Boston, Massachusetts, USA). Patients who developed a MRSA bacteremia, and who had no blood cultures positive for coagulase-negative staphylococci or known previous infections with *S. aureus* were included. The latter two adsorptions removed antibodies to *S. aureus* surface antigens except those to PNAG, or with *S. aureus* Δcap5 (strain Newman) or Δcap8 (strain MN8) when testing OPKA against a CP5 strain or a CP8 strain, respectively. These latter two adsorptions removed antibodies to *S. aureus* surface antigens except those to CP5 or CP8 (35, 36). Additionally, a double mutant of *S. aureus* MN8 deleted for both the ica and cap8 loci (strain MN8 Δica + Δcap8) was used to adsorb some sera, leaving in *S. aureus*-specific antibodies to both PNAG and CP antigens. White blood cells were prepared from fresh human blood collected from healthy adult volunteers under an approved protocol from the Partner’s Health Care System Institutional Review Board. All donors gave written informed consent to have blood taken. Blood was mixed with Mono-Poly Resolving Medium (MP Biomedicals) and centrifuged according to the manufacturer’s instructions, the layer containing the PMNs collected, and the cells pelleted by centrifugation; hypotonic lysis of any remaining erythrocytes was accomplished by resuspension of the cell pellet in 1% NH4Cl for 10 minutes at room temperature. PMNs were then washed and resuspended in MEM with 1% BSA. Using trypan blue staining to differentiate dead from live PMNs, the final cell count was adjusted to 2 × 10⁷ PMNs/ml.

Complement in rabbit serum (low cytotoxicity for use in human leukocyte antigen typing, final concentration 2.5% in the assay) was adsorbed with *S. aureus* strains to remove any reactive antibodies and served as the source of this opsonin.

The OPK assays followed published protocols (17, 20, 23), with one modification: in this study, *S. aureus* strains were grown overnight and in Columbia broth plus 2% NaCl (15, 18, 21). Sera were classified as positive if, after subtraction of the background killing in control sera using NRS or an irrelevant mAb, more than 30% of the bacterial CFU were killed, and classified as negative if 30% or less killing was obtained. This demarcation was based on prior experience with this assay and correlating OPKA with protective efficacy in animals, wherein sera with less than 30% killing do not have protective properties (15, 18).

**Murine bacteremia and skin infection models.** All animal studies were conducted under a protocol approved by the Harvard Medical Area Institutional Animal Care and Use Committee, Boston, Massachusetts, USA. The model of bacteremia was as described previously (15). Skin abscesses were induced by mixing 0.1 ml of a sterile suspension of dextran microbeads (131–220 μm diameter, Cytodex beads, Sigma-Aldrich) with *S. aureus* and inoculated under the skin (23). Twenty-four hours prior to infection, Swiss-Webster female mice, 4–6 weeks old, were injected by the i.p. route with 0.2 ml of immune sera to CP8 or PNAG mixed with either 0.2 ml of antiserum to the heterologous antigen (PNAG or CP8, respectively) or with 0.2 ml NRS. Controls received 0.4 ml NRS. Sera were adsorbed with 10¹⁰ CFU/ml of either WT or mutant strains of *S. aureus* to confirm antigenic specificity of protective antibodies.

**Complement deposition.** *S. aureus* PS80 cells (2 × 10⁸ CFU) were suspended in either antiserum to CP8 or PNAG alone or mixed together, with one serum held at a constant concentration (1:20 dilution in MEM–1% BSA) and the
other serum diluted and a 1:10 dilution of rabbit complement in MEM–1% BSA added for 30 minutes at 37°C with rotation. After 3 washes, the pelleted bacteria were suspended in goat antibody to rabbit C3c and incubated 1 hour at 37°C. Controls received MEM–1% BSA in place of the primary antibody. After 3 washes, the cells were suspended in rabbit antibody to goat IgG conjugated to alkaline phosphatase, and after 1 hour of incubation at 37°C, the bacterial cells were washed 3 times, then suspended in 100 μl alkaline phosphatase substrate and the entire contents transferred to an ELISA plate for reading of the OD at 405 nm.

Antibody binding to S. aureus cells. S. aureus PS80 was suspended in 1 mg trypsin/ml in PBS for 1 hour at 37°C to remove protein A and washed, and approximately 2 × 10^8 CFU/ml was suspended in 100 μl of a 1:10 dilution of either monospecific or a mixture of antibody to CP8 or PNAg. After 30 minutes at room temperature, cells were washed 3 times, then suspended in either donkey antibody to rabbit IgG conjugated to FITC or donkey antibody to goat IgG conjugated to phycocerythrin, both of which were adsorbed with S. aureus PS80 cells. After 30 minutes at room temperature, the S. aureus cells were washed 3 times, suspended in 2% paraformaldehyde overnight at 4°C, washed 3 times, and placed into flow cytometry tubes for FACS analysis as described previously (38).

Electron microscopy. Preparation of mouse mAbs for observation by electron microscopy was as described (39).

Isothermal titration calorimetry. Isothermal titration calorimetry experiments were performed using a VP-ITC microcalorimeter (MicroCal) available at the X-Ray Crystallography Facility, Harvard Medical School. A protein G column was used to prepare solutions containing 250 μM IgG purified from rabbit immune sera raised to either PNAg CP8, CP5 or from NRS. These preparations were dialyzed against MEM or PBS and added as titrants into calorimetry sample cells containing 5 μM of mouse mAb to CP8; human mAb to PNAg; Fab fragments purified from the human mAb to PNAg, prepared using papain conjugated to agarose beads (Sigma-Aldrich) as described (40); or MEM buffer. The heat of the reaction (microcalories per second) per injection of 10-μl volumes of titrant was determined by integration of the peak area using Origin Version 5.0 software (OriginLab) with background dilution heats subtracted. Analysis of the data was as described (41, 42).

SPR. Characterization of the binding between antibodies to CP antigens and antibody to PNAg was analyzed by SPR using a BIAcoreTM 3000 (BIAcore AB) machine. All experiments were performed at 25°C employing 10 mM HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% polysorbate 20, pH 7.4, as running buffer at a flow rate of 20 μl/min. Rabbit IgG was purified using a protein G column. Rabbit polyclonal IgG antibodies to PNAg (50 μg/ml in 10 mM phosphate buffer, pH 5.0) were coupled to CM-5 sensor chips utilizing an amine-coupling kit (BIAcore AB). The binding reactions were carried out by injecting various concentrations of antisera to CP8 or CP5 over the immobilized IgG on the chip surface. Background responses from a reference cell were subtracted from the experimental responses. All of the sensorsgrams were analyzed utilizing BioEvaluation software package version 3.0 (BIAcore AB).

Statistics. Normally distributed, paired data were analyzed by 2-tailed t tests; multigroup comparisons were analyzed by ANOVA and post hoc paired analysis analyzed using the TMCT. Categorical analysis used the Fisher’s exact test. A P value less than 0.05 was considered significant. Figure legends indicate the method used for data presentation.

Acknowledgments

We thank Casie Kelly-Quintos for preparation of the Fab fragments from mAb F598 and Michel Vidal for helpful discussions about SPR. We also thank Christian Garcia for contributions to specimen and plasma collection. This work was supported by NIH grant AI046706, the Fondation pour la Recherche Médicale, and a Collaborative Research Agreement with GlaxoSmithKline Biologicals.

Received for publication February 22, 2010, and accepted in revised form June 2, 2010.

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