Laboratory and Clinical Evaluation of Conjugate Vaccines Composed of *Staphylococcus aureus* Type 5 and Type 8 Capsular Polysaccharides Bound to *Pseudomonas aeruginosa* Recombinant Exoprotein A

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The synthesis, standardization, and immunogenicity in young outbred mice and clinical evaluation in adult volunteers of investigational vaccines designed to induce serum antibodies to the type 5 and type 8 capsular polysaccharides (CPs) of *Staphylococcus aureus* are described. Conjugates composed of the type 5 CP and a sonicated preparation of a high-molecular-weight type 8 CP bound to a nontoxic recombinant protein derived from *Pseudomonas aeruginosa* exotoxin A (rEPA) were synthesized. The conjugates were nontoxic and elicited serum CP antibodies after two subcutaneous injections into young outbred mice; a third injection elicited a booster response. The lower-molecular-weight type 8 CP was not immunogenic in the mice, and the high-molecular-weight type 8 CP elicited low levels of antibodies without a booster effect. In the volunteers, neither the conjugates nor the type 8 CP alone caused significant local reactions or fever. The conjugates elicited type-specific antibodies of both the immunoglobulin M (IgM) and IgG classes after the first injection; a second injection 6 weeks later did not stimulate a booster effect. The high-molecular-weight type 8 CP alone, injected once only, elicited levels of IgG and IgM type-specific antibodies similar to those of the conjugate. The vaccine-induced CP antibodies were mostly of the IgG1 and IgG2 subclasses and had opsonophagocytic activity. The conjugates elicited IgG antibodies to the native exotoxin A with neutralizing activity. In summary, the type 5 and type 8 conjugates were safe and elicited biologically active antibodies to both the CP and rEPA components.

Systemic infections due to *Staphylococcus aureus* are common and serious. Septicemia is ranked 13th as the cause of death in the United States by analysis of death certificates (5). In most series of severe nosocomial infections, *S. aureus* was the most common organism causing bacteremia, accounting for approximately one-fourth of the isolates (32, 33, 35). In addition, many patients suffer from the complications of bacteremia, such as endocarditis, arthritis, and osteomyelitis (18, 44). *S. aureus* may be isolated from the skin and nasopharynx of ~25% of healthy individuals and is considered to be an opportunistic pathogen because it rarely causes systemic infections in otherwise healthy individuals (6, 47). Patients with end-stage renal disease undergoing dialysis or after cardiac surgery or who have sustained severe trauma are particularly susceptible to bacteremia caused by *S. aureus* (1, 30, 39, 57). *S. aureus* is a common cause of systemic infection in infants (45, 54): in some series, this organism was the most common pathogen isolated from infants with systemic infections in developing countries (7). Recently, outbreaks of bacteremia caused by *S. aureus* resistant to methicillin have been reported (17, 18, 34). In one study, a predominance (87%) of methicillin-resistant *S. aureus* bacteria had the type 5 capsular polysaccharide (CP) (18). Systemic infections with this pathogen may occur occasionally in healthy individuals. *S. aureus* was a major cause of postinfectious pneumonia during pandemics of influenza (16, 29).

Epidemiologic and in vitro studies indicate that two CPs of *S. aureus* are both virulence factors and protective antigens. Briefly, surveys of bacteremia in hospitalized patients, conducted in several countries by different investigators, showed that only 2 of the 11 known CPs of *S. aureus*, types 5 and 8, accounted for ~80% of the isolates (1, 2, 19, 21, 24, 26, 47). These two types also constituted about 80% of the *S. aureus* isolated from goats, sheep, and cows with mastitis (38, 49, 50). In an in vitro assay as a correlate of virulence, noncapsulated *S. aureus* organisms are readily opsonized and killed (25, 56). Strains of type 5 and type 8 *S. aureus*, in contrast, resist opsonophagocytosis. The addition of serum type-specific antibodies facilitates specific opsonophagocytosis (14, 25, 56). These findings are similar to those for other capsulated pathogens such as pneumococci, meningococci, and *Haemophilus influenzae* type b (40, 41).

Active immunization with the *S. aureus* CPs would be intended mostly for patients with decreased antibody responsiveness. We assumed that the type 5 and 8 CPs of *S. aureus* would not be effective immunogens because of their small molecular sizes compared with those of the CP vac-
cines of meningococci, pneumococci, and *H. influenzae* type b (40). Because conjugates are more immunogenic than polysaccharides alone in both healthy and immunocompromised adults (40, 42, 43, 46, 48), we developed methods for synthesizing conjugates with the type 5 and 8 CPSs of *S. aureus*, using *Pseudomonas aeruginosa* exotoxin A (ETA) as the carrier protein (7, 13–15). We chose ETA because this protein is unrelated serologically to *S. aureus* and because there is evidence that antibodies to this ETA confer protection against *P. aeruginosa* (36, 37). In young outbred mice, these conjugates were more immunogenic than the CPS alone and elicited type-specific antibodies that facilitated opsonization as well as neutralizing antibodies to the ETA. The conjugation procedure reduced the toxicity of the ETA ~1,000-fold but did not eliminate its activity (14). For this reason, we used the recombinant cross-reacting mutant protein that has glutamic acid 553 and several adjacent residues deleted (3, 6, 23, 31). This recombinant protein, designated rEPA, has no detectable ADP-ribosyl activity and is indistinguishable antigenically from the native ETA (31).

In this study, we report the synthesis, safety, and some immunologic properties of conjugates, composed of *S. aureus* type 5 and 8 CPSs covalently bound to *P. aeruginosa* rEPA, in young outbred mice and in adult volunteers. In addition, we evaluated a high-molecular-weight type 8 CP from a newly discovered strain of *S. aureus* (25a).

**MATERIALS AND METHODS**

**Bacteria.** *S. aureus* type 5 (strain Lowenstein) and type 8 (strain Wright) were described previously (26). A mucoid strain of type 8, 013, was isolated from a patient with cystic fibrosis. The rEPA was prepared from a recombinant strain of *Escherichia coli*, BL21 (DE3), transfected with the expression vector pVC45DF4-T (6, 11, 23). A deletion mutant was prepared by oligonucleotide-directed mutagenesis of the cloned *P. aeruginosa* ETA gene in *E. coli*. This vector was constructed as described previously (23), resulting in a recombinant protein with the Glu-553 missing.

**Reagents.** Pyrogen-free water and pyrogen-free saline (Travenol Laboratories, Deerfield, Ill.) were used throughout these experiments. DNase, RNase, protease, lysostaphin, dithiothreitol (DTT), and 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide were from Sigma Chemical, St. Louis, Mo. Cystamine and cysteamine were from Fluka, Buchs, Switzerland. Bicinchoninic acid and N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) were from Pierce Chemical Co., Rockford, Ill. Hyperimmune rabbit sera, prepared by multiple intravenous injections of Formalin-inactivated *S. aureus* type 5 (strain Lowenstein) or type 8 (strain Wright), have been described (25, 26).

**Polysaccharides.** *S. aureus* type 5 (strain Lowenstein) was used to prepare the CPSs for the conjugates and the enzyme-linked immunosorbent assay (ELISA). Strain 013 was used to prepare the type 8 CP for the vaccines, and strain Wright was used to prepare the type 8 CP for ELISA. The strains were grown in 100 liters of ultrafiltered Columbia broth (Difco) supplemented with 2% NaCl at 37°C, with ~10% O2 saturation, in a 300-liter fermentor for ~18 to 22 h. When maximal agglutination of the cells with the homologous antiserum was observed, phenol-ethanol (1:1, vol/vol) was added to a final concentration of 2% and the temperature was brought to 20°C for 6 h. The culture was then centrifuged. We obtained ~1 kg of cell paste from each cultivation. The CPSs were purified from 500 g of cell paste by enzyme treatment, anion-exchange chromatography, and gel filtration as described previously (14, 19, 33). The yield was ~300 mg of type 5 CP and 150 mg of type 8 CP. The type 8 CP from strain 013 was passed through a column of Sepharose CL-2B (2.5 by 95 cm) in 0.2 M NaCl; all of the type 8 CP eluted in the void volume. This type 8 CP was sonicated for 40 min to reduce the size to a partition coefficient (Kd) of 0.25 on CL-4B Sepharose and used for synthesis of the conjugate lot 51008 (52, 53). Lot 51708 was the native type 8 CP from *S. aureus* 013.

**Proteins.** ETA was from List Biological Laboratories, San Francisco, Calif. *E. coli* BL21 (DE3), transfected by the mutagenized gene (vide supra), was cultivated as described previously (12). To isolate the rEPA from the cells, the culture was centrifuged at 8,000 × g for 15 min (10, 11). The pellet was suspended in 20% sucrose-20 mM Tris buffer (pH 7.6) (half the original volume) at 4°C. After incubation for 20 min, the cells were pelleted at 10,000 × g at 4°C for 15 min and suspended in cold water in one-third of the original volume. After mixing gently for 10 min, the mixture was centrifuged at 4,000 × g for 15 min. The supernatant, containing the rEPA, was adjusted to 2 mM Tris (pH 7.6), mixed with DEAE-Sepharose equilibrated with the Tris buffer overnight at 3 to 8°C, and packed into a column. The column was washed with 6 column volumes of the Tris buffer, and then a linear gradient of 8 column volumes, 0.0 to 0.5 M NaCl in 20 mM Tris (pH 7.6), was applied. Fractions that contained rEPA, as detected by capillary precipitation with ETA antiserum and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), were pooled and applied to a Mono Q column (Pharmacia, Piscataway, N.J.) (13). Gradient elution from this column yielded purified rEPA that was concentrated by vacuum dialysis and sterile filtered. The final product showed a distinct band corresponding to rEPA and an identical line of precipitation with ETA by immunodiffusion with ETA antiserum (results not shown). The rEPA had ~10% lower specific ADP-ribosyl transferase activity than the native ETA (15, 22).

**Immunization of mice.** Female 6- to 8-week-old general-purpose mice from the NIH colony were injected biweekly subcutaneously with 2.5 μg of the CP alone or as a conjugate in 0.1 ml; 10 mice from each group were bled before the second injection and 1 week after the last two injections.

**Analyses.** The nucleic acid contents of the CPSs were measured by their A260 as described previously (55). Sulfhydryl (SH) groups were measured by the method of Ellman, with cysteine as the standard (15). Protein was measured and SDS-PAGE was performed as described previously (15). Gel filtration and calculation of the partition coefficient of the CP and proteins has been described (55). High-performance liquid chromatography (HPLC) of the conjugates has been described (16). The effluent from column chromatography was monitored by refractometry because the *S. aureus* type 5 and 8 polysaccharides cannot be assayed by the colorimetric methods used for other polysaccharides (14, 15).

**Thiolation of the CPs.** The thiolation of the CPs and the derivatization of the rEPA were done by a previously published method (4, 14, 15). Briefly, 100 μg of the CP was dissolved in 10.0 ml of saline and brought to 0.25 M in cystamine. The pH was maintained at 4.75 with 0.1 N HCl, and 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide was added to a final concentration of 0.05 M. The reaction proceeded for 3 h at 37°C. The pH was brought to 7.0 with 0.1 M NaOH, and the reaction mixture was dialyzed against water at 3 to 8°C for 3 days. The dialysate was freeze-dried
TABLE 1. Composition of the type 5 and 8 CPs and the three conjugates with rEPA

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Lot no.</th>
<th>Nucleic acid (%)</th>
<th>Protein (%)</th>
<th>Kd</th>
<th>Cystamine/ saccharide (%)</th>
<th>SPDP/rEPA ratio (mol/mol)</th>
<th>rEPA/polysaccharide in conjugate (wt/wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 5 CP-rEPA</td>
<td>49704</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>0.36a</td>
<td>5.8</td>
<td>6.8</td>
<td>1.00</td>
</tr>
<tr>
<td>Type 5 CP-rEPA</td>
<td>50179</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>0.36a</td>
<td>5.8</td>
<td>6.0</td>
<td>0.97</td>
</tr>
<tr>
<td>Type 8 CP</td>
<td>51008</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>0.25c</td>
<td>5.7</td>
<td>5.5</td>
<td>1.00</td>
</tr>
</tbody>
</table>

a Partition coefficient for polysaccharide vaccines (55).
b Gel filtration through a column of CL-6B Sepharose (1 by 90 cm) in 0.2 M NaCl (55).
c Gel filtration through a column of CL-4B Sepharose (1 by 90 cm) in 0.2 M NaCl (55).
d NA, not applicable.

and is referred to as the CP-cystamine derivative. An aliquot was mixed with 100 mM DTT for 1 h at room temperature and passed through a P-6DG column in saline. The void volume peak was pooled and assayed for its content of SH groups. One hundred milligrams of the CP-cystamine derivative was dissolved in 8.0 ml of saline, brought to 0.1 M DTT, and allowed to stand for 1 h at room temperature. The reaction mixture was dialyzed against saline for 30 min, passed through a P-6DG column (2.5 by 40 cm), and eluted with phosphate-buffered saline (PBS)-0.001 M EDTA. The void volume fractions (60 to 80 ml) were pooled and sterile filtered.

**Derivatization of rEPA with SPDP.** SPDP, 40 mM in absolute ethanol, was added to rEPA in PBS to a final concentration of 10 mg of rEPA per ml and 4 mM SPDP, mixed at room temperature for 1 h, and passed through a P-6DG column (2.5 by 40 cm) in saline. The void volume fractions were treated with 0.04 M DTT at room temperature for 1 h and sterile filtered; the A280 of an aliquot was measured to determine derivatization with SPDP (5).

**Conjugation.** The reduced CP-cystamine derivative was added to a reaction flask and flushed with argon, an equal weight of rEPA-SPDP was added, and the reaction mixture (3.0 mg of each component per ml) was tumbled under argon for 3 days at 3 to 8°C. The reaction mixture was centrifuged at 10,000 x g at 3 to 8°C for 20 min, and the supernatant was passed through a column of S-300 Sephacryl (5 by 95 cm) in 0.2 M NaCl-0.01% thimerosal. A 0.5-ml aliquot was removed from every third fraction, and its A290 and A280 were recorded. The peak that emerged in the void volume was dispensed to 2.8-ml aliquots into 5.0-ml vials by the Pharmacy Branch, Clinical Center, National Institutes of Health. These vials were assayed for sterility, pyrogenicity, and general safety according to the U.S. Code of Federal Regulations. Two lots of type 5 CP-rEPA (lots 49704 and 50179) and one lot of type 8 CP-rEPA (lot 51008) were used.

**Toxicity of rEPA.** The rEPAs and the conjugates were assayed in vivo as described previously (22). General-purpose female mice from the NIH colony, 6 to 8 weeks old, were injected intraperitoneally with 0.5 ml of ETA, rEPA, or the conjugates in the final container, diluted twofold in 0.1% bovine serum albumin (BSA) in PBS. The surviving mice were exsanguinated after 48 h, and the sera were assayed for serum glutamic oxalacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) by Medpath Laboratories, Rockville, Md. The controls were mice injected with 0.1% BSA in PBS.

Toxicity was assayed in vitro with CHO cells grown in RPMI medium supplemented with 5% fetal calf serum for 3 days (14, 22). The cells were harvested and suspended at 10^6/ml, and 200-μl aliquots were added to the wells of a microtiter plate containing serial dilutions of the test samples. The plates were incubated at 37°C in 5% CO_2 for 2 days. The wells were emptied, washed, fixed, and stained with Giemsa stain. The end point was the lowest concentration of the sample that killed >95% of the cells.

**Vaccines.** These vaccines were approved for investigation by the National Institutes of Health (protocol 90 CH 176) and the Food and Drug Administration (protocol BB IND 3679).

**Serology.** Serum antibodies to the S. aureus type 5 and 8 CPs were measured by ELISA (13, 51). Biotin-labelled CPs

<table>
<thead>
<tr>
<th>Injection</th>
<th>Amt injected (μg)</th>
<th>No. of survivors/total no.</th>
<th>Geometric mean level (U/liter) (25th-75th centiles)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETA</td>
<td>0.2</td>
<td>4/10</td>
<td>3,577 (2,388-6,377)</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>9/9</td>
<td>383 (243-623)</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>9/9</td>
<td>313 (192-528)</td>
</tr>
<tr>
<td>rEPA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lot 1</td>
<td>200</td>
<td>9/9</td>
<td>128 (103-154)</td>
</tr>
<tr>
<td>lot 2</td>
<td>400</td>
<td>4/4</td>
<td>100 (98-102)</td>
</tr>
<tr>
<td>lot 3</td>
<td>200</td>
<td>5/5</td>
<td>96 (85-111)</td>
</tr>
<tr>
<td>BSA (0.1%) in PBS</td>
<td>8/8</td>
<td>145 (129-174)</td>
<td>47 (41-56)</td>
</tr>
</tbody>
</table>

Conjugate

| Type 5 CP-rEPA (lot 49704) | 20 | 10/10 | 88 (73-107) |
| Type 5 CP-rEPA (lot 50179) | 25 | 5/5   | 64 (49-80)  |
| Type 8 CP-rEPA (lot 51008) | 25 | 5/5   | 86 (75-97)  |

* Normal values in mice for this laboratory are 250 to 400 U/liter for SGOT and 50 to 175 U/liter for SGPT.
were used to coat the plates as described previously (51). Pooled sera, taken from mice hyperimmunized with type 5 or type 8 conjugates (14), were used as standards and assigned a value of 100 U/ml. For the assay of the human antibodies, standards were prepared from the plasma of volunteers whose postimmunization sera yielded a precipitin reaction with the type 5 or 8 CPs. These plasmas were precipitated with 60% saturated ammonium sulfate, and the immunoglobulin G (IgG) and IgM were purified by anion-exchange chromatography and gel filtration. The concentrations of type-specific antibodies in these immunoglobulin fractions were determined by quantitative precipitation for the IgM and IgG of type 5 and for the IgG of type 8 (20). There was insufficient anti-type 8 IgM fraction for precipitin analysis, and, accordingly, it was assigned a value of 100 U/ml.

A serum with a high titer of IgG antibody to ETA, from a volunteer injected with the type 5 conjugate, lot 50179, was used as a reference and assigned a value of 100 U/ml.

**Opsonophagocytosis.** Sera inactivated at 56°C for 30 min, taken before and 6 weeks after immunization of the volunteers, were assayed for their bactericidal activities with ~10⁷ organisms of the test strains for each type and ~10³ human peripheral blood polymorphonuclear leukocytes (25). The assays were done in duplicate on three different occasions with human peripheral blood polymorphonuclear leukocytes (from nonparticipants in the study), and the values are expressed as the averages of the determinations.

**Neutralization of ETA.** The neutralizing activity of sera against ETA was tested in vitro with CHO cells (22). We found that 10 to 15 ng of ETA killed >90% of cultured 2 × 10⁶ CHO cells per well in 96-well culture plates. Twenty-five microliters of test sera was serially two-fold diluted in the plate, and 25 ng of ETA in 25 µl was added to all wells. Two hundred microliters of medium containing 2 × 10⁴ CHO cells was then added to the wells and mixed, and the plates were incubated at 37°C in 5% CO₂, for 48 h. The medium was discarded, and the plates were washed, fixed with methanol, and stained. The results are expressed as the lowest dilution that killed >95% of the cells as determined visually.

**Clinical protocol.** Healthy adults of either sex, between 18 and 45 years of age, were recruited. Individuals with positive serologic tests for human immunodeficiency virus type 1, hepatitis B, or pregnancy or with higher-than-normal values of SGOT and SGPT were excluded. The volunteers were assigned one of the vaccines on a random basis. On the day of vaccination, volunteers were given a general physical examination. Those with normal temperature and vital signs were injected intramuscularly with 0.5 ml of one of the investigational vaccines, and their injection sites and temperatures were monitored at 6, 24, and 48 h. This process was repeated 6 weeks later for the volunteers assigned to receive a conjugate; only one injection of the type 8 CP was given. SGPT and SGOT were measured by the Clinical Laboratories, Clinical Center, National Institutes of Health, before and 2, 5, 7, and 14 days after injection. Serum antibodies were measured before each injection, 2 and 4 weeks after the first injection, and 2, 6, and 26 weeks after the second injection.

**Statistics.** Data analyses were performed by using the Statistical Analysis System. The logarithms of the antibody concentration were used for all calculations. Antibody concentrations below the sensitivity of the ELISA were assigned one-half of that value. Comparisons of geometric

### Table 3. Serum type-specific antibodies elicited in mice to the *S. aureus* CPs

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Coating antigen</th>
<th>Geometric mean antibody level (ELISA units) a (25th-75th centiles) after the:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1st injection</td>
</tr>
<tr>
<td>Type 5 CP</td>
<td>Type 5</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Type 5 CP-rEPA, lot 49704</td>
<td>Type 5</td>
<td></td>
</tr>
<tr>
<td>Type 5 CP-rEPA, lot 50179</td>
<td>Type 5</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Type 8 CP, lot 51708</td>
<td>Type 8</td>
<td>0.8 (0.5-1.5)</td>
</tr>
<tr>
<td>Type 8 CP-rEPA, lot 51008</td>
<td>Type 8</td>
<td>0.6 (0.4-0.7)</td>
</tr>
<tr>
<td>Type 5 Cs-rEPA, lot 50179</td>
<td>Type 8</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

a Mice were injected with 2.5 µg of CP alone or as a conjugate and exsanguinated before the second injection and 1 week after the last two injections. Antibodies were measured by ELISA with hyperimmune sera, assigned a value of 100 ELISA units, as a reference (14). An injection of saline did not elicit type 5 or 8 antibodies (data not shown).

b e versus d, P = 0.01; f versus e, P = 0.003; h versus g and g versus d, P = 0.0001.

c ND, not done.

### Table 4. Serum antibodies in mice to *P. aeruginosa* ETA

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Geometric mean antibody level (ELISA units) a (25th-75th centiles) after the:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st injection</td>
</tr>
<tr>
<td>rEPA</td>
<td></td>
</tr>
<tr>
<td>Type 5 CP</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Type 5 CP-rEPA, lot 49704</td>
<td>&lt;1 f</td>
</tr>
<tr>
<td>Type 5 CP-rEPA, lot 50179</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Type 8 CP, lot 51708</td>
<td>ND</td>
</tr>
<tr>
<td>Type 8 CP-rEPA, lot 51008</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

a Mice were injected subcutaneously with 2.5 µg of rEPA or CP, alone or as a conjugate. Ten mice from each group were exsanguinated before the second injection and 1 week after the last two injections. ETA antibodies were measured by ELISA and expressed as ELISA units in comparison with a reference serum with an assigned value of 100 ELISA units.

b e versus d, P = 0.01; f versus e, P = 0.003; h versus g and g versus d, P = 0.0001.

c ND, not done.
TABLE 5. Serum type 5 CP antibodies in volunteers injected with S. aureus type 5 CP-rEPAa

<table>
<thead>
<tr>
<th>Lot no.</th>
<th>n</th>
<th>Ig</th>
<th>Geometric mean antibody level (µg/ml) (25th–75th centiles) at weeks postimmunizationc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pre-imm.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IgM</td>
<td>2.5 (1.7–3.5)</td>
</tr>
<tr>
<td>50179</td>
<td>23</td>
<td>IgG</td>
<td>8.5 (6.1–16.3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IgM</td>
<td>3.6 (2.4–5.9)</td>
</tr>
</tbody>
</table>

a Volunteers were injected with 0.5 ml of S. aureus type 5 CP-rEPA containing 25 µg of CP on day 0 and 6 weeks later. Type 5 CP antibodies were measured by ELISA calibrated by a reference serum from a volunteer whose IgG and IgM antibody levels were measured by precipitin analysis.

b *P < 0.05.

c Pre-imm., preimmunization. Weeks postimmunization: 2 post 1 (for example), 2 weeks after first injection. Asterisk indicates second injection was given.

d Geometric mean.

e Geometric mean.

TABLE 6. Serum type 8 CP antibodies in volunteers injected with S. aureus type 8 CP-rEPAa

<table>
<thead>
<tr>
<th>Lot no.</th>
<th>n</th>
<th>Ig</th>
<th>Geometric mean antibody level (µg/ml) (25th–75th centiles) at weeks postimmunizationc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pre-imm.</td>
</tr>
<tr>
<td>51008</td>
<td>22</td>
<td>IgG</td>
<td>7.73 (3.72–12.1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IgM</td>
<td>7.56 (4.07–11.3)</td>
</tr>
<tr>
<td>51708</td>
<td>22</td>
<td>IgG</td>
<td>7.14 (5.08–12.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IgM</td>
<td>4.90 (3.28–10.8)</td>
</tr>
</tbody>
</table>

a Volunteers were injected with 0.5 ml of S. aureus type 8 CP-rEPA or type 8 CP containing 25 µg of CP on day 0 and 6 weeks later. Type 8 CP antibodies were measured by ELISA calibrated by a reference serum from a volunteer whose IgG was measured by precipitin analysis.

b Type 8 CP IgM is expressed in ELISA units; type 8 IgG is expressed in micrograms per milliliter.

c For abbreviations and symbols for weeks postimmunization, see Table 5, footnote c.
TABLE 7. IgG subclasses in volunteers immunized with S. aureus CP-rEPA conjugates or S. aureus type 8 CP alonea

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>n</th>
<th>IgG1</th>
<th></th>
<th>IgG2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pre-imm.</td>
<td>Post 6 wks</td>
<td>Pre-imm.</td>
<td>Post 6 wks</td>
</tr>
<tr>
<td>Type 5 CP-rEPA, lot 50179</td>
<td>15</td>
<td>0.4b (0.1-1.7)</td>
<td>14.9b (20-38)</td>
<td>1.7b (1.2-4.5)</td>
<td>139b (86-268)</td>
</tr>
<tr>
<td>Type 5 CP-rEPA, lot 49704</td>
<td>15</td>
<td>0.7b (0.5-1.5)</td>
<td>39.5b (20-76)</td>
<td>1.7b (1.1-4.2)</td>
<td>72.4b (31-117)</td>
</tr>
<tr>
<td>Type 8 CP-rEPA, lot 51008</td>
<td>10</td>
<td>3.1b (1.7-5.3)</td>
<td>36.5b (22-104)</td>
<td>11.1b (8.1-23)</td>
<td>68.1b (44-137)</td>
</tr>
<tr>
<td>Type 8 CP, lot 51708</td>
<td>10</td>
<td>2.6b (1.0-5.2)</td>
<td>16.6b (6.4-46)</td>
<td>10.5b (7.4-15)</td>
<td>91.6b (40-161)</td>
</tr>
</tbody>
</table>

a Only a few volunteers had rises in IgG subclasses 3 and 4; see the text. Serum from a high responder with the appropriate subclass was used as a reference in the ELISA and assigned a value of 100 ELISA units. c versus h, e versus d, g versus f, and i versus h, P = 0.0001; k versus j, m versus l, o versus n, and q versus p, P < 0.01. For abbreviations for weeks postimmunization, see Table 5, footnote c.

U/liter). The second volunteer had a rise from 42 to 85 U/liter in the SGOT level. Two volunteers in the group receiving lot 50179 had rises in SGPT levels after 2 days (from 49 to 81 and from 19 to 38 U/liter). None of the volunteers in this group had any rise in the level of SGOT. None of the volunteers immunized with the type 8 CP-rEPA (lot 51008) or the type 8 CP (lot 51708) had rises in the enzyme levels. No changes in the levels of enzymes were observed after the second injection of the three conjugates.

**Type 5 CP antibodies (Table 5).** The preimmunization levels of IgM and IgG type 5 CP antibodies were similar in both groups of volunteers. Two weeks after the first injection, both conjugates elicited significant rises in the geometric mean IgG and IgM levels in all of the recipients (P < 0.0001): all responded with a ≥4-fold rise in type 5 IgG antibodies. Six weeks after the first immunization, volunteers immunized with lots 49704 and 50179 had 33- and 37-fold rises of their type 5 CP IgG antibody levels and 15- and 10-fold rises of their IgM CP antibodies, respectively. A second injection of either of the type 5 conjugates did not elicit a rise of CP antibodies. About 6 months later, the levels of type 5 antibodies declined slightly: IgG levels decreased 26% (lot 49704) and 14% (lot 50179), and IgM antibodies decreased about 50% in both groups. Lot 50179 elicited higher levels of IgG type 5 antibodies after the first immunization, and, 26 weeks later, these levels remained about twofold higher than those of the volunteers injected with lot 49704 (274 μg of antibody per ml versus 166 μg of antibody per ml, P < 0.05); the IgM antibody levels were similar for both groups.

**Type 8 CP antibodies (Table 6).** Preimmunization levels of type 8 antibodies in both groups were similar. Immunization with the type 8 CP-rEPA (lot 51008) or with the type 8 CP alone (lot 51708) elicited about an eightfold increase of IgG antibodies and about a fivefold increase of IgM antibodies (both, P = 0.0001). These levels were observed 2 and 6 weeks after the first immunization and did not change after the second immunization or at the 6-month check-up. The type 8 CP conjugate (lot 51008) elicited higher levels of IgM antibodies than lot 51708 at all times, but these differences were not significant.

**IgG subclass composition of CP antibodies (Table 7).** The IgG subclasses of CP antibodies from the preimmunization and 6-week postimmunization sera were assayed. All volunteers responded with significant rises of IgG1 or IgG2 antibodies (P < 0.01); most responded with significant rises in both subclasses. Of those injected with lot 49704, 15 of 15 responded with ≥4-fold rises of both IgG1 and IgG2 type 5 antibodies; only 2 had a ≥4-fold rise of IgG3, and 3 had IgG4 type 5 antibodies. Among the volunteers injected with lot 50179, 12 of 15 had ≥4-fold rises of IgG1 and 15 of 15 had ≥4-fold rises of IgG2. One volunteer in this group responded with a ≥4-fold rise of IgG3, and four responded with ≥4-fold rises of IgG4. Among the 10 volunteers immunized with the type 8 conjugate, 8 had ≥4-fold rises of IgG1 and 9 had ≥4-fold rises of IgG2: one responded with a rise in IgG1 alone, and one responded with ≥4-fold rises of IgG1, IgG2, and IgG3. Of 10 volunteers injected with the type 8 CP alone, 7 responded with rises of IgG1 and 9 responded with rises of IgG2. In this group, 3 responded with rises of IgG1, IgG2, and IgG4.

**Opsonophagocytic activity of vaccine-induced CP antibodies (Fig. 1).** As reported, preimmunization sera from rabbits and the volunteers induced no or a slight (−1/2 log) reduction in the number of S. aureus bacteria of type 5 or 8 after incubation with human peripheral blood mononuclear leukocytes (14, 25). The hyperimmune rabbit serum and the postimmunization sera of the volunteers elicited a type-specific reduction in the number of S. aureus bacteria of from 1.5 to 4 logs. Not shown was the failure of CP-adsorbed sera to elicit this bactericidal effect.

**Anti-ETA antibodies (Table 8).** Most of the volunteers had nondetectable or low levels of ETA antibodies in their preimmunization sera. Immunization with the type 5 CP-rEPA conjugates elicited an increase in the geometric mean ETA antibody levels (P = 0.0001), and the levels in the two groups

**TABLE 8. Serum IgG antibodies to P. aeruginosa ETA in volunteers injected with S. aureus CP-rEPA vaccines**

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Geometric mean antibody level (ELISA units/ml) (25th-75th centiles) at weeks postimmunizationa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-imm.</td>
</tr>
<tr>
<td>Type 5 CP-rEPA, lot 49704</td>
<td>0.7 (0.4-1.7)</td>
</tr>
<tr>
<td>Type 5 CP-rEPA, lot 50179</td>
<td>0.5 (0.3-1.1)</td>
</tr>
<tr>
<td>Type 8 CP-rEPA, lot 51008</td>
<td>0.7 (0.5-1.0)</td>
</tr>
<tr>
<td>Type 8 PS, lot 51708</td>
<td>0.87 (0.05-1.0)</td>
</tr>
</tbody>
</table>

a For abbreviations for weeks postimmunization, see Table 5, footnote c. Asterisk indicates second injection was given.

b ND, not done.
were similar. The second immunization elicited a slight rise (not significant) in the ETA antibody levels, and 43 of 45 volunteers had ≥4-fold rises over their preimmune levels. Six months later, there was a ~50% decrease in the ETA antibodies in both groups.

The type 8 CP-rEPA elicited a lesser rise of ETA antibodies than the type 5 CP-rEPA conjugates (16.6 and 11.3 versus 3.4 ELISA units/ml; \( P < 0.05 \)). The second injection of type 8 CP-rEPA, lot 51008, elicited a significant rise of ETA antibodies to a geometric mean of 8.3 ELISA units/ml, but this was still less than the levels of the groups immunized with the type 5 CP conjugates. As expected, volunteers immunized with type 8 CP alone did not have a rise in their antibodies to ETA.

**Neutralizing antibodies to ETA (Table 9).** A serum, with a neutralizing titer (antitoxin) of 1/256 from a volunteer immunized with type 5 CP-rEPA, lot 50179, was assigned a value of 100 neutralization units and used as a standard. The geometric mean antibody levels of the volunteers injected with the type 5 CP-rEPA conjugates were similar and are combined. There were low levels of antitoxin after injection of either the type 5 or type 8 conjugates. The second injection of the type 8 conjugate elicited a statistically significant rise of antitoxin, and there was a correlation between the levels of ETA antibodies measured by ELISA and neutralization assay \( r = 0.7, P = 0.05 \).

**DISCUSSION**

The three conjugates and the high-molecular-weight type 8 CP alone did not elicit either local or systemic adverse reactions. Reinjection of the conjugates elicited some minor

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**TABLE 9. Neutralizing antibodies (antitoxin) to *S. aureus* ETA elicited in adult volunteers by *S. aureus* CP-rEPA conjugates**

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>( n )</th>
<th>Geometric mean antitoxin level (neutralization units/ml) (no. of volunteers with &gt;2 neutralization units/ml/total no.)( ^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pre-imm.</td>
</tr>
<tr>
<td>Type 5 CP-rEPA( ^c )</td>
<td>48</td>
<td>0.51 (1/48)</td>
</tr>
<tr>
<td>Type 8 CP-rEPA</td>
<td>21</td>
<td>0.67 (4/21)</td>
</tr>
</tbody>
</table>

\( ^a \) Representative sera from volunteers were assayed for their neutralizing activity (antitoxin) by the CHO cell assay (22).

\( ^b \) For abbreviations for weeks postimmunization, see Table 5, footnote c.

\( ^c \) Data from groups injected with lots 50179 and 49704 were pooled.
local reactions, which were likely due to vaccine-induced antibodies to the rEPA. We conclude that the assays of these vaccines were predictive of their safety.

Unlike the conjugates prepared with ETA as the carrier (14), the first injection of the S. aureus type 5 or 8 rEPA conjugates did not elicit CP antibodies in mice. Since the compositions and molecular sizes of these conjugates were similar, we suggest that the residual activity of the ETA exerted an adjuvant effect upon CP antibody synthesis over that of the rEPA. The three conjugates elicited CP antibodies in the mice after the second injection and a booster response after the third injection; the high-molecular-weight type 8 CP (lot 51708) elicited low levels of antibodies after the first injection, which did not change after the second and third injections. The lower-molecular-weight type 5 and 8 CPs in our previous work (14, 15) and the type 5 CP in this study did not elicit antibodies in the mice. We conclude that covalent binding to the rEPA both increased the immunogenicity and conferred T-cell-dependent properties upon the type 5 and 8 CPs. In addition, type 5-rEPA lot 50179 elicited higher levels of CP antibodies in the mice than lot 49704, which correlated with their molecular sizes by HPLC and was predictive of their immunogenicity in the volunteers. The only difference in the synthesis of these conjugates was the higher concentration of the type 5 CP-SH derivative used for the conjugation reaction of lot 49704 than of lot 50179. This difference may have resulted in the lower molecular weight of type 5 CP-rEPA lot 49704 compared with lot 50179. We have no explanation for this finding, but it does give us information about the requirements for future lots of S. aureus CP conjugates. Similarly, we found a greater immunogenicity of Vi protein conjugates of higher molecular weight prepared by different synthetic schemes (15, 52).

Unlike the sera of the mice, the preimmunization sera of the volunteers had low levels of both IgM and IgG antibodies to the type 5 and 8 CPs. The presence of these “natural” antibodies could be due to carriage of the homologous organisms and/or bacteria with cross-reacting antigens, as has been observed in the sera of adult humans and animals with other CPs (28, 40, 41). The presence of these natural antibodies could explain the failure of our conjugate vaccines to elicit a booster response to either of the two CPs in the volunteers; the initial response is likely a booster in a sensitized host (13, 40–43). The failure to elicit a booster response in adults has been observed with other capsular and O-specific polysaccharide conjugate vaccines (9, 13, 40).

In patients with AIDS, conjugates of H. influenzae type b CP elicited a lesser response than they did in healthy controls: the effect of reimmunization of these vaccines in patients with AIDS was not reported (48). Injection of the H. influenzae type b CP also yielded lower levels of serum antibodies in patients with human immunodeficiency virus type 1 infections than in normals. Further, the CP elicited lower levels of antibodies than the conjugate of this CP in patients with immunodeficiencies of different severities. Although the high-molecular-weight type 8 CP elicited levels of antibodies similar to those of the conjugate, we plan to study the latter vaccine in patients susceptible to S. aureus bacteremia. Because of its simplicity and comparative ease of production, the high-molecular-weight type 8 CP could be used to immunize volunteers to make hyperimmune globulin for passive immunization.

There were no differences in the serum antitoxin responses elicited by the two type 5 and the type 8 CP-rEPA conjugates. All three conjugates failed to elicit antitoxin after the first injection in the mice, and comparatively low levels were detected after the third injection. These conjugates elicited antitoxin responses in about two-thirds of the volunteers after the first injection and in all of them after the second injection. The levels in mice were lower than those we observed after injection of the S. aureus conjugates with ETA as the carrier protein, and, while it may be presumptuous to compare our results with other clinical studies, these antitoxin levels seemed to be lower than those elicited by the recombinant protein alone in volunteers (31). This lesser immunogenicity in mice could be due to the elimination of biological activity or a reduction in the immunogenicity of the native toxin by the genetically induced mutation. It has been reported that ETA has adjuvant-like properties in mice (27). The rEPA has no residual activity and, therefore, would not be expected to exert a modulating effect upon the immunologic properties of the conjugate.

The immunogenicity of the S. aureus CPs was enhanced after their covalent binding to the rEPA. These conjugates elicited no or minor side effects in all 70 volunteers. We plan to compare the levels of antibodies induced by these conjugates in patients at increased risk for bacteremia caused by S. aureus.

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