

A *Staphylococcus aureus* Capsular Polysaccharide (CP) Vaccine and CP-Specific Antibodies Protect Mice against Bacterial Challenge

ALI I. FATTOM,* JAWAD SARWAR, ALBERTO ORTIZ, AND ROBERT NASO

W. W. Karakawa Microbial Pathogenesis Laboratory, NABI—Rockville, Rockville, Maryland 20852

Received 18 December 1995/Returned for modification 1 February 1996/Accepted 20 February 1996

The efficacy of capsular polysaccharide (CP)-specific antibodies elicited by active immunization with vaccines composed of *Staphylococcus aureus* types 5 and 8 CP linked to *Pseudomonas aeruginosa* exoprotein A or with immune immunoglobulin G (I-IgG) obtained from vaccinated plasma donors was tested in lethal and sublethal bacterial mouse challenge models. A dose of 2×10^5 CFU of *S. aureus* type 5 CP per mouse administered intraperitoneally (i.p.) with 5% hog mucin was found to cause 80 to 100% mortality in BALB/c mice within 2 to 5 days. Mice passively immunized i.p. 24 h earlier or subcutaneously 48 h earlier with 0.5 ml of I-IgG showed significantly higher average survival rates than animals receiving standard IgG or saline ($P < 0.01$) following the bacterial challenge. Animals actively immunized with the monovalent type 5 CP-*P. aeruginosa* exoprotein A conjugate showed a survival rate of 73% compared with 13% in phosphate-buffered saline-immunized animals. The prechallenge geometric mean titer of type 5 CP antibodies in animals that died was significantly ($P < 0.05$) lower than that of animals which survived the challenge (95.7 versus 223.6 $\mu\text{g/ml}$, respectively). The IgG was further evaluated in mice challenged i.p. with a sublethal dose of 5×10^4 CFU per mouse. Serial blood counts were performed on surviving animals at 6, 12, 24, and 48 h. Surviving animals were sacrificed at 72 h, and bacterial counts were performed on their kidneys, livers, and peritoneal lavage fluids. Animals receiving I-IgG had lower bacterial counts in blood samples and lower bacterial densities in kidneys, livers, and peritoneal lavage samples than mice immunized with standard IgG ($P < 0.05$). These data suggest that *S. aureus* type 5 CP antibodies induced by active immunization or administered by passive immunization confer protection against *S. aureus* infections.

Despite an earlier demonstration of the role of opsonins in the phagocytosis of *Staphylococcus aureus* by Wright and Douglas in 1904 (59), uncertainty has prevailed about the role of antibodies in immunity to these organisms (52). The uncertainty has continued even in the face of a growing body of literature supporting immunological approaches for the prevention and treatment of *S. aureus* infections (5, 8, 11–13, 15, 19, 20, 24, 25, 35, 49, 53). This ambiguity was clarified by Arbeit et al. and Karakawa et al. (4, 28), who discovered at least eight different, immunologically distinct capsular polysaccharides (CPs) associated with clinical isolates of *S. aureus*. It was further shown that two *S. aureus* serotypes, types 5 and 8, comprise greater than 80% of all clinical isolates (4), and subsequent studies showed that this was true for isolates from human as well as animal infections (10, 46, 48). It was also demonstrated that these capsules are produced in vivo during *S. aureus* infections (2, 3, 7) and that these polysaccharides can induce type-specific opsonic antibodies. In in vitro opsonophagocytic assays, nonencapsulated strains were observed to be readily opsonized and killed by polymorphonuclear leukocytes (PMNs), while type 5 and type 8 encapsulated strains resisted opsonophagocytosis (29, 30). Monoclonal as well as polyclonal type-specific CP antibodies were shown to enhance the phagocytosis of *S. aureus*. In all respects described above, the serospecific opsonophagocytosis of *S. aureus* appears to be similar to that of other capsulated pathogens such as pneumo-

cocci, meningococci, and *Haemophilus influenzae* type b (50, 51).

Types 5 and 8 CPs have been purified, and their chemical structures have been elucidated (21, 22, 31, 42). They consist of nearly identical monosaccharides and differ only in the glycosidic linkages and the site of O acetylation on the mannose-aminouronic acid moieties. In spite of their striking structural similarity, no serological cross-reactivity was demonstrated between these two CPs.

A bivalent type 5 and type 8 polysaccharide conjugate vaccine which produces type-specific antibodies to both CPs in immunized animals and humans was developed (14–16). Antibodies from both vaccinated humans and animals have been shown to be biologically active; that is, they induce opsonophagocytosis (14).

In this report, we evaluate the protective value in mice of passively administered or actively elicited antibodies to *S. aureus* capsules against lethal *S. aureus* type 5 bacterial challenge. We also evaluate the passive immunization against bacteremia and organ seeding in a sublethal bacterial challenge mouse model.

MATERIALS AND METHODS

Bacteria. *S. aureus* Lowenstein (15), a capsular type 5 strain, was used for the preparation of the type 5 CP vaccine preparations and for assays. The challenge studies were performed with a newly isolated, highly virulent clinical *S. aureus* strain, ST021, a capsular type 5, methicillin-resistant strain which was repeatedly isolated from patients at the University of Maryland Shock Trauma and Intensive Care Units over a 2-year period. ST021 caused a variety of infections, including bacteremias, pneumonias, and wound infections, in the hospitalized patients (13a).

Mice. Female BALB/c mice, 6 to 8 weeks of age, weighing 15 to 22 g (Harlan Sprague Dawley, Indianapolis, Ind.), were housed 5, 10, or 15 animals per cage on the basis of cage size and study requirements. These mice were fed Purina

* Corresponding author. Mailing address: W. W. Karakawa Microbial Pathogenesis Laboratory, NABI—Rockville, 12280 Wilkins Ave., Rockville, MD 20852. Phone: (301) 770-3099, ext. 210. Fax: (301) 770-2014.

Certified Rodent Diet 5002 and provided with filtered tap water. Mice were housed in an environment of $72 \pm 6^\circ\text{F}$ ($22.2 \pm 3.4^\circ\text{C}$) with a 12-h light–12-h dark cycle. All mice were quarantined for 4 to 7 days prior to initiation of the study.

Vaccines. A bivalent *S. aureus* vaccine of type 5 and type 8 CPs conjugated to recombinant exoprotein A from *Pseudomonas aeruginosa* was prepared under good manufacturing procedures and used to immunize plasma donors for the preparation of human hyperimmune IgG (I-IgG). The monovalent type 5 conjugate component of the vaccine was also used to immunize mice in active immunization studies (14, 15).

Serology. *S. aureus* type 5 CP antibodies in mouse and human preparations were measured in an avidin-biotin-based enzyme-linked immunosorbent assay (ELISA) as described previously (39, 58). Reference antiserum, human or mouse, against type 5 CP was prepared as follows. Mice hyperimmunized with the vaccine were bled, and their sera were pooled, quantified for specific IgG in an ELISA (15, 63), and used as a reference. For the assay of human antibodies, a standard for type 5 CP was prepared from plasma donated by a high-responder volunteer immunized with a monovalent type 5 CP conjugate vaccine. IgG was purified by ammonium sulfate precipitation and gel chromatography as described previously (14) and quantified by immunoprecipitation (26). The human IgG preparations and the animal sera were analyzed in an avidin-biotin-based ELISA with the appropriate standard reference.

Opsonophagocytosis assay. The in vitro opsonophagocytosis assay was performed as described previously (29). Human PMNs were prepared from heparinized blood by sedimentation in 3% dextran T-250. The opsonic reaction mixture (1 ml) contained $\sim 10^6$ PMNs in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, $\sim 10^8$ CFU of *S. aureus*, and 0.1 ml of the test serum or IgG preparation. Hyperimmunized rabbit serum was used as a positive control, and 0.1 ml of nonimmune rabbit serum was used as a complement source for the IgG samples. The reaction mixtures were incubated at 37°C , and bacterial samples were transferred at 0, 60, and 120 min into water and subsequently diluted, spread on tryptic soy agar plates, and incubated at 37°C for bacterial count after overnight incubation.

Generation and quantification of immune immunoglobulins. Plasma from donors immunized intramuscularly with a single dose of the bivalent conjugate vaccine containing 25 μg each of type 5 and type 8 CP were collected, pooled, and fractionated by standard operating procedures (Rh Pharmaceutical, Winnipeg, Canada) to produce I-IgG preparations. Plasma from donors immunized with a nonrelated bacterial polysaccharide, *P. aeruginosa* mucopolysaccharide, was fractionated by the same process and used to produce standard IgG (S-IgG) to passively immunize control animals. Total IgG was quantified by absorption at 280 nm, and the type 5-specific IgG concentration was quantified by an ELISA as described previously (14, 39, 58).

Distribution of antibodies. Pharmacokinetic studies were carried out to determine *S. aureus* type 5 CP antibody titers in sera from animals that were passively immunized with IgG preparations via the intraperitoneal (i.p.) or subcutaneous (s.c.) route or animals that were actively immunized s.c. three times (14, 15) with the vaccine.

Mouse lethal challenge model. Data generated in a series of preliminary experiments showed that 5% hog mucin potentiated the lethality of *S. aureus* but did not induce mortality in mice when injected alone. With this concentration of hog mucin, the bacterial challenge dose which caused >80% mortality (>80% lethal dose) within 5 to 6 days was determined to be approximately 2×10^5 CFU. The bacterial challenge for the subsequent experiments was prepared as follows: *S. aureus* type 5 strain ST021 was grown overnight (18 to 24 h) in modified Columbia broth medium (Difco Laboratories, Detroit, Mich.) supplemented with 0.15 M CaCl_2 and 0.05 M MgCl_2 . Bacterial cells were suspended to an optical density of 0.02 at 600 nm, corresponding to 4×10^6 CFU/ml, and then adjusted to 8×10^5 CFU/ml with phosphate-buffered saline (PBS). The challenge dose, 2×10^5 CFU, was prepared by mixing an equal volume of sterile (autoclaved) 10% hog mucin in PBS with an equal volume of the adjusted bacterial suspension immediately before injection. Mice were challenged by administering i.p. 500 μl of the bacterium-mucin mixture. Nonimmunized control mice were challenged with the 500 μl of 5% hog mucin in PBS.

Passive immunization and challenge of animals. Pharmacokinetic studies of human IgG preparations in mice revealed that maximum concentrations of circulating antibodies were achieved at 24 h after passive immunization regardless of the route of administration (s.c. or i.p. [results not shown]). The peak antibody levels persisted for 3 days, and by day 6, titers were 50% of maximum levels.

Two sets of experiments were performed utilizing the i.p. and s.c. routes for immunization. In the first set of experiments, 500- μl volumes containing I-IgG, S-IgG, or PBS were administered i.p. to the mice, which were then challenged with bacteria via the same route 24 h later. In the second set of experiments, in which mice were to be immunized s.c., we observed that the mice developed a lethargy following the administration of higher doses of human IgG and that this lethargy persisted for 24 h. In addition, animals receiving S-IgG showed an earlier and enhanced mortality (within 12 h of challenge) compared with that of the i.p. immunized animals. We therefore modified the s.c. procedure to include a postimmunization 48-h rest period and administration of 500 μl of PBS prior to the administration of the bacterial challenge. The protocol for the s.c. series was as follows. Test IgG preparations were administered s.c. to the mice, and this was followed by i.p. administration of 500 μl of sterile PBS 24 h later. The

bacterial challenge was administered at 48 h postimmunization. The i.p. injection of sterile PBS 24 h after immunization was found to delay mortality to a level similar to that obtained in the i.p. model. For both the i.p. and s.c. versions of the model, two control groups were run simultaneously: one group received PBS instead of the IgG preparation, and the other group was immunized with an equivalent amount of standard human IgG (S-IgG) from *P. aeruginosa* mucopolysaccharide-immunized donors. Mortality was recorded twice per day for the first 48 h and once a day for the next 4 to 7 days.

Active immunization. Mice were immunized as described previously (15, 54). Briefly, mice were immunized s.c. on days 0, 14, and 28 with 2.5 μg of *S. aureus* type 5 CP-*P. aeruginosa* recombinant exoprotein A conjugate vaccine in 0.1 ml of PBS. A serum sample was obtained from each mouse 5 days after the last immunization, and serum samples were analyzed in an ELISA for type 5 CP-specific antibodies. Animals were challenged with the regular challenge dose of 2×10^5 CFU 5 to 6 days following their test bleeds. A control group of mice injected simultaneously with PBS was also bled concurrently and challenged.

Bacteremia and organ abscess model. Since almost all mice receiving S-IgG died within 24 h of the lethal challenge, a bacteremia model was developed with sublethal doses of bacteria. It was found that >85% of nonimmunized animals routinely survived a challenge dose of 5×10^4 CFU of *S. aureus*. In subsequent bacteremia and organ abscess studies, animals received either I-IgG or S-IgG preparation via the s.c. route. The bacterial challenge of 5×10^4 CFU of *S. aureus* was administered 48 h later. Mice were randomly selected and removed from cages at the designated time points after challenge. Selected mice were exsanguinated, and a sample of their blood was cultured for bacterial counts. Animals exsanguinated at 72 h for blood counts were also evaluated for liver, kidney, and peritoneum bacterial counts. Organs were excised, weighed, washed with ethyl alcohol to eliminate surface-attached organisms and then with sterile PBS, homogenized in 1 ml of PBS, and cultured on Columbia salt agar plates. For peritoneal counts, 2 ml of PBS was injected into the peritoneal cavity of each mouse, the abdomen of each mouse was massaged for 2 min, and a sample of the lavage fluid was drawn by a syringe and cultured. The plates were counted 24 h later and expressed as CFU per gram of tissue or CFU per milliliter of lavage solution.

Statistical analysis. Data analysis was performed by use of the Statistical Analysis System. Survival analysis of challenged animals was performed by Wilcoxon analysis. Comparison of the mean surviving values in multiple repeats was performed by analysis of variance and chi-square tests. Antibody titers were compared by Student's *t* test.

RESULTS

IgG preparations and characterization. I-IgG preparations were obtained from the plasma of human donors immunized with *S. aureus* bivalent type 5 and type 8 conjugate vaccines. The I-IgG preparation contained 1,800 μg of type 5 CP IgG antibodies per ml, which was equivalent to 4% of the total IgG in the preparation. In all of the mouse protection experiments, the efficiency of this antibody preparation was compared with that of a control or S-IgG obtained from the plasma of donors immunized with *P. aeruginosa* mucopolysaccharide. S-IgG contained 50 μg of type 5 CP-specific antibodies per ml, which was equivalent to 0.1% of the total IgG in the preparation. The results of the opsonophagocytic tests on the I-IgG and S-IgG preparations used at similar total IgG concentrations are shown in Fig. 1. The type 5 *S. aureus* counts in the opsonophagocytosis assay fell 2.5 logs with I-IgG, whereas the S-IgG did not significantly reduce bacterial counts. Type 5 CP-specific rabbit antiserum generated by vaccination of rabbits with vaccine was employed as a positive control.

Passive immunization (i.p.) with immune and nonimmune IgG. The passive protection obtained with I-IgG was compared with that obtained with S-IgG. Other control mice were given PBS or saline instead of IgG. As indicated in Table 1, the trials were repeated four times with I-IgG and the PBS-saline controls and three times with S-IgG. The i.p. dose of I-IgG contained 130 μg of type 5 CP-specific antibody in 4 mg of total IgG. The mice in the control groups were injected with an S-IgG preparation which contained 5 μg of type 5 CP antibody in 4 mg of total IgG. Serum antibody after passive immunization was measured in pharmacokinetic studies prior to the protection trials. The geometric mean titer of specific antibodies to type 5 CP in the pharmacokinetic study was 31 $\mu\text{g}/\text{ml}$ of serum

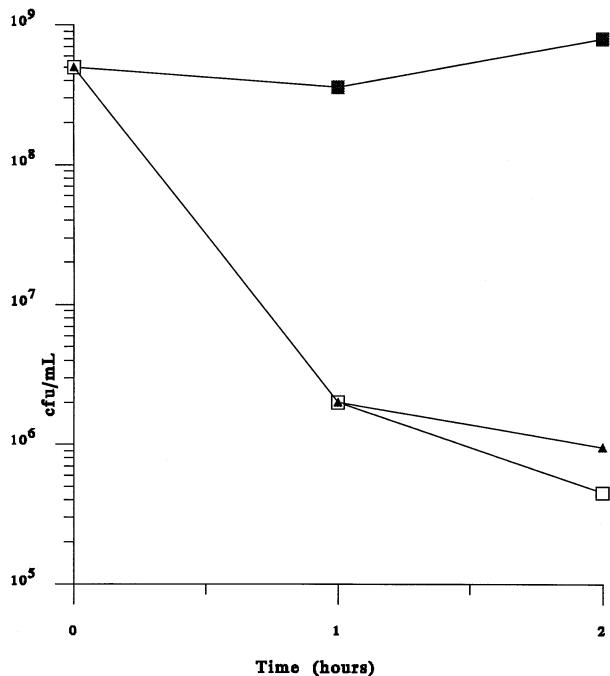


FIG. 1. Opsonophagocytosis of *S. aureus* type 5 (strain Lowenstein) by conjugate-induced type-specific human IgG. *S. aureus* organisms were incubated with 10^6 to 10^7 human PMNs at 37°C in the presence of IgG from conjugate vaccine-immunized plasma donors (\square), IgG from donors immunized with *P. aeruginosa* mucoexopolysaccharide (\blacksquare), or serum from a type 5 CP conjugate-immunized rabbit (\blacktriangle).

in mice receiving the I-IgG, $<1 \mu\text{g/ml}$ in mice receiving S-IgG, and $<0.1 \mu\text{g/ml}$ in mice receiving PBS or saline.

The survival of mice after challenge with 2×10^5 CFU of *S. aureus* is shown in Table 1. Death occurred most commonly within the first 24 h after bacterial challenge. Only a few deaths occurred thereafter. The control mice appeared lethargic during the first day after challenge. The I-IgG-immunized mice were active and appeared healthy. The survival rates ranged from 80 to 100% in the I-IgG-treated mice in the four experiments. This is in contrast to the survival of the mice receiving S-IgG, where the range of survival for the three trials was 20 to 46%. The difference in survival between the I-IgG- and the S-IgG-treated mice was statistically significant ($P < 0.05$). The survival of the mice which had received PBS or saline i.p. was essentially the same as that of the mice that received S-IgG.

A comparison of the protection obtained with i.p. versus s.c. administration of IgG. In the experiments described above, the antibody was delivered i.p., and this administration was fol-

TABLE 1. Protection of mice against i.p. *S. aureus* type 5 challenge by i.p. administration of human immune and nonimmune IgG

Material for passive protection	No. of trials	Total no. of mice	Range of survival rates (%) ^a	No. of surviving animals/total no. at ^b :			
				Day 1	Day 2	Day 4	Day 6 to 7
I-IgG	4	50	80–100	45/50	45/50	45/50	45/50
S-IgG	3	40	20–46	12/40	12/40	12/40	12/40
PBS	4	50	10–40	13/50	11/50	11/50	11/50

^a Range of survival rates observed at the end of each individual experiment.

^b I-IgG versus S-IgG or PBS, $P < 0.05$ and $P < 0.0001$, respectively; PBS versus S-IgG, $P > 0.05$.

TABLE 2. Protection of mice against i.p. *S. aureus* type 5 challenge by s.c. administration of human immune and nonimmune IgG

Immunization ^a	No. of trials	Total no. of mice	Range of survival rates (%) ^b	No. of surviving mice/total no. at ^c :			
				Day 1	Day 2	Day 4	Day 7
I-IgG	3	40	66–100	37/40	37/40	36/40	33/40
S-IgG	3	40	15–33	25/40	18/40	12/40	9/40
PBS	3	40	10–20	15/40	9/40	6/40	6/40

^a Mice received 9.6 mg of total IgG per mouse. I-IgG contained 400 μg of IgG to type 5 CP; S-IgG contained 5 μg of IgG to type 5 CP.

^b Range of survival rates observed at the end of each individual experiment.

^c I-IgG versus S-IgG or PBS, $P < 0.05$ and $P < 0.0001$, respectively; PBS versus S-IgG, $P > 0.05$.

lowed 24 h later by the i.p. *S. aureus* type 5 challenge in mucin. It could be argued that the protective effect was due primarily to the local interaction of the antibody and the bacteria in the peritoneum and not a consequence of systemic action of antibody at distal sites. To evaluate this, additional experiments in which mice were given antibody s.c. and then challenged i.p. 48 h later were carried out. The s.c. dose of I-IgG was 130 μg , and the challenge dose was 2×10^5 CFU of *S. aureus* type 5 CP. The geometric mean serum antibody level in mice passively immunized s.c. that received the I-IgG in the pharmacokinetic study was 20 $\mu\text{g/ml}$. Although 5 of 15 mice immunized s.c. survived for 48 h, only 1 of 15 control mice survived. These results suggested partial protection, and a second series of experiments was undertaken to evaluate higher doses of I-IgG.

In the second series of experiments, the protective effect of a s.c. injection of 400 μg of specific I-IgG was determined. Control animals were given a level of total IgG (S-IgG) equivalent to that of the I-IgG-dosed group; other control groups of mice were given either PBS or saline. The geometric mean level of specific serum I-IgG antibody at the time of challenge was established in the pharmacokinetic studies to be 135 $\mu\text{g/ml}$ in the I-IgG-immunized mice and $<1 \mu\text{g/ml}$ in the S-IgG-immunized mice. A comparison of the three groups of mice, each consisting of three trials, is shown in Table 2. A total of 40 mice were used in each group. A total of 37 of 40 mice receiving I-IgG s.c. survived for 7 days, with a range of 66 to 100% survival among the three trials. Nine of 40 mice receiving the S-IgG survived 7 days, with a range of 15 to 33% survival for the three trials. Only 6 of the 40 control mice which received PBS or saline survived, with a range in survival of 10 to 20% for the three trials. The I-IgG-immunized mice appeared active and healthy throughout the study. Most deaths in the other two groups of mice occurred in the first 2 days postchallenge, and during that time, the animals, even those that ultimately survived for 7 days, appeared stressed as evidenced by lethargy and ruffled coats. As noted, the differences in survival between the I-IgG-immunized group and the two control groups were statistically significant ($P < 0.05$).

Mouse protection obtained with active immunization with the monovalent type 5 *S. aureus* conjugate vaccine. Mice were given three immunizations with type 5 CP monovalent conjugate vaccine as indicated in Materials and Methods and bled 4 to 5 days after the last injection. The mice were challenged i.p. with 2×10^5 CFU of *S. aureus* type 5 and observed for 7 days. As shown in Table 3, 33 of 45 immunized mice survived 7 days, whereas only 4 of 30 of the mice that were sham immunized with PBS survived. The data shown in Table 3 represent three separate trials with survival rates that ranged from 73 to 80%. The difference in survival between mice that were immunized

TABLE 3. Protection of mice against lethal *S. aureus* type 5 challenge by active immunization with *S. aureus* type 5 conjugate vaccine^a

Vaccine	Challenge conditions		No. of surviving mice after challenge ^b					
	Geometric mean CP 5 IgG ($\mu\text{g/ml}$) ^b	Bacterial challenge	16 h	Day 1	Day 2	Day 3	Day 6	Day 10
<i>S. aureus</i> type 5 conjugate	172.1 (40–528) ^c	2×10^5 CFU	42/45	41/45	37/45	37/45	36/45	33/45
PBS	<1		23/30	18/30	6/30	4/30	4/30	4/30
None	<1	5% Mucin	15/15	15/15	14/15	14/15	14/15	14/15

^a Data represent three separate animal trials.

^b *S. aureus* type 5 conjugate versus PBS, $P < 0.01$.

^c Values in parentheses indicate ranges of values.

with vaccine and those that were sham immunized was statistically significant ($P < 0.05$).

Serum levels of type 5 CP-specific antibody were measured prior to challenge to determine the relationship between protection and antibody levels achieved in challenged animals. As expected, there was a variation in antibody concentration from animal to animal and from one trial to the next. Nevertheless, a comparison of the serum antibody concentrations between immunized mice which survived and those which ultimately died was revealing. The peak geometric mean antibody concentration for all immunized mice was 172.1 $\mu\text{g/ml}$ of serum (range, 40 to 528 $\mu\text{g/ml}$). Concentrations were 223.6 $\mu\text{g/ml}$ (range, 53 to 528 $\mu\text{g/ml}$) for the 33 surviving mice and 95.7 $\mu\text{g/ml}$ (range, 40 to 173.5 $\mu\text{g/ml}$) for the mice that eventually died; the difference in concentrations was statistically significant ($P < 0.05$). (These data are from two animal trials, with 30 and 15 mice per group in each trial.)

In all of the active vaccination studies reported here, hog mucin was used with the bacterial i.p. challenge. Control mice were given a similar quantity of hog mucin alone to determine its effect on survival; only 1 of 15 mice treated in this way died by day 7 postchallenge.

Protection of mice against bacteremia from sublethal bacterial challenge following immunization with I-IgG. In the experiments, animals were given s.c. equal amounts (9.6 mg of total IgG per mouse) of either I-IgG or S-IgG 48 h prior to the bacterial challenge. The data presented in Table 4 show that in the first 12 h following the bacterial challenge, >60% of the animals in both I-IgG and the S-IgG groups were bacteremic ($>10^2$ CFU/ml of blood). However, animals in the I-IgG group had lower bacterial counts per milliliter than those of the S-IgG group. While the number of bacteremic animals in the I-IgG group dropped from 60% (12 of 20 mice) to 20% (4 of 20 mice) at 24 h following the bacterial challenge, the counts increased slightly from 75% (15 of 20 mice) to 85% (17 of 20 mice) in the S-IgG group. Consequently, the geometric mean bacterial counts decreased more rapidly in animals receiving I-IgG than those in animals receiving S-IgG. At 48 h post-

challenge, an increase in the number of animals clearing bacteremia was observed in both the I-IgG and S-IgG groups. However, there continued to be a greater reduction in bacteremia in the I-IgG- than in the S-IgG-treated animals. As shown in Table 5, passive immunization with I-IgG also reduced the number of mice with *S. aureus* counts in their kidneys, livers, and peritoneal lavage fluids. *S. aureus* was found in only 1 of 20 lavage fluids from the I-IgG-treated group, compared with 7 of 18 in the S-IgG-treated group. The numbers of animals with detectable liver or kidney bacterial counts were significantly lower for animals receiving I-IgG, i.e., 5 of 20 and 3 of 18 (for liver and kidney, respectively), than for those receiving S-IgG, i.e., 14 of 20 and 17 of 18, respectively.

DISCUSSION

Previous studies have shown that clinical isolates of *S. aureus* can be classified into at least eight serologic types of chemically distinct CPs (4, 28, 30). Seroepidemiology studies revealed that 80% of the clinical isolates, including those from nosocomial infections, were either type 5 or type 8 (2, 4, 7, 27). Opsonophagocytic tests revealed that phagocytosis and bacterial killing are dependent on the presence of capsular type-specific antibodies, whereas heterologous antibodies did not promote phagocytosis (29, 30). The advent of multiple drug resistance among severe *S. aureus* infections prompted efforts to develop a conjugate bivalent vaccine covering types 5 and 8 CPs (15, 16). The vaccine has been shown to be safe and highly immunogenic in both mice and rabbits. More recently, the vaccine was shown to be safe and to elicit high immune responses in humans (14). The successful mouse protection studies reported here lend additional support for continuing vaccine trials in humans to determine whether the vaccine induces protection against clinical *S. aureus* infections.

The mouse models employed here demonstrate that i.p. and s.c. passive immunization of mice with human type 5-specific IgG antibodies protects mice against i.p. challenge with encapsulated type 5 *S. aureus*. Furthermore, active immunization

TABLE 4. Effect of s.c. passive immunization on the blood counts of mice challenged with 5×10^4 CFU of *S. aureus* type 5 strain ST021^a

Time postchallenge (h)	I-IgG		S-IgG	
	CFU/ml ^b (range)	No. of positive mice/total no.	CFU/ml ^b (range)	No. of positive mice/total no.
6	1.8×10^2 B (4×10^2 to 2.1×10^6)	13/20	1.6×10^3 C (2.5×10^3 to 2.2×10^5)	16/20
12	1.2×10^2 B (1.2×10^2 to 2.3×10^4)	12/20	5.8×10^2 B (1×10^2 to 1×10^6)	15/20
24	0.5×10^1 A (5.4×10^2 to 1×10^5)	4/20	1.4×10^3 C (2.4×10^3 to 6.3×10^5)	17/20
48	0.13×10^1 A (4×10^2)	1/20	1.7×10^1 A (2×10^2 to 2.3×10^5)	7/17

^a Mice were randomly selected at each datum point and exsanguinated, and the blood sample was cultured.

^b Results are expressed as geometric mean values for all animals, with ranges given for positive animals ($\geq 10^2$ CFU/ml). Values followed by the same letter are not significantly different at $P < 0.05$. A versus B, A versus C, and B versus C, $P < 0.05$.

TABLE 5. Effect of s.c. immunization on bacterial counts from livers and kidneys and from peritoneal lavage fluids of mice challenged with 5×10^4 CFU of *S. aureus* ST021

Organ or site	I-IgG		S-IgG ^a	
	CFU (range) ^b	No. of positive mice/total no.	CFU (range) ^b	No. of positive mice/total no.
Lavage fluid ^c	7×10^1 (1.4×10^3)	1/20	0.28×10^2 (1.2×10^2 to 2.1×10^5)	7/18
Liver ^d	0.37×10^1 (1×10^2 to 8.2×10^2)	5/20	4.03×10^2 (1.1×10^2 to 1.5×10^4)	14/18
Kidney ^d	0.28×10^1 (6.5×10^2 to 3.2×10^3)	3/20	7.20×10^2 (1.3×10^2 to 1×10^6)	17/18

^a All bacterial counts for S-IgG were significantly different from those for I-IgG at $P < 0.05$.

^b Values are per milliliter for lavage fluid and per gram for liver and kidney. Results are expressed as geometric mean bacterial counts for all animals, with ranges given for positive animals ($\geq 10^2$ CFU/ml).

^c Two milliliters of PBS was injected into the peritoneal cavity of mice, and a sample of the lavage fluid was drawn and cultured for bacterial counts.

^d Kidneys and livers were excised, weighed, washed with 70% ethanol, homogenized in 1 ml of PBS, and cultured for bacterial counts.

with a conjugate vaccine induces protection to subsequent i.p. challenge. As expected, actively immunized mice that survived the bacterial challenge had higher geometric mean antibody levels than those that did not, i.e., 223.6 versus 95.6 $\mu\text{g/ml}$, respectively. In all of these protection studies, there was statistically significant protection afforded by the active immunization with vaccine and the passive immunization with vaccine-induced antibodies ($P < 0.05$).

Blood samples from passively immunized, challenged animals showed that $>90\%$ of the animals develop bacteremia ($>10^2$ CFU/ml) within 6 to 12 h following i.p. bacterial challenge, regardless of the IgG preparation they received. Since most of the control animals receiving S-IgG were dead within 24 h following the bacterial challenge, it was difficult in the lethality model to comparatively evaluate bacteremia. We therefore developed a bacteremia model in which mice were challenged with a sublethal *S. aureus* dose of 5×10^4 CFU per mouse. Individual animals were exsanguinated at each datum point and tested. Compared with the S-IgG-treated animals, significantly fewer animals receiving I-IgG developed bacteremia, and the geometric mean bacterial counts were significantly lower in the I-IgG-treated animals. Furthermore, the I-IgG-treated animals were protected against kidney and liver seeding, as evident by visual examination of abscesses and quantitative bacterial counts. These data suggest that the protection conferred by the type 5 CP-specific antibodies was through clearance of the challenge from the bloodstream and not solely by trapping or localization of the bacterial challenge in the peritoneum. The results are consistent with the interpretation that antibody-mediated clearance of the bacteria in both the peritoneum and the bloodstream reduces blood bacterial counts to sublethal levels. This reduction in bacteremia presumably allows immunologic mechanisms such as opsonophagocytosis to overcome the bacterial challenge, thereby conferring protection from lethality and preventing seeding of organs with bacteria and the formation of organ abscesses.

In these studies, the serum antibody concentrations required for protection when animals were immunized via the s.c. route were observed to be higher than that required in animals immunized i.p. An i.p. dose of 130 μg of type 5 CP IgG antibodies yielded a type 5-specific geometric mean serum antibody titer of 31 $\mu\text{g/ml}$. This level of antibody was evidently sufficient to protect mice challenged i.p. To obtain similar levels of protection upon s.c. passive immunization, the antibody dose had to be increased to about 400 μg , which resulted in a geometric mean serum antibody concentration of 135 $\mu\text{g/ml}$. Lower doses of I-IgG resulted in delayed mortality among immunized animals compared with that of animals receiving equal amounts of S-IgG, but the reduced doses did not result in higher survival rates. We postulate that mice immu-

nized via the i.p. route maintain some of that antibody in the peritoneal cavity. Although antibody in the peritoneal cavity appears not be sufficient to affect the initial bacteremia observed after challenge, it appears to eventually slow the seeding of the blood with more bacteria, perhaps by confining bacteria to the peritoneal cavity or by promoting efficient opsonophagocytosis in the peritoneal cavities of immunized animals. This effect, combined with the higher clearance rate of the bacteria from the blood in the presence of specific antibodies, may result in containment of bacteremia at sublethal levels and clearance thereafter.

Historically, the vast majority of work done on the role of CPs in the virulence of *S. aureus* was performed with strains that were isolated only rarely from clinically significant *S. aureus* infections (e.g., strain Smith and strain M) (32, 37, 41, 43, 45). In these cases, capsules were found to confer resistance on these microorganisms to opsonophagocytosis (40), thus rendering them virulent in animals. Isolates that lost their ability to produce capsules were found to lose their ability to infect animals as well (36). Immunization of animals with whole, killed-cell bacteria or with purified CP elicited antibodies that facilitated in vitro opsonophagocytosis and, as expected, protected animals against lethal bacterial challenge (17, 18, 40). Similar protection was achieved in animals passively immunized with immune sera prior to the bacterial challenge (17, 18, 33, 34, 40, 61, 62). These data supported the conclusion that CPs are virulence factors and protective bacterial antigens.

For various reasons, there has been reluctance to accept the application of the conclusions of these extensive studies on the clinically significant *S. aureus* types 5 and 8 capsules. In particular, several investigators reported results of opsonophagocytosis which were at variance with the work reported by Karakawa and collaborators (29). A difficulty also arose in the interpretation of the results from various animal models of *S. aureus* infection. For example, Xu et al. (60) reported that strains Reynolds (type 5) and Becker (type 8) were opsonized and phagocytosed by human PMNs in the presence of heat-inactivated sera from rabbits immunized with the appropriate killed whole-cell vaccines or with sera from rabbits immunized with a nonencapsulated strain of *S. aureus*, suggesting that CPs have no advantage over other surface components in the generation of opsonic antibodies. Further complicating the story, a previous study by Albus et al. (1) showed that two capsule-deficient type 5 mutants were as virulent for mice as the encapsulated parent strains and caused equal mortality rates in challenged mice. In these experiments, the mice were challenged with logarithmic-growth bacteria at 10^8 to 10^9 CFU per mouse. Using these mutants, Baddour et al. (6) reported that type 5 and type 8 capsulated strains as well as their nonencapsulated mutants were all virulent in an experimental endocar-

ditis rat model. They concluded that expression of CPs by types 5 and 8 *S. aureus* attenuated the bacterial virulence in this animal model.

Some of these seemingly conflicting results may be ascribed to inconsistencies in the production of CPs by *S. aureus* when grown in culture under suboptimal conditions (e.g., high phosphate). Failure to optimize capsule production may indeed make encapsulated challenge bacteria appear to be equivalent to nonencapsulated mutants, since suboptimal production of capsule makes *S. aureus* susceptible to complement-mediated killing and such bacteria are likely to be equally affected in in vitro opsonophagocytosis assays and, as described below, in vivo virulence models as are unencapsulated mutants.

The protective efficacy of the vaccine in our studies was related to the levels of the antibodies achieved by passive or active immunization. The geometric mean CP-specific antibody titer of the animals that died was significantly lower than that of the surviving animals. These protection data are in contrast with data generated with active immunization in other animal models. Greenberg et al. (23) evaluated the effect of immunization with killed whole-cell vaccine on the occurrence of endocarditis in a rabbit endocarditis model. They showed that a high titer of agglutinating antibodies in immunized animals did not result in protection from endocarditis. In a catheter-induced endocarditis rat model, Nemeth and Lee (44) reported the failure of type 5 CP-specific antibody to protect rats from developing infected heart valve vegetation following intravenous challenge with *S. aureus* type 5. Taken together, these data implied that, contrary to the virulence-promoting role of CPs in the case of other bacterial pathogens, including *S. aureus* types 1 and 2 (17, 33, 34, 36, 40), the newly described type 5 and type 8 capsules did not play a significant role in pathogenesis or immunity.

We assume that our positive results reported here on the type-specific protection of mice were due to the following. (i) The bacterial challenge was made from a fresh clinical isolate that had been grown for a sufficient time (i.e., to late log or early stationary phase) and under appropriate culture conditions (i.e., low phosphate) to achieve full encapsulation (thus utilizing the accumulated experience regarding the physiology and ecology of *S. aureus* capsulation [9, 38, 47, 55–57]). (ii) The quantitative determination of the amount of specific IgG type 5 CP antibodies employed in passive protection enabled us to measure and adjust the levels of antibodies achieved in immunized animals. (iii) The use of a highly immunogenic, purified, conjugate vaccine induced antibodies in active immunization which achieved protective levels that often are not obtained with CP alone or with killed whole-cell vaccines. (iv) Our mortality and bacteremia animal models used an i.p. route of challenge rather than the intravenous challenge used in most previous studies. We presume that the i.p. challenge enabled a more gradual and perhaps more clinically relevant generation of bacteremia and organ seeding than that obtained with bolus intravenous challenge. Finally, (v) our models employed the use of hog mucin, which effectively reduced the effective dose of challenge bacteria required to achieve lethality, or, in the sublethality model, bacteremia and organ seeding, while not totally overwhelming the ability of the immune system and immune mechanisms to mitigate the infection.

It is expected that it will be possible to demonstrate protective immunity for the several different animal models of *S. aureus* infections noted above if attention is given to the technical aspects of antibody levels, challenge dose, and route of administration. Indeed, data generated in a rabbit experimental endocarditis model, using conjugate vaccine-induced rabbit IgG and challenging with the *S. aureus* ST021 used in our

studies, showed protection against late bacteremia as well as lower vegetation bacterial counts in immune IgG-receiving rabbits compared with those receiving nonimmune rabbit IgG (6a). Furthermore, preliminary results of studies, carried out in collaboration with J. Lee at Brigham and Women's Hospital, Boston, Mass., have shown that CP conjugate vaccine-induced antibodies produced in rabbits protected rats against experimental endocarditis when the *S. aureus* Reynolds bacterial challenge was administered i.p. instead of intravenously. In this modified model, passively immunized animals also had significantly lower bacterial counts in their blood, valve vegetations, and kidneys than S-IgG-receiving animals (35a).

In summary, we have demonstrated that active immunization with an *S. aureus* type 5 and type 8 CP conjugate vaccine and passive immunization with antibodies elicited by the vaccine are protective in both lethality and sublethality animal models. These results confirm the role of these polysaccharides as virulence factors and protective antigens and provide an impetus for further development of the vaccine and vaccine-induced antibodies for the prevention and treatment of *S. aureus* infections in humans. The efficacy of this vaccine and of antibodies derived from vaccinated plasma donors in the appropriate human trials will determine their usefulness and the validity and applicability of protective immunity in *S. aureus* pathogenesis and infection.

ACKNOWLEDGMENTS

We thank Mitch Katz for plasma collection, Deborah Sinclair for assistance in animal handling throughout the study, and Abbas Hawari for statistical analysis of the data. We are grateful to Richard Krause for critical review of the manuscript.

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