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Protection by Serum Antibodies in Experimental Nontypable *Haemophilus influenzae* Otitis Media

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The chinchilla experimental model of otitis media was used to examine the importance of serum antibodies in protection against disease caused by nontypable *Haemophilus influenzae*. An immune serum pool was prepared by immunizing chinchillas with killed bacterial cells of nontypable *H. influenzae* 3245. Pooled preimmune or immune serum from these immunized animals was administered intravenously to a group of nonimmune chinchillas 1 day before intrabullar challenge with strain 3245. Of 5 animals receiving preimmune serum, 5 developed otitis media compared with 0 of 10 animals receiving immune serum ($P = 0.008$). The immune serum pool contained antibodies directed against both surface-exposed outer membrane proteins and lipopolysaccharide (LPS). The 39-kilodalton major outer membrane protein was the immunodominant surface protein. Anti-LPS antibodies were removed from the immune serum pool by affinity chromatography, and affinity-purified anti-LPS antibodies were recovered. Immune serum, immune serum absorbed of LPS antibodies, or affinity-purified LPS antibodies were then administered to another group of experimental animals 1 day before bacterial challenge. Of four animals that received the affinity-purified LPS antibodies, four developed otitis compared with zero of four animals that received the immune serum or zero of four animals that received the LPS-absorbed immune serum ($P = 0.028$). These studies indicate that passive immunization with immune serum is protective in experimental nontypable *H. influenzae* otitis media and that bacterial outer membrane proteins may be the principal targets of protective antibody.

Unencapsulated (nontypable) strains of *Haemophilus influenzae* are a major cause of middle ear infections in young children (13, 25, 30). The role of antibody in prevention of middle ear disease is unclear. Preliminary evidence suggests that strain-specific immune responses develop after natural infection and that such responses could be important in protection. Shurin et al. reported that children recovering from *H. influenzae* otitis develop increases in serum bactericidal activity (28). Furthermore, the absence of serum bactericidal activity could be correlated with susceptibility to *H. influenzae* otitis. The bacterial surface antigens recognized by bactericidal antibodies were not identified in this work. Recent studies suggest that the bacterial outer membrane proteins of nontypable *H. influenzae* are important targets for human bactericidal antibodies (8).

The chinchilla model of otitis media has been useful in investigations of the pathogenesis of otitis media and the importance of host immunity in prevention of disease (6, 7, 15). Karasic and co-workers reported the development of strain-specific protective immunity in chinchillas after experimentally induced *H. influenzae* otitis (15). Coincident with the development of immunity, animals demonstrated increases in serum antibody directed predominantly against outer membrane protein determinants. However, the role of antibody in protection was not examined.

The purpose of the present investigation was to determine whether serum antibodies administered by passive immunization could protect animals against development of *H. influenzae* otitis media. An effort also was made to identify the specific bacterial surface antigens recognized by protective serum antibodies. Our results indicate that passive immunization provides protection against experimental otitis in the chinchilla model and that bacterial outer membrane proteins appear to be the principal targets of protective antibody.

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MATERIALS AND METHODS

Bacterial strain. Nontypable *H. influenzae* 3245, originally isolated from the middle ear fluid of a child with acute otitis media, was identified by standard methods (16). The organism failed to agglutinate with a panel of typing antisera a to f (Burroughs Wellcome Co., Research Triangle Park, N.C.). Additionally, saline suspensions of the organism failed to show lines of precipitation with these antisera in counterimmunoelectrophoresis assays (32). This strain was biotype II (16). The organism was stored at −70°C in skim milk after two subpassages from the initial clinical isolation.

Growth conditions of bacteria. Bacteria were recovered from skim milk stocks by transfer of a loopful of thawed organisms to a chocolate agar plate and incubation for 16 h at 37°C. Five to ten colonies were then isolated with a sterile loop and used to inoculate 50 ml of brain heart infusion broth supplemented with NAD and hemin, each at 4 μg/ml. Growth proceeded for approximately 12 h overnight at 37°C, at which time the bacteria were in the stationary phase of growth. A 2-ml portion of the overnight suspension was then inoculated into 50 ml of fresh supplemented brain heart infusion broth, and the bacteria were grown to mid-log phase at 37°C in 250-ml Erlenmeyer flasks with a shaker-incubator (model number G25; New Brunswick Scientific Co., Inc., Edison, N.J.). Bacterial cells were harvested by centrifugation at 12,000 × *g* at 4°C and washed twice with phosphate-buffered saline (PBS) before further use.

Preparation of immune serum pool. Freshly grown and washed logarithmic-phase cells of strain 3245 were fixed in a 0.4% formaldehyde solution (Fisher Scientific Co., Pittsburgh, Pa.), diluted to a concentration of 10^6 to 10^7 cells per ml in PBS, and emulsified in Freund incomplete adjuvant (Difco Laboratories, Detroit, Mich.). Nine chinchillas were immunized subcutaneously with approximately 10^7 cells in Freund adjuvant every 3 to 4 weeks over the course of 4 months. Preimmune samples were collected before immunization. Immune serum samples were collected after the third immunization and then after each subsequent immunization. Serum samples from all bleedings were pooled to generate the immune serum pool used throughout the subsequent experiments.

Absorption of anti-LPS antibodies. The technique of Darveau and Hancock was used to purify lipopolysaccharide (LPS) from strain 3245 (4). When examined by polyacrylamide gel electrophoresis and silver staining (12), this LPS preparation demonstrated a pattern similar to that observed with other nontypable *H. influenzae* LPS (14). Protein content was 5% as determined by the modified Lowry technique of Peterson (21), but no discrete protein bands were visible by silver staining after gel electrophoresis (12).

The LPS preparation was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N.J.) and used as a solid-phase absorbant (27). In brief, 10 mg of LPS was dissolved in 5 ml of coupling buffer (0.1 M sodium bicarbonate, 0.5 M NaCl, 0.2% sodium deoxycholate, pH 8.3) and reacted for 2 h at room temperature with 1 g of activated Sepharose 4B. Residual reactive groups were then blocked with ethanolamine. The matrix was washed extensively with coupling buffer to remove non-covalently bound LPS before equilibration with PBS–0.5% bovine serum albumin.

The chinchilla immune serum pool (45 ml) was passed through the column. Anti-LPS antibody, as measured by enzyme-linked immunosorbent assay (ELISA; see below), was completely removed by a single pass. The column was washed with 2 column volumes of PBS–0.5% bovine serum albumin, then anti-LPS antibodies were eluted from the solid phase with 3.5 M $MgCl_2$ and immediately diluted with 1 volume of PBS–0.5% bovine serum albumin. The eluted antibodies were dialyzed exhaustively against PBS at 4°C before further use.

Measurement of anti-LPS antibodies. Serum binding activity against LPS antigens was determined by ELISA. The technique previously used in this laboratory for the measurement of antibodies to LPS of *H. influenzae* type b (27) was modified slightly for this assay. In brief, 96-well flat-bottom enzyme immunoassay microtitration plates (Linbro/Titertek; Flow Laboratories, Inc., McLean, Va.) were coated for 16 h at 4°C with purified LPS (10 μg/ml) in $NaCO_3$ buffer (pH 9.6). After overnight incubation, the plates were washed three times with PBS–0.05% Tween 20 and then incubated for 1 h with PBS–0.5% bovine serum albumin at room temperature (blocking step). The plates were again washed three times with PBS–0.05% Tween 20. The serum specimens, diluted in PBS–0.5% Tween 20, were added and incubated for 1 h at room temperature. After an additional wash, affinity-purified rabbit anti-chinchilla immunoglobulin G (IgG) or IgM conjugated with alkaline phosphatase (see below) was added at a 1:500 or 1:250 dilution, respectively. After a final wash, the substrate, disodium *p*-nitrophenylphosphate (1 mg/ml in 10% diethanolamine buffer, pH 9.8), was added, and the reaction was allowed to proceed at room temperature. A_{405} was monitored with a Titertek Multiskan reader (Flow Laboratories). Serial dilutions of an immune serum pool were run as a control with each LPS ELISA experiment. The test reactions were read when a 1:1,000 dilution of this control reached an optical density of 0.3. Specimen titers were expressed as the limiting dilution that gave an optical density of 0.3. Negative controls included wells coated with antigen and reacted with conjugate, mock-coated wells reacted with serum and conjugate, and mock-coated wells reacted with conjugate alone. All negative controls gave optical density readings of <0.1.

Preparation of anti-chinchilla IgG and IgM conjugates for ELISA. Chinchilla IgG and IgM fractions were prepared by the techniques of Wanatabe et al. (33), slightly modified. In brief, a crude immunoglobulin pool was prepared from a chinchilla serum pool by two successive precipitations with ammonium sulfate at 40% final saturation. The precipitate was resuspended in 1/20 of the original volume of serum, dialyzed against PBS, pH 7.4, and applied to a Sephacryl S-300 column which was equilibrated with PBS. Fractions were assayed for protein content by the modified Lowry method (21). The first protein peak was used as a source of IgM, and the second peak was used as a source of IgG.

The IgM peak was concentrated and rechromatographed twice through a Sephacryl S-300 column equilibrated with PBS. The IgG peak from the Sephacryl S-300 column was concentrated, dialyzed against 0.005 M sodium phosphate buffer, pH 8.0, and then applied to a DEAE-Sephacel ion-exchange column equilibrated with 0.005 M sodium phosphate buffer. The proteins were eluted by a continuous gradient of 0.005 to 0.5 M sodium phosphate. When examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie blue staining, the purified IgG and IgM fractions demonstrated protein bands which migrated with apparent molecular weights typical of immunoglobulin heavy and light chains of the respective classes. The immunoglobulins appeared to be free of other protein contamination.

To prepare antisera, 100 μg of purified chinchilla IgG or IgM was emulsified in Freund complete adjuvant (Difco Laboratories) and administered subcutaneously to rabbits in a series of four injections over 3 months. Rabbit immunoglobulins were recovered from the immune serum by precipitation with 40% saturated ammonium sulfate. The ammonium sulfate-precipitated fractions were subjected to affinity chromatography to obtain antibodies specifically recognizing either chinchilla IgG or IgM. In brief, solid-phase affinity columns were prepared with either purified chinchilla IgG or IgM by coupling to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals) (22). The ammonium sulfate preparations from the rabbit immune serum were passed over the respective heterologous chinchilla immunoglobulin columns (e.g., anti-chinchilla IgG over the IgM column) to remove cross-reacting antibodies. To generate IgG- or IgM-specific preparations, the column fall-throughs were next applied to the respective homologous immunoglobulin column. Adherent antibodies were eluted from the homologous column with 3.5 M $MgCl_2$, dialyzed against PBS, and concentrated before coupling with alkaline phosphatase.

The technique of Avrameas was used to couple alkaline phosphatase to the affinity-purified antibodies (2). In brief, 0.5 mg of purified antibody in PBS was mixed with 1.5 mg of alkaline phosphatase (Sigma) and dialyzed for 16 h at 4°C against PBS. The next day, 25% glutaraldehyde (Sigma) was added to the antibody-enzyme mixture to achieve a final concentration of 0.2%, and the mixture was incubated for 2

h at room temperature. The mixture was dialyzed for an additional 16 h at 4°C against PBS, diluted in 10 ml of PBS–0.5% bovine serum albumin with 0.02% azide, and stored for later use.

Detection of antibodies directed against surface-exposed outer membrane proteins by whole-cell radioimmunoprecipitation. The whole-cell immunoprecipitation assay was performed by the technique of Hansen et al. (10, 11). In brief, strain 3245 cells were grown to early log phase in supplemented brain heart infusion broth, and the cells were harvested by centrifugation and washed in PBS. Outer membrane proteins of intact cells were then extrinsically radiolabeled with [^{125}I]iodine by the lactoperoxidase method. Intact labeled cells were washed with PBS three times and resuspended at a concentration of approximately 10^{10} cells per ml (specific activity, 0.01 cpm per CFU). Labeled cells (250 μl) were then mixed with an equal volume of serum or a serum fraction diluted in PBS, and the mixtures were rocked at 4°C for 90 min. Unattached antibodies were removed from the cell suspension by a washing of the cells once with PBS. Antigen-antibody complexes were extracted from the cells by incubation of the cells in "solubilization buffer" consisting of 10 mM Tris buffer (pH 7.8) containing 150 mM NaCl, 10 mM EDTA, 1% (vol/vol) Triton X-100, 0.2% (wt/vol) sodium deoxycholate, and 0.1% (wt/vol) sodium dodecyl sulfate for 1 h at 37°C. The resultant preparation was centrifuged at 45,000 × *g* for 1 h at room temperature to remove insoluble material. The supernatant was then incubated overnight at 4°C with 4 mg of protein A-Sepharose beads (Sigma) to bind soluble antigen-antibody complexes (predominantly IgG-containing complexes). The protein A-Sepharose antibody-antigen complexes were washed five times in solubilization buffer and dissociated by boiling for 5 min in a "dissolution buffer": 0.0625 M Tris buffer (pH 6.8) containing 2% sodium dodecyl sulfate, 10% glycerol, and 0.001% bromphenol blue tracking dye. The Sepharose beads were removed by centrifugation, and the immune precipitates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by autoradiography to identify radiolabeled outer membrane proteins present in the immune precipitates.

Detection of antibodies directed against surface-exposed LPS determinants by the indirect immunofluorescence assay. Strain 3245 cells were grown to early log phase in supplemented brain heart infusion broth, and the cells were harvested by centrifugation and washed three times in sterile PBS with 1% albumin. The bacteria were maintained at 4°C throughout the remainder of the experiment. Bacterial cells were adjusted to a final optical density of 0.5 (approximately 5.10^8 CFU per ml), and 500 μl of the suspension was incubated with 500 μl of the affinity-purified anti-LPS antibody preparation for 60 min with rocking. Bacterial cells were then pelleted and washed twice with PBS-albumin. The washed cells were next incubated for 30 min with 100 μl of fluorescein-conjugated rabbit anti-chinchilla IgG antibody prepared from the anti-chinchilla IgG we had purified. Bacterial cells were washed three times with PBS-albumin, and the final pellet was resuspended in PBS-albumin diluted 1:1 with glycerol (Sigma). Cells were monitored for fluorescence by using an incident light fluorescent microscope (Nikon). Negative controls included strain 3245 cells incubated with nonimmune serum and fluorescein-conjugated antibody or with fluorescein-conjugated antibody alone. Positive controls included strain 3245 cells incubated with the unabsorbed chinchilla immune pool or the LPS-absorbed immune pool.

TABLE 1. Effect of intravenous pretreatment with immune serum on the development of *H. influenzae* otitis media

Serum	Dose (ml/kg)	No. of animals with culture-positive otitis/total no.
Preimmune	5	5/5[a]
Immune	5	0/5[a]
	10	0/5

[a] $P = 0.008$ by the Fisher exact two-tailed test.

Detection of antibodies directed against LPS by presolubilized cell radioimmunoprecipitation. For radioimmunoprecipitation analysis of anti-LPS antibodies (9), strain 3245 cells were intrinsically radiolabeled with [^{32}P]phosphoric acid by the method of Hitchcock and Brown (12). In brief, the culture medium for the experiment was depleted of P_i by raising the pH to 10 with NaOH, adding 3.5 M $MgCl_2$ until precipitation ceased, and removing the precipitates by centrifugation. The medium was adjusted to pH 7.3 with HCl and filter sterilized before use. Strain 3245 cells were grown to stationary phase overnight in standard supplemented brain heart infusion broth, and then 1 ml of overnight growth was used to inoculate 25 ml of the P_i-depleted medium containing 2 mCi of [^{32}P]phosphoric acid. Cells were harvested, washed three times with PBS, solubilized,and then used as antigen in the immunoprecipitation system.

Experimental animals. Healthy adult chinchillas 1 to 2 years of age with weights of 350 to 500 g were used for preparation of the immune serum pool and for the challenge experiments. The animals were obtained from an outbred chinchilla colony maintained at Washington University School of Medicine by the Department of Otolaryngology. The animals were housed in separate cages, and noninfected animals were kept in rooms separate from animals receiving bacterial challenges.

Experimental infection. Experimental animals received intravascular infusions of the test serum or serum fractions 24 h before bacterial challenge. Serum samples were obtained from each animal just before serum administration to monitor for any preexisting antibody directed against the challenge strain. The next day, freshly grown strain 3245 cells were prepared as detailed above and diluted to a concentration of 10^4 CFU per ml in PBS–0.5% bovine serum albumin (pH 7.4). The colony count was confirmed by plating appropriate dilutions. The left middle ear space of each animal was inoculated via the epitympanic bulla with 0.1 ml (containing 10^3 CFU). In preliminary experiments, this bacterial inoculum reproducibly caused middle ear infection in nonimmune experimental animals. Additional serum samples were collected from each animal at the time of challenge to monitor serum antibodies.

The course of middle ear disease was monitored by periodic otoscopy, aspiration of fluid from the middle ear space via the epitympanic bullae, and semiquantitative culture of collected fluid. Middle ear aspiration was performed every 2 to 3 days after bacterial challenge, irrespective of whether or not otoscopic examination demonstrated the presence of tympanic membrane inflammation or middle ear effusion. In preliminary experiments, daily blood cultures were obtained after middle ear challenge with strain 3245. However, bacteremia was not observed, and blood cultures were therefore not included as part of the specimen collection for the animals described in the following experiments.

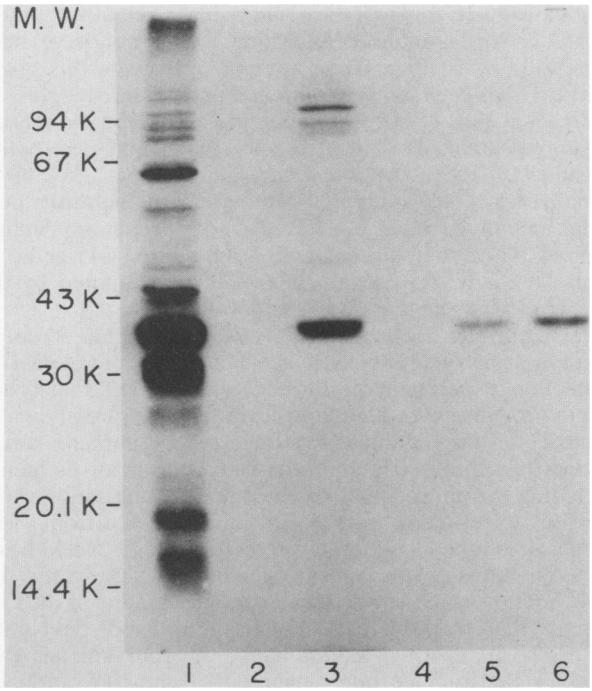

FIG. 1. Whole-cell radioimmunoprecipitation assay with serum samples used in the passive protection experiment comparing preimmune and immune serum (Table 1). Iodinated strain 3245 cells were used as the test strain. Lane 1 demonstrates the iodinated proteins present in the cells labeled for this experiment. Proteins immunoprecipitated by the individual serum samples are shown as follows: lane 2, preimmune serum; lane 3, immune serum; lanes 4 to 6, serum collected immediately before experimental challenge from individual animals receiving 5 ml of preimmune serum per kg of body weight or 10 ml of immune serum per kg of body weight, respectively. Molecular masses (in kilodaltons) are listed to the left of the figure. Molecular weight standards were visualized by Coomassie blue staining before gel autoradiography.

RESULTS

Passive immunization with immune serum. The effect of pretreatment of animals with immune serum on the development of experimental *H. influenzae* otitis media was assessed (Table 1). All five control animals that received the preimmune pool developed culture-positive otitis by 2 days after experimental challenge. Middle ear fluid cultures remained positive for each of the animals for approximately 21 days. Bacterial densities in middle ear fluid samples were generally $>10^5$ CFU per ml except for the last positive culture, from which lower counts were observed.

In contrast, none of the 10 animals that received immune serum developed culture-positive otitis. A few showed evidence of mild inflammation of their tympanic membranes and small middle ear effusions. The middle ear effusions were aspirated and cultured but were uniformly culture negative. All signs of tympanic membrane inflammation in these animals had resolved by 7 days after experimental challenge.

Characterization of antibodies present in immune serum. Serum antibodies likely to be important in protection are those which interact with antigenic surface molecules of the bacteria. Two major classes of surface molecules on nontypable *H. influenzae* are the outer membrane proteins and LPS. To identify the surface-exposed outer membrane proteins of strain 3245 recognized by antibodies in the immune serum, we performed a whole-cell radioimmunoprecipitation assay with strain 3245 cells and the serum samples used in the passive protection experiment (Fig. 1). The preimmune pool did not precipitate any of the surface proteins (Fig. 1, lane 2). In contrast, the immune serum pool precipitated several proteins (Fig. 1, lane 3). Predominant activity was directed against a 39-kilodalton (kDa) protein, one of the major outer membrane proteins of strain 3245. Serum samples collected from individual experimental animals that received either preimmune or immune serum demonstrated similar results: lane 4, from an animal that received preimmune serum, had no demonstrable activity; lanes 5 and 6, from animals that received immune serum, had activity directed primarily against the 39-kDa protein (Fig. 1).

Serum antibody activity directed against LPS determinants was monitored by ELISA. The preimmune serum pool had no detectable IgG or IgM anti-LPS activity. In contrast, the immune serum pool had an IgG anti-LPS titer of 1:1,000, but no IgM anti-LPS activity was demonstrable. Animals receiving 5 ml of the immune pool per kg of body weight had a mean prechallenge serum anti-LPS titer of 1:162; those receiving 10 ml of the immune pool per kg of body weight had a titer of 1:186.

Preparation and characterization of LPS-absorbed immune serum and affinity-purified anti-LPS antibodies. We next wanted to assess the protective ability of the immune pool absorbed of anti-LPS antibodies and of affinity-purified

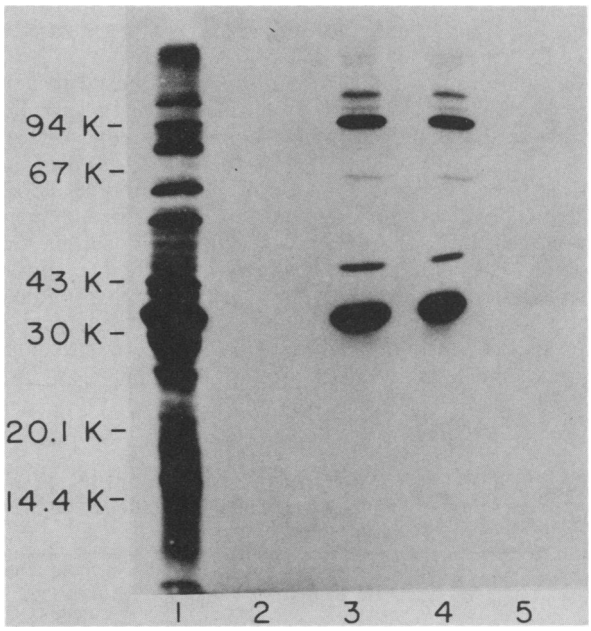

FIG. 2. Whole-cell radioimmunoprecipitation assay with serum samples used in the passive protection experiment comparing immune serum, LPS-absorbed immune serum, and affinity-purified LPS antibodies (Table 2). Iodinated strain 3245 cells were used as the test strain. Lane 1 demonstrates the iodinated proteins present in the cells labeled for this experiment. Proteins immunoprecipitated by the individual serum samples are shown as follows: lane 2, preimmune serum; lane 3, immune serum pool before absorption with LPS; lane 4, immune serum pool after absorption with LPS; lane 5, affinity-purified anti-LPS antibody. Molecular masses (in kilodaltons) are listed to the left of the figure. Molecular weight standards were visualized by Coomassie blue staining before gel autoradiography.

anti-LPS antibodies derived from the pool. The immune serum pool was absorbed of all ELISA-detectable anti-LPS activity by affinity chromatography. Anti-LPS antibodies were then recovered from the affinity column. Approximately 50% of the anti-LPS antibodies present in the original immune serum pool were recovered. The anti-LPS antibodies were concentrated so that the IgG anti-LPS ELISA titer of the affinity-purified antibodies was equivalent to the 1:1,000 titer of the original immune serum.

The whole-cell radioimmunoprecipitation assay was used to characterize the surface-exposed outer membrane proteins recognized by antibodies in the different fractions. The protective immune pool (lane 3) and immune pool absorbed to remove anti-LPS antibodies (lane 4) precipitated several proteins (Fig. 2). The proteins precipitated by the absorbed pool were virtually identical to those precipitated by the unabsorbed immune pool. As demonstrated previously (Fig. 1), the predominant activity appeared to be directed against a 39-kDa protein. However, notable activity was also present against a protein of approximately 50 kDa and another of approximately 100 kDa. The differences observed in the patterns of immunoprecipitation produced by the immune pool in Fig. 1 and 2 were quantitative rather than qualitative. More efficient radiolabeling of the cells used in Fig. 2 permitted the visualization of protein bands only faintly observed in Fig. 1. When affinity-purified anti-LPS antibodies were assayed, very slight activity was noted in the region of the 39-kDa protein (Fig. 2, lane 5). This finding is thought to represent a small amount of coprecipitation of the protein by bound LPS. With *H. influenzae* type b strains, small amounts of LPS remain complexed with a protein of similar molecular weight, and the protein can be precipitated by anti-LPS monoclonal antibodies (9).

An indirect immunofluorescent assay was used to determine whether or not the affinity-purified anti-LPS antibodies recognized antigenic determinants exposed on the surface of strain 3245. Cells incubated with the affinity-purified anti-LPS antibodies demonstrated bright membrane fluorescence, as did cells incubated with the unabsorbed chinchilla immune serum or the LPS-absorbed immune serum. Cells incubated with nonimmune serum demonstrated no activity.

An additional concern was that the LPS preparation used to prepare the affinity column and as the sensitizing antigen in the ELISA assay may not have been representative of all the antigenic LPS moieties present in strain 3245. Thus, residual anti-LPS antibodies might have remained in the LPS-absorbed immune pool which would not have been detectable by ELISA. In an attempt to exclude this possibility, we independently monitored our serum samples for anti-LPS activity with the radioimmunoprecipitation assay with presolubilized ^{32}P-labeled cells. In cells labeled by this technique, LPS incorporates the radioisotope and can be visualized by autoradiography. Both the immune serum pool and the affinity-purified anti-LPS antibody preparation immunoprecipitated radiolabeled LPS from strain 3245 cells. In contrast, there was no detectable LPS precipitated by the LPS-absorbed immune pool (data not shown).

Passive immunization with LPS-absorbed immune serum or affinity-purified anti-LPS antibody. The results of the passive protection experiment performed with the LPS-absorbed serum and affinity-purified anti-LPS antibody are shown in Table 2. All three animals that received preimmune serum developed culture-positive otitis media with courses identical to those of animals that received preimmune serum in the previous experiment. Middle ear fluid collected from these animals remained culture positive for 2 to 3 weeks, and bacterial densities in samples of middle ear fluid were $>10^5$ CFU per ml. As in the previous experiment, none of the four animals that received the immune serum pool developed culture-positive otitis. Additionally, the four animals that received the immune pool absorbed of anti-LPS antibody were protected against culture-positive otitis. Small transient effusions were observed in three of the animals, but cultures of these fluids were uniformly sterile.

All four animals that received the affinity-purified anti-LPS antibody developed culture-positive otitis and had courses indistinguishable from those of the controls. Specifically, middle ear fluid samples remained culture positive for 2 to 3 weeks, and colony counts were uniformly $>10^5$ CFU per ml until just before becoming sterile. When anti-LPS titers in the sera of individual experimental animals were assayed, the geometric mean titer in animals receiving the nonabsorbed immune serum pool was 1:112, and the geometric mean titer in animals receiving the affinity-purified anti-LPS antibody preparation was 1:122.

TABLE 2. Effect of intravenous pretreatment with LPS-absorbed immune serum or affinity-purified anti-LPS antibody on the development of otitis media

Serum or serum fraction[a]	Anti-LPS ELISA titer	No. of animals challenged	No. of animals with culture-positive otitis
Preimmune	<10	3	3[b]
Immune	1,000	4	0[b,c]
Immune LPS-absorbed	<10	4	0[b,c]
Affinity-purified anti-LPS antibody	1,000	4	4[c]

[a] All animals received 5 ml of the indicated serum or serum fraction per kg of body weight.

[b] $P = 0.057$ by the Fisher exact two-tailed test for preimmune serum versus immune serum or preimmune versus immune LPS-absorbed serum.

[c] $P = 0.028$ by the Fisher exact two-tailed test for immune serum versus affinity-purified anti-LPS antibody or LPS-absorbed immune serum versus affinity-purified anti-LPS antibody.

DISCUSSION

In an effort to define more precisely the role of serum antibodies in protection against *H. influenzae* otitis media, we performed a series of passive immunization experiments using the chinchilla model of otitis media. This animal model has been widely used in studies of the pathogenesis of otitis media (6, 7, 15), and the immunologic data collected from the model appear to correlate well with human data (6, 17). Previous work using the chinchilla model suggested that protective immunity developed after experimentally induced *H. influenzae* otitis media and coincided with the appearance of antibodies to the infecting strain in convalescent serum (15). These observations suggested that serum antibodies might be important in protective immunity. Although serum antibodies might simply be markers for the presence of other host responses in the middle ear space, the antibodies themselves may be able to enter the middle ear space and interact with bacterial pathogens during the course of an acute inflammatory response (29). In our study, we were able to demonstrate that passive immunization with an immune serum pool prepared against the *H. influenzae* challenge strain protected against experimental disease, thereby proving the protective role of serum antibodies. Transient culture-negative middle ear effusions developed in

a few animals that received the immune serum, but this finding was not unexpected. Other investigators have demonstrated that inoculation of the middle ear space of chinchillas with nonviable *H. influenzae* leads consistently to the development of sterile middle ear effusions, presumably on the basis of inflammation induced by bacterial products such as LPS (5).

The immune serum pool used in our experiments contained antibodies against both surface-exposed outer membrane proteins and LPS determinants. Antibodies recognizing either of these classes of bacterial surface antigens may be important in activating complement-mediated bactericidal or opsonic activity in vitro and in conferring protection in experimental models (1, 8, 20, 23, 24, 26). In the present investigation, animals that received either the immune serum pool or the immune serum pool absorbed of LPS antibody appeared to be protected equally against development of otitis. In contrast, animals that received affinity-purified anti-LPS antibodies were not protected. They demonstrated a course of infection indistinguishable from that of control animals. Because only 50% of the anti-LPS activity in the chinchilla immune pool was recovered in the affinity-purified antibody fraction, it would be premature to conclude that anti-LPS antibodies serve no protective role. It is conceivable that the anti-LPS antibodies which were not recovered from the column may represent a high-affinity subset of molecules which are potentially protective.

Use of the whole-cell radioimmunoprecipitation assay allowed us to identify specific outer membrane proteins recognized by the serum antibodies in the immune serum pool. Predominant activity appeared to be directed against a 39-kDa outer membrane protein, and lesser activity was directed against a few higher-molecular-mass species (Fig. 2). The surface accessibility of the 39-kDa protein of strain 3245 has also been demonstrated by using the indirect immunofluorescence assay described above. Affinity-purified anti-39-kDa protein antibody prepared from the chinchilla immune pool demonstrated bright membrane fluorescence when tested against strain 3245 (unpublished observations). The 39-kDa protein is one of the major outer membrane proteins of strain 3245 (data not shown) and appears to be analogous to protein P2, a porin protein purified from *H. influenzae* type b (18, 31). In our previous investigations of experimental *H. influenzae* type b disease, antiserum raised against purified protein P2 protected infant rats against bacteremia with the homologous strain (18).

Recent reports suggest that monoclonal antibodies directed against LPS determinants of *H. influenzae* type b may coprecipitate outer membrane protein P2 when assayed by the whole-cell radioimmunoprecipitation assay we used (9). Although we observed a minimal amount of activity in the region of the 39-kDa protein when affinity-purified anti-LPS antibodies were analyzed, the spectrum of proteins immunoprecipitated by the immune serum pool before and after LPS absorption was virtually identical (Fig. 2). Furthermore, when ^{32}P-labeled cells were used in the radioimmunoprecipitation assay, no labeled LPS was immunoprecipitated by the LPS-absorbed immune serum pool, whereas significant activity was present in the nonabsorbed immune pool and in the affinity-purified anti-LPS antibody pool.

Nontypable *H. influenzae* demonstrates substantial strain heterogeneity when examined by serologic and gel electrophoretic techniques (3, 19). Although we demonstrated that serum antibodies protected against infection by the homologous strain, the degree of protection conferred against infection by heterologous organisms is unknown. In this investigation, we did not challenge animals with heterologous organisms in our passive protection experiments. However, Karasic and co-workers reported that protective immunity after active infection may be strain specific (15). Further investigation is needed to identify the specific surface antigens important in eliciting protective antibody and to characterize the immunologic relatedness of these antigens in different nontypable *H. influenzae* isolates. Such studies may provide insight into possible vaccine candidates for prevention of *H. influenzae* otitis media in children.

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