Passive Immunization Against *Pseudomonas* with a Ribosomal Vaccine-Induced Immune Serum and Immunoglobulin Fractions

MICHAEL M. LIEBERMAN,* DONNA C. MCKISSOCK, AND GWENDOLYN L. WRIGHT

Clinical Investigation Service, Brooke Army Medical Center, Fort Sam Houston, Texas 78234

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Passive protection of mice against *Pseudomonas aeruginosa* using specific antisera and immunoglobulin fractions induced by immunizing rabbits with a ribosomal vaccine is reported. The results demonstrated that protection by the ribosomal vaccine against challenge with live organisms can be serum mediated. Previous work has shown that the vaccine can be separated into two components on the basis of molecular weight and that both the higher (peak A)- and lower (peak B)-molecular-weight fractions were capable of inducing active immunity in mice. The present report indicates that both fractions are also capable of eliciting the production of mouse-protective antibody in rabbits. Agar gel diffusion with antisera to peaks A and B or unfraccionated vaccine indicated a common antigenic component among them in addition to an extra antigen in unfraccionated vaccine not present in peak B. Passive hemagglutination with antisera to peaks A and B demonstrated high-titer agglutinating antibody only with antiserum to peak A when a method of erythrocyte sensitization for lipopolysaccharide antigens was used. Also, passive hemagglutination was greatly inhibited by small amounts of lipopolysaccharide prepared from the same organism from which the vaccine was made. Both antisera to peaks A and B fixed complement with either A or B antigens. Antisera to peaks A and B, when reacted with peak B antigen, had about the same complement fixation titer (as determined by a quantitative complement fixation test). However, when peak A antigen was used, antiserum to peak A had about twice the complement fixation titer that antiserum to peak B had. These results are consistent with previous observations which suggest that the ribosomal vaccine contains lipopolysaccharide in addition to an unidentified immunogenic principle associated with ribosomes. Furthermore, this immunogen was present in both peaks A and B, but detectable amounts of lipopolysaccharide were present only in peak A. The relative importance of the immunoglobulin G (IgG) and IgM classes of antibodies was also compared. The results indicated that both IgG and IgM isolated from immune rabbit serum are protective in mice. Only IgG precipitated with the vaccine in agar gel diffusion, but both IgG and IgM were active in passive hemagglutination and in complement fixation. The passive hemagglutination titer of the IgM was higher than that of the IgG, but the complement fixation titer of the IgG was higher than that of the IgM. The mouse-protective capability of the IgG and IgM was about the same.

*Pseudomonas* bacteremia presents a particularly difficult problem of medical management because of the high associated patient mortality and the frequent occurrence of multiple-antibiotic-resistant organisms. The ability to predict specific populations at risk for *Pseudomonas* infections, such as the oncology patient on intensive chemotherapy, patients with cystic fibrosis, or patients with extensive thermal injury, makes the prospect of active immunization appealing.

Previous studies from this laboratory have described the preparation, properties, and immunogenicity of ribosomal vaccines from *Pseudomonas aeruginosa* (25). In these studies active immunization of mice with ribosomal vaccines provided effective protection against challenge with live organisms.

The effect of active immunization is limited, however, by the time required to develop and the host's ability to make specific protective antibody. Thus, passive immunization with hyperimmune globulin might be critical in the management of certain patient populations. This hyperimmune globulin could be used as prophylactic.
laxis in high-risk patients, especially those in an immunosuppressed state, as well as for administration to patients with known Pseudomonas infections, e.g., burn patients in which it might also provide passive "protection" or enhanced host resistance. Thus, the ability of a Pseudomonas vaccine to induce humoral immunity to allow for passive immunization with hyperimmune serum or globulin would be an important property of the vaccine.

Other studies of passive immunity to Pseudomonas have utilized immunoglobulin or antiserum to lipopolysaccharides (LPS) (1, 16), "slime" polysaccharides (2, 6), glycolipoproteins (33), or "core" glycolipids (8, 46). In addition, specific antitoxin (antitoxin A) has been tested for the capacity to provide effective mouse protection by passive immunization (26, 31, 35). Investigations concerning the immunogenicity of ribosomal vaccines from several bacterial species have primarily utilized the active mode of immunization (3, 5, 15, 18–21, 27, 30, 32, 34, 37, 39, 42–44). Passive immunization using antiserum or immunoglobulins prepared against ribosomal vaccines has received relatively little attention (36, 38, 40). This report describes the passive immunization of mice against Pseudomonas using specific antiserum and immunoglobulin fractions produced in rabbits with a ribosomal vaccine.

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MATERIALS AND METHODS

Bacteria and mice used, determination of mouse lethal titers of Pseudomonas cultures, and preparation of ribosomal vaccines were all described previously (25).

Rabbits. White, New Zealand rabbits, 4 to 8 pounds (ca. 1.8 to 3.6 kg; male or female), were used.

Vaccination of rabbits. Before vaccination, all rabbits were bled by cardiac puncture to obtain preimmune sera. Rabbits were then injected subcutaneously with aqueous vaccine preparations at the various doses described in the text. No adjuvants were used. Ten to 14 days after the initial vaccination, the rabbits were given a booster dose of the same vaccine. Fourteen to 20 days after the booster vaccination, the rabbits were again bled by cardiac puncture to obtain the immune sera. Groups of five to eight rabbits were used for each vaccine, and sera from the individual rabbits in each group were pooled.

Passive mouse protection. Aliquots, 0.5 ml, of serially diluted immune or preimmune sera were injected intraperitoneally into groups of five mice per dilution. Mice were challenged 3 h after administration of serum (unless otherwise stated in the text) by injection of an aliquot of whole (live) culture of the appropriate organism exactly as described previously (25) for direct challenge of vaccinated mice.

AGD. Agar gel diffusion (AGD) was performed using either MCI-Microfilms (Marine Colloids, Inc.) or Immuno-plates (Hyland Laboratories, Inc.). After the wells in the plate were filled with 0.005 ml (Immunoplates) or 0.010 ml (MCI-Microfilms) of the appropriate antigen or antiserum, the plates were incubated at 37°C for 24 h. The plates were then stained by immersion in 1% tannic acid for 15 min followed by immersion in distilled water. It was found that better results were obtained (less background) when antisera were precipitated with 50% saturated (NH₄)₂SO₄ before use. Equal volumes of antiserum and saturated (NH₄)₂SO₄ (pH 7.3) were mixed in an ice bath for 10 min and then centrifuged at 12,000 x g for 15 min. The supernatant was discarded, and the pellet was drained well and resuspended to the original volume of anti-serum in normal saline.

PHA. Passive hemagglutination (PHA) was performed essentially as described by Kabat and Mayer (23), using both the method for LPS antigens (method 1) and the method for protein antigens (method 2). Briefly, these are as follows. For method 1, an aqueous preparation of antigen was adjusted to 0.25 N NaOH and incubated at 37°C for 3 h. The material was then neutralized with HCl and dialyzed against at least 100 volumes of normal saline. Saline-washed sheep erythrocytes (obtained from Microbiological Associates) were then resuspended in the antigen preparation at a concentration of about 3% (packed cell volume/total volume) and incubated at 37°C for 4 h with periodic mixing. The sensitized cells were then washed twice with saline and once with a buffer consisting of 0.075 M KHP₂O₄-N₂HPO₄, 0.075 M NaCl-0.2% bovine serum albumin, pH 7.2 (PBS-BSA), and then resuspended in the same buffer at a concentration of 10%. Antisera were heat inactivated (65°C, 30 min) and then serially diluted in PBS-BSA, pH 7.2. To 0.4 ml of diluted antiserum was added 0.05 ml of the suspension of sensitized cells (or a suspension of control cells treated with saline instead of antigen). The tubes were mixed and allowed to incubate overnight at room temperature.

For method 2, saline-washed sheep erythrocytes were resuspended at a 2.5% concentration in PBS, pH 7.2 (the same buffer as above but without BSA), and then added to an equal volume of 1:20,000 tannic acid in PBS, pH 7.2. The cells were incubated at 37°C for 10 min, centrifuged, washed with PBS, pH 7.2, and resuspended in the same buffer to a 20% concentration. One milliliter of the tanned cells was then added to 4.0 ml of a buffer consisting of 0.075 M KH₂PO₄, Na₂HPO₄, 0.075 M NaCl, pH 6.4 (PBS), plus 1.0 ml of the antigen (not alkali treated) in normal saline. The sensitization mixture was then incubated at room temperature for 15 min, and the cells were centrifuged, washed twice with PBS-BSA (pH 7.2), and resuspended in the same buffer at a 10% concentration. The remaining steps in method 2 are the same as in method 1.

CF. The complement fixation (CF) test was adapted from Marucci and Fuller (29). Complement (lyophilized guinea pig serum) and hemolysin (rabbit anti-sheep erythrocyte stroma serum), both obtained from Microbiological Associates, were titrated, and the sheep erythrocytes were standardized at 10⁶ cells
per ml, according to Kabat and Mayer (23). The highest dilution of hemolysin yielding nearly maximal hemolysis and the dilution of complement yielding about 90% hemolysis (the top of the linear part of the S-shaped curve) obtained when hemolysis is determined as a function of complement concentration, were chosen for use in the CF test. CF buffer (29) was made up as follows: 8.30 g of NaCl, 1.019 g of sodium-5,5-diethylbarbiturate, 0.1015 g of MgCl2-6H2O, and 0.01667 g of CaCl2 were dissolved in 193 ml of distilled water, and 6.92 ml of 0.5 N HCl was added. (This comprises the CF stock solution.) The CF stock solution was then diluted fivefold with distilled water, and BSA was added to yield a 0.1% BSA concentration. The resulting CF buffer had a pH of 7.3 and was used for diluting all reagents in the CF test. The CF test was performed as follows: (i) 0.1 ml of appropriately diluted antigen was mixed with 0 to 0.3 ml of diluted antisera in an ice bath; (ii) 0.1 ml of diluted (1:100) complement was added, and the volumes were adjusted to 0.5 ml with CF buffer (tubes in which one or two of the reagents were omitted were included as controls); (iii) the tubes were mixed and kept on ice for 16 h; (iv) the sheep erythrocytes were sensitized by mixing equal volumes of the cell suspension standardized to 10⁶ cells per ml and hemolysin (diluted 1:1,000) and incubated for 10 min at 37°C; (v) 0.1 ml of the sensitized sheep cells was then added to each CF tube, and the contents of the tubes were mixed and incubated at 37°C for 60 min with periodic gentle shaking; (vi) 2.0 ml of normal saline was then added to each tube, and the tubes were centrifuged at 700 x g for 15 min; (vii) the supernatants were decanted, and their absorbances at 413 nm were read; (viii) the percentage of complement fixed (i.e., inhibition of hemolysis) in the presence of both antigen and antibody relative to hemolysis obtained when antigen was omitted was then plotted as a function of antisera concentration. (The degree of "anti-complementarity" shown by the antisera, i.e., inhibition of complement-dependent hemolysis by antisera without antigen, was less than 20% at the antisera dilutions used.) This plot results in a straight line in the region of 50% CF (see Results). From the plot, the volume of antisera of a given dilution yielding 50% CF can be determined. Thus, the number of 50% complement-fixing units per milliliter of antisera (CH50 units per milliliter) can be calculated. It should be noted that this value also depends on the antigen concentration, and thus a comparison of the number of CH50 units per milliliter for two different antisera is valid only when the antigen concentration is the same in the CF test for both antisera.

Preparation of LPS. LPS was prepared by the phenol-water technique (41) as follows. A 500-ml culture of bacteria was grown in brain heart infusion broth at 37°C with aeration for 18 h. The cells were harvested by centrifugation and washed three times with 0.05 M Na2HPO4, pH 7.2. The cells were then resuspended with 5 ml of distilled water, and 5 ml of 90% phenol was added. The mixture was stirred vigorously in a 65°C water bath for 20 min and then cooled on ice and centrifuged at 4°C and 1,000 x g for 20 min. The aqueous (top) layer was removed, and 5 ml of distilled water was added to the phenol layer. The mixture was again stirred vigorously at 65°C for 20 min, cooled on ice, and centrifuged as above. The aqueous layer was removed, combined with the previous aqueous phase, and clarified by centrifugation at 12,000 x g for 20 min. The clear supernatant was removed and then centrifuged in the Beckman L-5-50 ultracentrifuge at 131,000 x g (maximum) for 6 h with an SW27.1 rotor. The supernatant was discarded, and the colorless, opalescent pellet was drained well, suspended in 5 ml of normal saline by manual homogenization, and dialyzed against 1 liter of normal saline.

Chemical analyses. Protein, ribonucleic acid, and phosphate were estimated as described previously (25). Total carbohydrate was determined by the phenol-H2SO4 method (14), and methyl pentose was determined by the method of Dische and Shettles (13).

Separation of IgG and IgM. Five milliliters of antisera to a ribosomal vaccine was precipitated with 50% saturated (NH4)2SO4, and the supernatant was removed, and the precipitate was dissolved with 5 ml of a buffer consisting of 0.1 M tris(hydroxymethyl)aminomethane and 0.2 M NaCl at pH 8.0 (TS buffer). A glass column (2.54 by 100 cm) was packed with Sepharose 6B (Pharmacia Fine Chemicals) and equilibrated with TS buffer at 4°C. The (NH4)2SO4-precipitated antisera was applied to the column and the column was run at about 25 ml/h in TS buffer. The elution profile was monitored by reading the absorbance at 280 nm of the fractions in a Beckman Acta III spectrophotometer. Fractions were collected up to the two-thirds of the immunoglobulin M (IgM) peak and those within the trailing two-thirds of the IgG peak were pooled. (The remaining fractions in the peaks were not pooled to avoid cross-contamination.) The pooled fractions were precipitated with 50% saturated (NH4)2SO4, redissolved in 5.0 ml (original volume of antisera) of normal saline, and dialyzed against 100 volumes of saline.

β-ME treatment of antisera. Antisera was treated with β-mercaptoethanol (β-ME) by a method modified from Deutsch and Morton (12). Equal volumes of antisera and a buffer consisting of 0.2 M β-ME, 0.05 M Na2HPO4, and 0.1 M NaCl at pH 7.2 were mixed and incubated at 4°C for 3.5 h. The mixture was then dialyzed against 100 volumes of a buffer consisting of 0.02 M isoascetic acid, 0.08 M Na2HPO4, and 0.1 M NaCl at pH 7.2 for 4 h at 4°C with stirring, followed by dialysis against 200 volumes of normal saline at 4°C overnight.

RESULTS

Passive mouse protection with antisera to ribosomal vaccines. Ribosomal vaccines were prepared from P. aeruginosa serotypes 1, 8, and 13 as previously described (25). Vaccines were diluted to 1.0 mg of ribonucleic acid per ml (corresponding to 1.6, 1.4, and 1.8 mg of protein per ml for types 1, 8, and 13, respectively), and 1.0-ml doses were used to vaccinate rabbits as described above (after obtaining preimmune sera). After the vaccination schedule was completed, rabbits were bled for immune sera, which were then tested for passive mouse protection (along with the preimmune sera). The results
TABLE 1. Passive protection of mice with immune rabbit serum to ribosomal vaccine

<table>
<thead>
<tr>
<th>Rabbit antiserum to P. aeruginosa serotype no. *</th>
<th>Dilution</th>
<th>Mice survival</th>
<th>P value *</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>1:4</td>
<td>4/5 80</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>13</td>
<td>1:8</td>
<td>5/5 100</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>13</td>
<td>1:16</td>
<td>0/5 0</td>
<td></td>
</tr>
<tr>
<td>Preimmune</td>
<td>1:4</td>
<td>0/5 0</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1:4</td>
<td>4/5 80</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>8</td>
<td>1:8</td>
<td>4/5 80</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>8</td>
<td>1:16</td>
<td>0/5 0</td>
<td></td>
</tr>
<tr>
<td>Preimmune</td>
<td>1:4</td>
<td>0/5 0</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Undiluted</td>
<td>2/5 40</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1:4</td>
<td>0/5 0</td>
<td></td>
</tr>
<tr>
<td>Preimmune</td>
<td>Undiluted</td>
<td>0/5 0</td>
<td></td>
</tr>
</tbody>
</table>

* Serum was obtained from rabbits either before immunization (preimmune) or after two immunizations with the ribosomal vaccine at a dose of 1 mg of ribonucleic acid.

** Mice were given 0.5 ml, intraperitoneally, of the immune or preimmune serum at the stated dilution 2 h before challenge with live organisms (about 50% lethal doses of homologous serotype).

*** Mice were scored after 48 h for survivors.

**** P value was calculated by the chi-square test (11).

TABLE 2. Passive mouse protection versus time of administration of antiserum

<table>
<thead>
<tr>
<th>Time (h) of antiserum administration *</th>
<th>Expt 1</th>
<th>Expt 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-72</td>
<td>3/5</td>
<td>60</td>
</tr>
<tr>
<td>-48</td>
<td>2/5</td>
<td>40</td>
</tr>
<tr>
<td>-24</td>
<td>4/5</td>
<td>80</td>
</tr>
<tr>
<td>-8</td>
<td>5/5</td>
<td>100</td>
</tr>
<tr>
<td>-4</td>
<td>5/5</td>
<td>100</td>
</tr>
<tr>
<td>-2</td>
<td>5/5</td>
<td>100</td>
</tr>
<tr>
<td>0</td>
<td>5/5</td>
<td>100</td>
</tr>
<tr>
<td>+1</td>
<td>2/5</td>
<td>40</td>
</tr>
<tr>
<td>+2</td>
<td>1/5</td>
<td>20</td>
</tr>
<tr>
<td>+3</td>
<td>0/5</td>
<td>0</td>
</tr>
<tr>
<td>+4</td>
<td>0/5</td>
<td>0</td>
</tr>
</tbody>
</table>

* Mice were scored after 48 h for survivors.

* Antiserum was diluted 1:2 in normal saline and administered at the times indicated before or after challenge.

The 0-h group was given antiserum immediately before challenge. The challenge inoculum contained 8 50% lethal doses of type 13 culture.

** P values were calculated by the chi-square test (11).

(Table 1) demonstrated that administration of immune rabbit serum to types 8 and 13 protected mice against challenge with live, homologous organisms, even at a 1:8 dilution of antiserum. Antiserum to type 1 vaccine showed only marginal protection when used undiluted. Preimmune serum did not afford any protection.

Passive mouse protection as a function of time of administration of antiserum. Antiserum to type 13 vaccine was administered to mice at various times before and after challenge. The results (Table 2) indicated that antiserum is effective in passive mouse protection for at least 72 h before challenge. However, delay in administration of antiserum by 1 h after challenge decreased the protection to a marginal level, whereas further delay resulted in no protection at all.

Passive mouse protection with antiserum to Sepharose-fractionated ribosomal vaccine. A type 13 ribosomal vaccine was separated into two components by chromatography on Sepharose 4B as described previously (25). The elution profile (Fig. 1) showed two peaks, A and B, eluting in the excluded volume and at about 300 ml, respectively. (Results of chemical analyses of these peaks have been published [25].) Material from each of these peaks was used to vaccinate rabbits, and the immune sera obtained were assayed for passive mouse protection. The results (Table 3) demonstrated that both peaks A and B are immunogenic in rabbits, i.e., are capable of eliciting the production of mouse protective antibody.

Effect of β-ME treatment on passive mouse protection with antiserum to ribosomal vaccine. Antiserum to a type 13 ribosomal vaccine was treated with β-ME and assayed for passive mouse protection in comparison with untreated antiserum. The results (Table 4) indicated that β-ME treatment of antiserum significantly decreased passive mouse protection.

Passive mouse protection with isolated IgG and IgM. IgG and IgM fractions isolated...
from antiserum to a type 13 vaccine preparation, as described above, were analyzed by AGD against antiserum to rabbit IgG (heavy chain specific; Cappel Laboratories) and antiserum to rabbit IgM (heavy chain specific; Miles Laboratories). The resulting precipitin patterns are shown in Fig. 2 and demonstrated that the IgG reacts with antiserum to IgG but is free of IgM and the IgM reacts with antiserum to IgM but is free of IgG. The isolated IgG and IgM fractions were tested for passive mouse protection, and the results are presented in Table 5. It is evident that both IgG and IgM are protective. The highest dilutions at which effective protection was achieved were about the same (1:4 or 1:8), which was somewhat lower than the highest dilution of whole antiserum yielding effective protection (1:16).

Serological analysis of antisera to the ribosomal vaccine and subfractions. AGD with the serotype 13 vaccine and corresponding antiserum (Fig. 3a) resulted in a double precipitin line, suggesting the presence of two separate antigenic components in the vaccine. Antisera to Sepharose peaks A and B and unfractionated vaccine showed lines of identity (Fig. 3b, pattern A) indicating a common antigenic component among them. However, when antiserum to unfractionated vaccine was run against peak B and unfractionated vaccine (Fig. 3b, pattern B, wells 6, 5, and center well, respectively), a spur was evident with unfractionated vaccine, indicating the presence of an antigenic component not present in peak B.

Antisera to Sepharose peaks A and B were

![Graph](https://via.placeholder.com/150)

**Fig. 1. Molecular-sieve chromatography of the ribosomal vaccine preparation from *P. aeruginosa* serotype 13 on a Sepharose 4B column. The excluded volume (Ve) is approximately 150 ml, and the total volume (Vt) is approximately 500 ml. The first peak eluting at Ve is peak A, and the second peak eluting at about 300 ml is peak B. A260, Absorbance at 260 nm.**

Serum was obtained from rabbits either before immunization (preimmune) or after two immunizations with Sepharose 4B-fractionated ribosomal vaccine (from serotype 13) at doses of 200 μg of protein (peak A) or 550 μg of protein (peak B).

Mice were given 0.5 ml, intraperitoneally, of the immune or preimmune serum at the stated dilution 2.5 h before challenge with live organisms (about 50% lethal doses) of homologous serotype.

Mice were scored after 48 h for survivors.

*P value was calculated by the chi-square test (11).
TABLE 5. Passive mouse protection with IgG and IgM fractions of antiserum

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Dilution</th>
<th>Survivors</th>
<th>No.</th>
<th>%</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>1:4</td>
<td>3/5</td>
<td>60</td>
<td></td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IgG</td>
<td>1:8</td>
<td>3/5</td>
<td>60</td>
<td></td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IgG</td>
<td>1:16</td>
<td>1/5</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgM</td>
<td>1:4</td>
<td>5/5</td>
<td>100</td>
<td></td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>IgM</td>
<td>1:8</td>
<td>2/5</td>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgM</td>
<td>1:16</td>
<td>1/5</td>
<td>20</td>
<td></td>
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<tr>
<td>Whole antiserum</td>
<td>1:4</td>
<td>4/5</td>
<td>80</td>
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<tr>
<td>Whole antiserum</td>
<td>1:8</td>
<td>3/5</td>
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<tr>
<td>Whole antiserum</td>
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<td>60</td>
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<td>&lt;0.05</td>
</tr>
<tr>
<td>Whole antiserum</td>
<td>1:32</td>
<td>2/5</td>
<td>40</td>
<td></td>
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<tr>
<td>Controls</td>
<td>0/5</td>
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<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a Mice were given 0.5 ml, intraperitoneally, of the IgG, IgM, or whole antiserum at the stated dilution 3 h before challenge with 8 50% lethal doses of live culture.

*b Mice were scored after 48 h for survivors.

*c P value was calculated by the chi-square test (11).

FIG. 2. AGD with isolated IgG and IgM fractions. (A) Center well: antiserum to rabbit IgG (heavy chain specific); wells 1 and 2: IgG fraction of rabbit antiserum; wells 3 and 4: IgM fraction. (B) Center well: antiserum to rabbit IgM (heavy chain specific); wells 1 through 4: same as (A).

assayed by PHA with the corresponding antigens, using both methods 1 (alkali-treated antigen) and 2 (tanned erythrocytes) described above. The results are given in Table 6 and show that a relatively high PHA titer (1:128) was obtained only with antiserum to peak A using method 1. Antiserum to peak A using method 2 and antiserum to peak B using either method failed to agglutinate sensitized cells at dilutions greater than undiluted.

A quantitative CF test was also used to analyze antisera to peaks A and B. Both antisera were assayed for CF titer against each antigen. The percentage of complement fixed as a function of antiserum concentration is shown in Fig. 4. The data show that with appropriate antigen concentrations these plots are linear in the region of 50% CF, and thus the number of CH50 units per milliliter of antiserum (CF titer) can be calculated. The calculated CF titers for both antisera against each antigen are given in Table 7. The data show that both antisera have about
the same CF titer against peak B antigen (about 600 CH₅₀ units/ml). However, antiserum to peak A has about twice the CF titer that antiserum to peak B has when measured with peak A antigen. This difference suggests that there is an additional antigenic component in peak A not present in peak B.

**Effect of LPS on PHA.** Since a relatively high PHA titer was obtained (Table 6) only with a method used for LPS antigens but not protein antigens (23), a preparation of LPS was made from *P. aeruginosa* type 13 and used for inhibition of PHA. The LPS prepared by phenol–water extraction, as described above, contained the following (micrograms per milliliter): total carbohydrate (with glucose as reference), 260; methyl pentose (rhamnose), 64.9; protein, 77.5; phosphate, 339.

PHA with antiserum to peak A and alkali-treated antigen (peak A) was then repeated in the presence or absence of the LPS. These results are presented in Table 8 and clearly demonstrate that the presence of LPS inhibits PHA.

**Serological analysis of isolated IgG and IgM.** The isolated IgG and IgM fractions were analyzed by AGD, PHA, and CF. The IgG formed a well-defined precipitin line with the ribosomal vaccine in AGD but the IgM did not
TABLE 6. Passive hemagglutination with immune rabbit serum to Sepharose-fractionated ribosomal vaccine

<table>
<thead>
<tr>
<th>Rabbit antiserum to Sepharose peak&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Tanned sheep erythrocytes, sensitized with Sepharose peak&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Sheep erythrocytes (not tanned) sensitized with alkali-treated Sepharose peak&lt;sup&gt;c&lt;/sup&gt;</th>
<th>PHA titer&lt;sup&gt;d&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>A</td>
<td>A</td>
<td>Not sensitized</td>
<td>128</td>
</tr>
<tr>
<td>A</td>
<td>A</td>
<td>Not sensitized</td>
<td>1</td>
</tr>
<tr>
<td>A</td>
<td>A</td>
<td>Not sensitized</td>
<td>128</td>
</tr>
<tr>
<td>B</td>
<td>B</td>
<td>Not sensitized</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>B</td>
<td>Not sensitized</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>B</td>
<td>Not sensitized</td>
<td>1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Serum was obtained from rabbits after two immunizations with Sepharose 4B-fractionated ribosomal vaccine (serotype 13) at doses of 200 µg of protein (peak A) or 550 µg of protein (peak B).

<sup>b</sup> Both peak A and B antigens were at the same concentration (185 µg of protein per ml) during sensitization of tanned erythrocytes.

<sup>c</sup> Both peak A and B antigens (at 185 µg of protein per ml) were treated with 0.25 N NaOH at 37°C for 3 h, neutralized, and dialyzed against saline.

<sup>d</sup> Highest dilution of antiserum (1 = undiluted) yielding clumped erythrocytes: <1 = no clumping with undiluted antiserum.

yield a detectable precipitin line (data not shown). However, both fractions were active in PHA (Table 9) and CF (Table 10). The IgM had the same PHA titer that the original antiserum had (1:256), whereas the IgG titer was lower (1:64). On the contrary, the CF titer of IgG was higher than that of IgM with peak A antigen (9,600 compared to 800 CH<sub>50</sub> units/ml), or with
peak B antigen (3,570 compared to 910 CH50 units/ml). The CF titer of the whole antiserum was 17,900 CH50 units/ml with peak A antigen and 5,700 CH50 units/ml with peak B antigen. The fact that the CF titer of the whole antiserum is higher than the combined CF titers of both the IgG and IgM fractions with either antigen is reasonable, since only about two-thirds of each immunoglobulin peak was pooled and the volumes were adjusted to the original volume of antiserum (see above).

**DISCUSSION**

The results presented in this report describe the mouse-protective capability and other serological properties of immune serum elicited in rabbits in response to a *Pseudomonas* ribosomal vaccine. Previous work in this laboratory has shown that such vaccines were effective in producing active immunity in mice (25). The present report provides evidence to demonstrate that

**TABLE 8. Inhibition of PHA with LPS**

<table>
<thead>
<tr>
<th>Addition</th>
<th>PHA titer of antiserum to peak A</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1:64</td>
</tr>
<tr>
<td>LPS, 1.3 μg</td>
<td>1:4</td>
</tr>
<tr>
<td>LPS, 6.5 μg</td>
<td>1:2</td>
</tr>
</tbody>
</table>

a PHA was performed by method 1 as described in the text.

b Highest dilution of antiserum yielding clumped erythrocytes.

c A 0.005-ml amount of LPS (1.3 μg of total carbohydrate) was added to each tube of serially diluted antiserum before addition of sensitized cells.

d A 0.025-ml amount of LPS (6.5 μg of total carbohydrate) was added, as in footnote c.

**TABLE 9. PHA with IgG and IgM fractions of antiserum**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>1:64</td>
</tr>
<tr>
<td>IgM</td>
<td>1:256</td>
</tr>
<tr>
<td>Whole antiserum</td>
<td>1:256</td>
</tr>
</tbody>
</table>

a PHA was performed by method 1 (alkali-treated peak A antigen).

b Highest dilution of antiserum yielding clumped erythrocytes.

**TABLE 10. CF with IgG and IgM fractions of antiserum**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Titer (CH50 units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak A antigen</td>
<td></td>
</tr>
<tr>
<td>Peak B antigen</td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>9,600</td>
</tr>
<tr>
<td>IgM</td>
<td>800</td>
</tr>
<tr>
<td>Whole antiserum</td>
<td>17,900</td>
</tr>
</tbody>
</table>

a CH50 units per milliliter were calculated from quantitative CF assays as described in the text.

b Sepharose-fractionated ribosomal vaccine from serotype 13: peak A used at 1 μg of protein per ml; peak B used at 7.5 μg of protein per ml.

**FIG. 4. CF as a function of antiserum concentration.** Symbols: (●) peak A antigen (1 μg of protein per ml) + antiserum to peak A (diluted 1:400); (○) peak A antigen (1 μg of protein per ml) + antiserum to peak B (diluted 1:200); (□) peak B antigen (7.5 μg of protein per ml) + antiserum to peak B (diluted 1:100); (Δ) peak B antigen (7.5 μg of protein per ml) + antiserum to peak A (diluted 1:100). The dilution of antiserum used divided by the volume (milliliters) of antiserum giving 50% CF yields the CF titer.

**TABLE 7. CF with immune rabbit serum to Sepharose-fractionated ribosomal vaccine**

<table>
<thead>
<tr>
<th>Antigen (Sepharose peak)</th>
<th>Antiserum to Sepharose peak</th>
<th>CH50 units/ml of antiserum</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>A</td>
<td>2,660</td>
</tr>
<tr>
<td>A</td>
<td>B</td>
<td>1,200</td>
</tr>
<tr>
<td>B</td>
<td>B</td>
<td>540</td>
</tr>
<tr>
<td>B</td>
<td>A</td>
<td>700</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Expt 1</th>
<th>Expt 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2,700</td>
</tr>
<tr>
<td></td>
<td>1,450</td>
</tr>
<tr>
<td>B</td>
<td>610</td>
</tr>
<tr>
<td>B</td>
<td>580</td>
</tr>
</tbody>
</table>

a Sepharose-fractionated ribosomal vaccine from serotype 13: peak A used at 1 μg of protein per ml; peak B used at 7.5 μg of protein per ml.

b Serum was obtained from rabbits immunized two times with either peak A at a dose of 200 μg of protein or peak B at a dose of 550 μg of protein.

c CH50 units per milliliter were calculated from quantitative CF assays as described in the text.
the immunity produced can be serum mediated. Immune rabbit serum was effective in passive immunization of mice against challenge with live organisms (Table 1). Furthermore, antiserum remained effective in mice for at least 3 days before challenge (Table 2). However, once infection was established subsequent administration of antiserum was not effective in protection, probably due to the large inoculum of organisms required to produce the lethal challenge (about 10^6 cells per 8 50% lethal doses) and the rapidity of bacterial growth in the animals.

Previous work also demonstrated that the ribosomal vaccine could be fractionated into two components on the basis of molecular weight (25). Each of these components was immunogenic in mice, although the higher-molecular-weight fraction (peak A) had greater immunogenicity (i.e., was effective at much lower doses) than the lower-molecular-weight fraction (peak B). The results described in this report showed that both fractions are also capable of eliciting the production of mouse-protective antibody in rabbits (Fig. 1, Table 3). However, the differences in immunogenicity were not as apparent from these data. Serological analysis of these antisera revealed several interesting properties related to their precipitating, agglutinating, and complement-fixing activities. AGD (Fig. 3) demonstrated precipitating antibody to two antigenic components in unfractionated vaccine. Antiserum to unfractionated vaccine, peak A, and peak B showed a line of identity indicating a common antigen among them in addition to an extra antigenic component in unfractionated vaccine not present in peak B.

PHA with antiserum to peaks A and B demonstrated high-titer agglutinating antibody only with antiserum to peak A when a method of erythrocyte sensitization for LPS antigens was used (Table 6). Furthermore, PHA was greatly inhibited by small amounts of LPS prepared from the same organism from which the vaccine was made (Table 8). Both antiserum to peaks A and B fix complement and cross-react with the heterologous antigens (Fig. 4, Table 7). Antiserum to peaks A and B, when reacted with peak B antigen, had about the same CF titer (CH50 units per milliliter), but when reacted with peak A antigen, antiserum to peak A had about twice the CF titer that antiserum to peak B had (Table 7). The above results are consistent with previous observations which suggest that the ribosomal vaccine contains LPS in addition to an unidentified immunogenic principle associated with ribosomes. Previously, the presence of components of LPS was demonstrated in unfractionated vaccine by radiological means (24). Subsequently, the presence of these components was also demonstrated in the peak A fraction of the vaccine, but the peak B fraction was shown to be free of LPS by both radiological and chemical means (25). The AGD and CF data indicate the presence of a shared component in both peaks A and B and in unfractionated vaccine. Furthermore, an additional component in unfractionated vaccine not present in peak B was shown by the AGD, whereas the CF data suggested an additional component in peak A not present in peak B. The reason for both antiserum to peaks A and B failing to show a PHA titer when an erythrocyte sensitization method for protein antigens was used is unknown. However, the PHA data clearly demonstrated the presence of LPS in peak A material. Other authors have suggested that the ribosomal vaccine from Salmo-nella contained LPS (15, 18), flagellin-like contaminants (9), or an unspecified glycoprotein or mucopolysaccharide (19).

The relative importance of the IgG and IgM classes of antibodies in mouse-protective capability and other serological activities of the anti-serum was investigated. β-ME inactivation of the IgM in the antiserum prior to testing for passive mouse protection suggested that both IgG and IgM were active in protection (Table 4). Thus, IgG and IgM fractions were isolated from antiserum to the ribosomal vaccine and tested for passive mouse protection. Both antibody classes provided effective mouse protection (Table 5). In addition, both IgG and IgM were active in PHA (Table 9) and in CF (Table 10). However, the relative activity of the two classes was different for PHA and CF. The PHA titer of the IgM was higher than that of the IgG (1:256 compared to 1:64), as might be expected, whereas the CF titer of the IgG was higher than that of the IgM with both peaks A and B antigens (Table 10). The mouse-protective capability of the two classes of antibody did not appear to be significantly different, but only the IgG was precipitated with the vaccine in AGD. Thus, there appeared to be little correlation of in vitro serological activity with passive mouse protection.

The question as to whether IgG or IgM antibody classes are protective against Pseudo-monas has been debated in the literature for some time. Sensakovic and Bartell (33) found that IgM from antiserum to Pseudomonas glycolipoprotein was protective, but IgG was not. Bjornson and Michael (7) found that IgG was more protective than IgM from antiserum raised against Pseudomonas polysaccharide. Crowder et al. (10), in a study of sera from patients with Pseudomonas infections, concluded that IgG
was more important than IgM for protection, whereas Alexander and Fisher (1) and Fisher (16) believe that IgG against O-antigen is the important antibody in vivo activity against *Pseudomonas*. Zinner and McCabe (47) and McCabe et al. (28) feel that for infections with gram-negative bacilli in general, IgM and IgG may exert complementary effects. IgM might prevent infection with "sensitive-strain" infections initially, whereas IgG would be primarily responsible for protection against established infections with "resistant-strain" infections (47). Young and Armstrong (45), using an opsonization assay, found that "natural" antibody activity from normal serum against *Pseudomonas* was primarily due to IgM, but convalescent sera contained both IgG and IgM activities. Jones et al. (22), in a study of vaccinated mice, also found protective antibody in both IgG and IgM fractions. The results presented in this report demonstrate that both IgG and IgM are protective. It seems probable that the particular response obtained in immune serum would depend on the nature of the antigens present in the vaccine and the vaccination and bleeding schedule used. The findings reported here extend the observations of the authors cited above concerning the properties of IgG and IgM to the immunoglobulin classes obtained from antisera to a ribosomal vaccine.

The concept of passive immunotherapy for gram-negative infections in general and *Pseudomonas* infection in particular is an attractive one as evidenced by the literature. Approaches utilized include an immunoglobulin prepared against a polyvalent LPS vaccine (16), antisera to the core glycolipid from an *Escherichia coli* mutant, J-5 (8), or a similar Salmonella rough mutant (46), and antisera to the "common antigen" (OEP) of *P. aeruginosa* (17), as well as antisera to *Pseudomonas* exotoxin A (anti-toxin A) (26, 31, 35). Each of these approaches appears to have certain advantages and disadvantages. Immunoglobulin to the type-specific LPS is protective and when prepared against a polyvalent vaccine would be effective against a number of immunotypes (16). However, the vaccine is relatively toxic (1), and thus preparation of a human hyperimmune globulin might be difficult. Antiserum to the core polysaccharide from the rough mutants of *E. coli* and *Salmonella* would appear to have good prospects for broad-spectrum, gram-negative immunity but yielded contrasting results. Braude et al. (8) claimed that antiserum to *E. coli* J-5, a uridine-diphosphate galactose epimerase-deficient mutant, was very effective in protecting against *Pseudomonas* infection in rabbits. In addition, the J-5 vaccine was claimed to be safe for human administration in spite of the fact that this core polysaccharide also contains lipid A, the well-documented toxic component of LPS. On the contrary, Young and Stevens (46) found that antiserum to *Salmonella* Re mutant core glycolipid provided significantly less protection against *E. coli* than type-specific LPS immunization against *E. coli* and failed to protect against *Pseudomonas* challenge as well. Antitoxin A in the hands of Liu and Hsieh (26) appeared to effectively protect against challenge with certain strains of *Pseudomonas*, but Snell et al. (35) found that antitoxin A could not protect mice against lethal *Pseudomonas* infection. Pavlovskis et al. (31) showed that antitoxin protected against a high-toxin, low-protease-producing strain of *Pseudomonas* but not against other strains.

Thus, passive protection with antiserum to ribosomal vaccines appears to be a viable alternative to the approaches discussed above. Although antiserum to ribosomal vaccines does not appear to have broad-spectrum effectiveness (preliminary experiments indicate that immunity is type specific), nevertheless, a polyvalent antiserum or immune globulin could be prepared which would protect against a number of serotypes. Furthermore, ribosomal vaccines are relatively less toxic than the LPS vaccines (4). Moreover, the toxicity would be even further reduced if an LPS-free fraction of the vaccine is used. This would facilitate vaccination of human volunteers in order to produce a human hyperimmune globulin.

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