INFECTION AND IMMUNITY, Nov. 1987, p. 2612–2618
0019-9567/87/112612-07$02.00/0

Vol. 55, No. 11

Protection of Infant Rats from *Haemophilus influenzae* Type b Infection by Antiserum to Purified Outer Membrane Protein *a*

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Received 20 April 1987/Accepted 6 August 1987

Protein *a* (46,000 molecular weight [46K]) was purified from outer membranes of *Haemophilus influenzae* type b by a relatively simple procedure. Spontaneously shed outer membranes from a 24-h, 12-liter culture of an unencapsulated variant of strain Eag were combined with outer membranes released from the cells by Tris buffer and extracted with the nonionic detergent octylpolyoxyethylene. The extract was then subjected to open column chromatography on Sephacryl S-200 and Trisacryl-carboxymethyl to yield 7.5 mg of protein *a* from 180 mg of outer membrane protein. Approximately 99% of the protein in this preparation was protein *a*; in addition, the preparation contained 1.25% (wt/wt) lipopolysaccharide and had a residual detergent/protein ratio of 1.6:1 (wt/wt). Antibodies to the preparation were induced in rabbits by using alum as an adjuvant. As determined by immunoblotting, the great preponderance of antibodies induced were specific for protein *a*. However, very low levels of antibodies to several other outer membrane components, which were not apparent on gels of the pure preparation of protein *a*, were also induced. Preimmune and postimmune sera, after depletion of antibodies to capsular polysaccharide and lipopolysaccharide, were tested for biological activity against *H. influenzae* type b. Compared with preimmune serum, postimmune serum was bactericidal in vitro against strain Eag (the only strain tested) and offered significant protection ($P < 0.01$) to infant rats against infection by all four strains tested, two of which had a protein *a* that was larger (47K) than the 46K protein *a* in the preparation. These results indicate that protein *a* should be considered as a vaccine to prevent *H. influenzae* type b disease.

Haemophilus influenzae type b, a human pathogen primarily of infants and young children, causes systemic infections, the most serious being meningitis. Although a vaccine consisting of the type b purified capsular polysaccharide has recently been licensed (12), it is effective in inducing protective levels of antibodies only in children 24 months old and older, a group which accounts for less than 30% of *H. influenzae* type b disease in the United States (9). Because of this delayed maturation of the immune response to the capsule, a more effective vaccine is needed.

Alternatives that appear promising because they induce higher levels of capsular antibodies in very young children (9) are conjugates of carrier protein either to capsular polysaccharide (5, 7) or to oligosaccharide subunits of the capsular material (2). However, it remains to be shown that these semisynthetic antigens are safe in the long term and can consistently induce protective levels of anticapsular antibodies sufficiently early in infancy to protect all of the susceptible population.

Another possible source of vaccine candidates are the outer membrane proteins of *H. influenzae* type b. The outer membrane is typical of gram-negative bacteria and consists of phospholipids, lipopolysaccharide (LPS), and about 24 proteins, 6 of which, the major outer membrane proteins, account for about 80% of the total protein (18). There are currently two nomenclatures for the six major proteins (9, 17): protein *a* is equivalent to P1, protein *b/c* to P2 (a doublet), protein *d/f* to P5 (two conformers of the same protein), protein *e* to P4 and protein *g* to P6.

There are good reasons to consider outer membrane proteins as vaccine candidates. Consistent with the finding that the human antibody response to proteins develops earlier than it does to polysaccharides (27), humans of all ages convalescing from *H. influenzae* disease were found to make antibodies to a spectrum of outer membrane proteins (11, 16). There is considerable cross-reactivity among the analogous proteins from different isolates (16), although data on cross-reactivity of exposed epitopes has been published only for a minor 98,000-molecular-weight (98K) protein (14). The purification of proteins *b/c* (P2) and *d/f* (P5) and the partial purification of *g* (P6) have been described by Munson and colleagues (22, 23). Rabbit antibodies to *b/c* (P2) and to *g* (P6), but not to *d/f* (P5), protected infant rats from infection by *H. influenzae*. It has also been reported without presentation of experimental data that antibodies to *e* (P4) were not protective (9) but that antibodies to *a* (P1) were protective (9). Lastly, a mouse monoclonal antibody to a 98K protein was also protective for infant rats (14).

This laboratory selected protein *a* to study as a possible vaccine candidate because it appears to have limited heterogeneity among *H. influenzae* type b strains (8), it is readily extracted from the outer membranes by nonionic detergents under mild conditions (see below), and it is surface exposed (M. R. Loeb and K. A. Woodin, Infect. Immun., in press). In this communication are described the purification of protein *a* from strain Eag and the ability of rabbit antibodies to this protein to protect infant rats from disease due to *H. influenzae* strains containing either a homologous protein *a* or a heterologous variant of protein *a*.

MATERIALS AND METHODS

Bacteria. The type b strains used were Eag, our standard laboratory strain (3); S2, a capsule-deficient mutant of Eag (21, 31); H113 (3); 1054; and 1079. The last two strains are more recent isolates from the collection of D. Granoff (Washington University) and were obtained with his permission from J. Musser, University of Rochester.

Purification of protein *a*. Strain S2 was grown at 37°C with aeration for 24 h in brain-heart infusion medium (BHI; Difco Laboratories) containing hemin (1 μg/ml) (Sigma Chemical

Co.) and NAD (2 μg/ml) (Sigma) prepared as described previously (18). The cells and medium were concentrated to 400 ml by a Pellicon apparatus (Millipore Corp.) with a filter (PTHK 00005) having a 100K cutoff. The concentrate was chilled, and all subsequent operations were performed in the cold. Cells and supernatant fluid were obtained as separate fractions after centrifugation at 10,000 × *g* for 20 min. Both were used as sources of outer membranes. The cells (85 g, wet weight) were suspended in 300 ml of 0.05 M Tris hydrochloride, pH 7.8, and centrifuged as above to yield a supernatant rich in outer membrane. (Apparently, washing late-stationary-phase cells with Tris causes release of outer membrane.) This supernatant and the medium supernatant were centrifuged at 45,000 rpm (158,000 × *g*) in a 45 Ti rotor (Beckman Instruments, Inc.) for 2 h to pellet the membranes; these preparations were designated TWOM and SNOM, respectively. Each preparation was suspended with a homogenizer in 65 ml of buffer A (0.01 M Tris, 0.1 M NaCl, pH 7.5, 10 μM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 1.5 mM azide) and repelleted as above. The final pellets were suspended in 20 ml of buffer A and frozen at −80°C. The total yields from 85 g (wet weight) of cells were 100 mg of protein in SNOM and 80 mg of protein in TWOM. To isolate protein *a*, the two preparations were defrosted, combined, and adjusted with buffer A to a protein concentration of 2.3 mg/ml. NaCl was added to a final concentration of 0.25 M, and a nonionic detergent, octylpolyoxyethylene (OPOE), was added to a final concentration of 2.3% (vol/vol). This detergent, the very generous gift of J. Rosenbusch (Basel University), is a nonionic polydisperse detergent consisting of an octyl group linked to 3 to 12 oxyethylene units per octyl unit, with a mean of 6 units; it has a critical micelle concentration (6.6 mM) which is sufficiently high to permit its effective removal by dialysis (24). After incubation at 37°C with gentle stirring for 1 h, the mixture was centrifuged at 255,000 × *g* for 3 h. The extract was concentrated to about 20 ml under nitrogen with a stirred cell (Amicon Corp) containing a 62-mm-diameter YM10 filter and then applied to a column of Sephacryl S-200 (69 by 2.5 cm; Pharmacia Fine Chemicals) in buffer A containing 1% OPOE. Fractions (3.1 ml) were collected and analyzed for protein *a* by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein *a*-rich fractions were pooled and dialyzed against 1.7 liters of buffer B (0.02 M MES [potassium-2-(*N*-morpholino)ethanesulfonate], pH 6.0, 1% OPOE, 10 μM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 1.5 mM azide). The dialysate was applied to a column (28.9 by 2.5 cm) of Trisacryl-carboxymethyl (LKB-Produkter), which was eluted first with 180 ml of buffer B and then with a 400 ml gradient of 0 to 0.5 M KCl in buffer B. Fractions of 2 ml each were collected. Protein *a*-rich fractions eluted at approximately 168 to 187 ml after the start of the gradient. These were pooled, concentrated to 5 ml, dialyzed against 2.5 liters of phosphate-buffered saline (PBS; 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.2) for 16 h, and then divided into portions and stored at −80°C. The yield was 7.5 mg of protein.

Immunization of rabbits. Rabbit antibodies to purified protein *a* were induced by two different immunization schedules. In one case, two rabbits (R1 and R2) were each inoculated subcutaneously (s.c.) in several dorsal sites with a mixture of 0.2 ml (300 μg) of protein *a* and 0.44 ml of alum [3 mg of $Al(OH)_3$] (30). A boost of 300 μg of protein *a* in 1 ml of PBS was given s.c. 63 days later, and the rabbits were exsanguinated 7 days later. Two other rabbits (R3 and R4) received the same type of first inoculation as above, then a repeat inoculation of protein *a* in alum at 63 days, and lastly a third inoculation 44 days later of 300 μg of protein *a* in PBS s.c., followed by exsanguination 7 days later. All boosts were given after antibody levels started to decline, as determined by enzyme-linked immunosorbent assay (ELISA) with outer membrane as the antigen.

Depletion of antibodies to capsule and LPS. Antisera were depleted of antibodies to capsule and LPS by affinity chromatography on Sepharose 4B (Pharmacia Fine Chemicals) to which either capsular polysaccharide or LPS was covalently bound (26). Alternatively, depletion was performed with alum to which capsular polysaccharide or LPS had been absorbed as follows. Alum [4 ml; 27 mg of $Al(OH)_3$] was mixed with 0.4 ml (1.7 mg) of capsular polysaccharide or with 0.4 ml (0.4 mg) of LPS in PBS from strain Eag and kept at 0°C for 30 min. The alum absorbents were then washed three times with PBS, combined, and used to absorb 4 ml of antisera, usually at 0°C for 90 min. Serum was freed of absorbent by centrifugation.

The capsular polysaccharide used in the absorptions was generously donated by P. Anderson and was prepared as described elsewhere (1). LPS was prepared from strain S2 by hot phenol-water extraction (32).

Immunoassays. Induction of serum antibodies resulting from immunization with protein *a* was measured by several assays. (i) ELISA, for assay of antibodies to LPS, outer membranes, or protein *a* (13). LPS (10 μg/ml) from strain Eag was assayed on Linbro microtiter plates (Flow Laboratories, Inc.); strain Eag outer membranes (6 μg of protein per ml) (18) and protein *a* (10 μg/ml) were assayed on Immulon 1 plates (Dynatech Laboratories, Inc.). The isotype response by immunoglobulin M (IgM) and IgG was determined with protein *a* as the antigen and was modified to include biotin-labeled goat anti-rabbit secondary antibodies specific for the heavy chains of IgM and IgG (Fisher Scientific) and an enzyme conjugate of avidin-biotin-horseradish peroxidase (Vector Laboratories, Inc.). (ii) Immunoblot for assay of antibodies to individual outer membrane proteins and LPS. Briefly, outer membranes from strain Eag were subjected to SDS-PAGE, and the resolved outer membrane antigens were electroblotted from the gel onto nitrocellulose, which was incubated with sera and then with ^{125}I-staphylococcal protein A and autoradiographed. This procedure has been described in detail (15). However, as used here, the sensitivity was greatly increased by using sera at a dilution of 1:100 rather than 1:10,000. (iii) Radioimmunoassay for detection of antibodies to capsular polysaccharide (1), modified for increased sensitivity to low-avidity antibodies (6). The radioantigen consisted of the high-molecular-weight fraction of ^3H-labeled capsule and was generously donated by P. Anderson. The standard serum, SK, was kindly donated by R. Schneerson of the National Institute of Child Health and Human Development.

Protection assay. The ability of the antiserum to protect against type b disease was tested in the infant rat (20). Timed-pregnant rats (Sprague-Dawley; Charles River Breeding Laboratories) were received 8 days before term and cared for at the University of Rochester vivarium. At 4 days of age the baby rats were collected, randomly redistributed in equal numbers to the mother rats, and inoculated s.c. on the dorsum with 0.1 ml of serum. On day 5 the rats were challenged by inoculation intraperitoneally with 220 to 900 CFU of *H. influenzae* type b (0.1 ml) freshly grown in BHI plus cofactors to 5×10^8 CFU/ml and diluted in PBS containing 0.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$. On day 6, 0.01-ml samples of blood were collected from a tail blood

vessel and plated undiluted and after 1:100 dilution in PBS–0.1% gelatin.

The statistical significance of differences in levels of bacteremia was analyzed by Student's *t* test, based on the geometric mean. Rats that were not bacteremic were assigned a value of 0.5 CFU/10 μl of blood. The chi-squared test with Yates' adjustment for continuity was used to determine the significance of differences in the proportion of bacteremic and nonbacteremic rats.

Bactericidal assay. The bactericidal activity of the antisera to protein *a* against strain Eag was determined as described previously (3); precolostral calf serum was used as the complement source, kindly donated by G. Siber (Harvard University). The concentration of complement used in the assay was the highest concentration that did not kill the bacteria, as determined by a checkerboard assay.

Other procedures. SDS-PAGE (10% gels) (18) and immunoblotting (15) were performed as described. The LPS content of the purified protein was determined by comparing the intensity of measured amounts of protein *a* and LPS on SDS-urea-polyacrylamide gels subjected to silver staining (28). The detergent content was similarly determined by thin-layer chromatography on silica gel-coated plates (Si 250-PA; J. T. Baker Chemical Co.) followed by development with iodine vapors. Protein was assayed by the method of Lowry et al. (19).

RESULTS

Purification of protein *a*. The procedure for obtaining washed outer membranes takes about 10 h. The yield, approximately 180 mg of outer membrane protein from 12 liters of late-stationary-phase cells, is about the same as that which can be obtained from log-phase cells by using a longer procedure involving breakage of the bacteria in a French press and subsequent isolation of outer membranes by equilibrium sucrose density gradient centrifugation (18). Although this latter procedure does yield a preparation that would be more ideal as the starting material because of its simpler protein composition (Fig. 1; compare lanes 1 and 2 with lanes 3 to 8), this method would be unwieldy for large-scale purification as used here.

Protein *a* is readily apparent in Fig. 1 in spite of the presence of other proteins of similar mobility because of its property of heat modifiability, i.e., if the sample is not heated prior to SDS-PAGE, the mobility of protein *a* (and protein *d*/*f*) is faster than if the sample is heated at 100°C (17).

Extraction of combined SNOM and TWOM with the detergent OPOE in 0.25 M NaCl is selective, i.e., extraction is highly efficient for proteins *a* and *e* as well as some other proteins found in stationary-phase but not log-phase outer membranes and partially effective for protein *d*/*f*, but almost completely ineffective for proteins *n*, *k*, and *b*/*c* (Fig. 1, lanes 8 to 13; the OPOE caused distortion of the gel bands in lanes 8 to 10). This is in contrast to a different nonionic detergent, octyl β-glucoside, which in addition extracts proteins *n* and *b*/*c* (data not shown). Ion-exchange chromatography of the protein *a*-rich fractions obtained by gel filtration on Sephacryl S-200 (Fig. 2, lanes 1 and 2) yields pure protein *a* as determined by Coomassie blue staining of SDS-polyacrylamide gels (Fig. 2, lanes 5 to 8). Silver staining of the same amount of protein showed very low levels of two protein contaminants and about 0.01 mg of LPS per mg of protein *a* (data not shown). Most of the LPS was removed during gel filtration (it eluted just after the protein *a*-rich fractions, and almost all the remainder was in the unbound fractions of ion-exchange chromatography). The OPOE content was about 0.25% (vol/vol) or 1.6:1 (wt/wt) (data not shown).

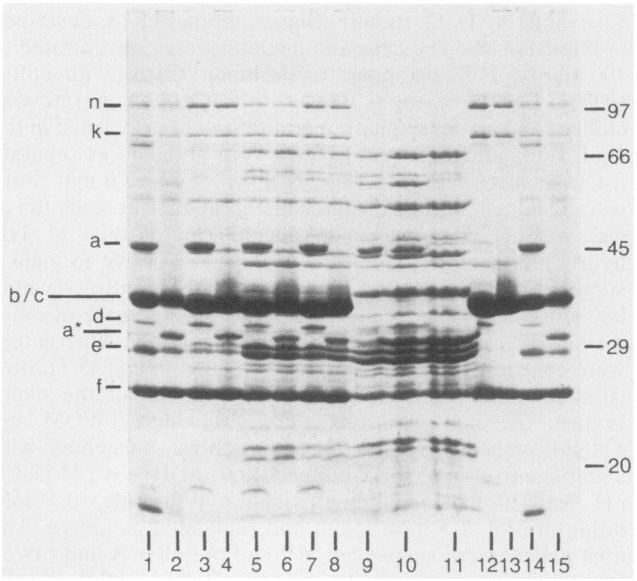

FIG. 1. SDS-PAGE profiles of outer membrane preparations and detergent extracts. Outer membranes of *H. influenzae* type b, strain S2, before and after extraction with OPOE were subjected to SDS-PAGE. Approximately 20 μg of protein was applied to each well. Samples in sample buffer were either unheated (lanes 2, 4, 6, 8, 11, 13, and 15) or heated (lanes 1, 3, 5, 7, 9, 10, 12, and 14) prior to SDS-PAGE. Coomassie-blue stained gel. Lanes: 1, 2, 14, and 15, log-phase outer membranes obtained from a sucrose density gradient (18); 3 and 4, SNOM; 5 and 6, TWOM; 7 and 8, combined SNOM and TWOM; 9, OPOE extract of combined SNOM and TWOM; 10 and 11, concentrate of OPOE extract; 12 and 13, material not extracted by OPOE. The letters on the left indicate outer membrane proteins; a* is the unheated form of protein *a*. The numbers on the right indicate the positions of molecular weight markers (10^3).

Antibody levels in preimmune and postimmune sera. Induction of rabbit antibodies as a result of immunization with protein *a* was measured by several assays, as noted above. The ELISA with outer membranes as the antigen was used primarily to follow development of antibody at several time points after inoculation and showed at least a 20-fold increase in titer, with no differences between the two inoculation schedules. Thus, the use of alum in the second boost did not yield increased antibody even though protein *a* binds tightly to the alum (data not shown). With protein *a* as the antigen, similar results were obtained, with the bulk of the increase being due to IgG rather than IgM (data not shown).

The ELISA with LPS as the antigen and the radioimmunoassay with capsule antigen were used primarily to measure depletion of antibodies as a result of absorption with LPS and capsule either bound to alum or covalently coupled to Sepharose. Typical results appear in Table 1. Anticapsular antibodies were depleted to levels just barely detectable by this assay. It should be noted that the assay as used in these experiments is especially sensitive for low-avidity antibodies to capsule. Rats injected with 0.1 ml of these adsorbed sera would receive 4 to 6 ng of anticapsular antibodies. After subsequent dilution in the blood of the animals, this would result in circulating anticapsular antibody levels much below the reported protective levels of about 30 to 50 ng/ml (20, 25).

Although the titer of anticapsular antibodies did not rise as

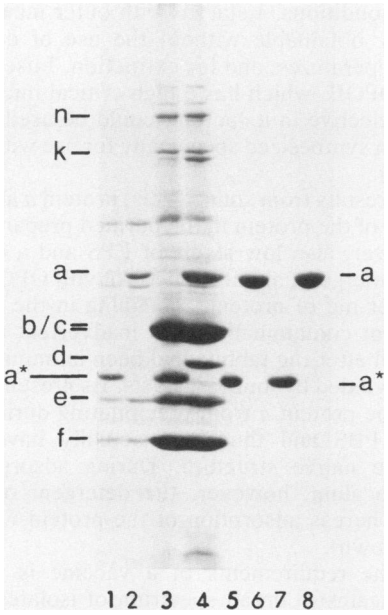

FIG. 2. Purification of protein *a*. Coomassie-blue stained gel. Lanes: 1 and 2, protein *a*-rich fractions from gel filtration; 3 and 4, 25 μg of outer membranes from log-phase cells of strain Eag; 5 and 6, dialyzed protein *a*-rich fractions from ion-exchange chromatography; 7 and 8, 5 μg of concentrated protein *a* fractions. Samples in lanes 1, 3, 5, and 7 were unheated; samples in lanes 2, 4, 6, and 8 were heated. See Fig. 1 legend for details.

a result of inoculation with protein *a*, low levels of anti-LPS antibodies were induced, as would be expected from the slight contamination of protein *a* with LPS (Table 1). Adsorption depleted these to near baseline levels, a result that was also obtained when more concentrated sera were used in the assay (data not shown). Data are not available on protective levels of LPS antibodies for the infant rat. In one study, high levels of polyclonal rabbit antibodies were not protective (26); in another study, a mouse monoclonal antibody specific for LPS was protective (10).

TABLE 1. Assay of antibodies to capsule and LPS in unabsorbed and absorbed[a] preimmune and postimmune sera

Rabbit no. and serum	Anticapsule antibodies (ng/ml)		Anti-LPS antibodies (OD_{400})	
	Preimmune	Post-immune	Preimmune	Post-immune
R1				
Unabsorbed	>1,440	>1,440	0.004	0.674
Absorbed	56	40	0.023	0.073
R2				
Unabsorbed	320	230	0.025	0.111
Absorbed	61	50	0.005	0.042
R3				
Unabsorbed	330	205	0.065	0.125
Absorbed	58	40	0.073	0.073
R4				
Unabsorbed	320	380	0.048	0.303
Absorbed	54	50	0.031	0.047

[a] Sera were absorbed by the alum procedure for 90 min at 0°C. Sera assayed for anticapsule antibodies were undiluted; sera assayed for anti-LPS antibodies were diluted 1:1,000.

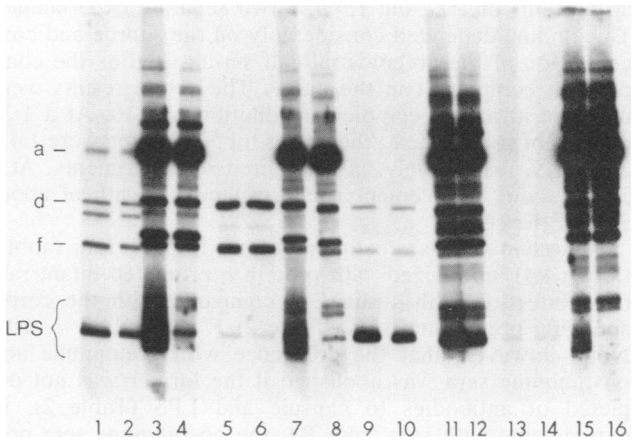

FIG. 3. Immunoblot of rabbit sera. Outer membranes of log-phase strain Eag were subjected to SDS-PAGE and immunoblotted onto nitrocellulose. The nitrocellulose was cut into strips (20 μg of outer membrane protein per strip), incubated with different sera (1:100 dilution) and then with 0.2 μCi of ^{125}I-protein A per strip, and autoradiographed, as described (15). Exposure with an intensifying screen was for 17 h. Lanes: 1 and 2, preimmune sera, R1; 3 and 4, postimmune sera, R1; 5 and 6, preimmune sera, R2; 7 and 8, postimmune sera, R2; 9 and 10, preimmune sera, R3; 11 and 12, postimmune sera, R3; 13 and 14, preimmune sera, R4; 15 and 16, postimmune sera, R4. Sera in even-numbered lanes had been depleted of antibodies to LPS and capsular polysaccharide.

The immunoblot assay was used primarily to follow the specificity of the induced antibodies. The results shown in Fig. 3 are for preimmune and postimmune sera before and after absorption with the alum-LPS-capsular absorbent. The data show that the preponderant antibody specificity in all four postimmune sera was to protein *a*. On less-exposed films, with which comparisons can more accurately be made, the levels in all four sera were about equal in spite of differences in the inoculating regimen. Preimmune serum contained various low amounts of antibodies to different proteins. R1, for example, had activity for proteins *a*, *d/f*, and others, with levels of antibodies to *d/f* increasing in post-immune serum. R2 also had antibodies to *d/f*, but these did not increase. Pre-immune R4 serum almost completely lacked antibodies. For this reason R4 serum was the most frequently used serum in the protection assays described below. Although antibodies to protein *a* predominated in postimmune sera, antibodies to other outer membrane components were also present, albeit at very low levels. A component just above protein *f* and a doublet near the bottom of the strips were present in all four sera; other components varied in their appearance. The identity of these antigens is unknown; they represent components that were not visible on stained gels of purified protein *a*. They may be very minor contaminants of the protein *a* preparation or dimers or breakdown products of protein *a*. Absorption with the alum reagent removed antibodies to LPS, in agreement with the ELISA results.

In addition in other immunoblot assays with sera obtained at different time points in the immunization schedule, it was apparent that this assay and the ELISA were in qualitative agreement, demonstrating the usefulness of the ELISA for following antibody development.

Bactericidal activity of antibodies to protein *a*. Absorbed preimmune serum from R4 did not kill strain Eag in vitro, whereas absorbed postimmune serum resulted in 50% killing

at dilutions of 1:17 and 1:9.6 in two separate experiments. This finding depended considerably on the source and concentration of the precolostral calf serum used as the complement component in the assay. The above results were obtained at a final complement dilution of 1:10. At a 1:20 dilution of complement, the values for 50% killing were 1:4.0 and 1:2.9, respectively, in the same two experiments. At a 1:5 dilution, complement itself resulted in death of about 25% of the cells.

Protection studies. Postimmune sera from all four rabbits (R1 to R4) immunized with protein *a* protected infant rats from infection with strain Eag compared with the corresponding preimmune sera (Table 2; $P < 0.001$ by *t* test). Note, however, that the difference with preimmune and postimmune sera was abolished if the former was not depleted of antibodies to capsule and LPS (Table 2). In experiments with sera from R4, the postimmune sera protected against *H. influenzae* strains having a protein *a* with the same mobility (strain 1079) as protein *a* of Eag or a slightly decreased mobility (strains H113 and 1054) (Table 2). Although in all experiments the differences in the degree of bacteremia were significant, in a few experiments the differences in the proportion of bacteremic rats were also significant (Table 2). Results were essentially the same whether the sera had been absorbed with the alum or Sepharose absorbent. In summary, sera highly enriched in antibodies to protein *a* from strain Eag were protective against strains with a homologous or heterologous protein *a*.

DISCUSSION

The goal of this research was to determine whether protein *a* of *H. influenzae* type b can induce protective antibodies. Although the results are not completely unambiguous (see below), they do suggest that protein *a* merits consideration as a vaccine candidate.

The challenge in purifying outer membrane proteins for use in a vaccine is to obtain a product that retains enough native structure so that the induced antibodies can be effective. The challenge is particularly difficult because there are no convenient assays for retention of native structure throughout the purification process. Therefore, to avoid denaturing conditions, I started with outer membranes that were readily obtainable without the use of detergents or elevated temperatures, and for extraction, I used a nonionic detergent, OPOE, which has a high critical micelle concentration, is selective in its action, could be used in the cold, and had been synthesized specifically for use with membrane proteins (24).

Based on results from stained gels, protein *a* accounted for at least 99% of the protein in the purified preparation. There were, however, also low levels of LPS and a considerable amount of detergent, about 0.25% (vol/vol) OPOE or 1.6 mg of OPOE per mg of protein, remaining in the preparation. The detergent contamination was inadvertent and was not assayed until after the rabbits had been immunized; it could have been avoided by longer dialysis. Its presence may have prevented the protein *a* from precipitating during the dialysis against PBS and thus may actually have helped to preserve the native structure. During adsorption of the protein onto alum, however, the detergent remained unabsorbed, whereas adsorption of the protein was complete (data not shown).

One of the requirements of a vaccine is that it offer protection against a broad spectrum of isolates. Protein *a*, from its mobility on SDS-PAGE gels, is not identical in all isolates of *H. influenzae* type b. According to the outer membrane protein subtyping scheme of Granoff et al., there are three analogs, designated H, L, and U, with the last accounting for only a very small number of strains (8). On our gels the H form is 47K and the L form is 46K. Although strains S2 and Eag, from which protein *a* was purified, have the L form, antibodies to this protein protected infant rats from strains containing either the L form (Eag and 1079) or the H form (H113 and 1054). In protection studies in other laboratories with antibodies to different outer membrane proteins (98K and P2), protection did not extend to strains having an analogous protein of different molecular weight (14, 23), because either the animal response was not to cross-reactive surface epitopes, cross-reactive surface epitopes did not survive the purification, or cross-reactive surface epitopes do not exist for these proteins. The full extent of protection afforded by antibodies to protein *a* requires further investigation.

From other data obtained in this laboratory, the protection

TABLE 2. Ability of rabbit antiserum to protein *a* to protect infant rats from challenge by homologous and heterologous *H. influenzae* type b

Expt no.	Serum (day 4)		Challenge (day 5)		Bacteremia (CFU/10 μl of blood, day 6)[b]		
	Rabbit	Absorbed[a]	Strain	CFU	Preimmune serum	Postimmune serum	*P*
1	R1	+	Eag	860	570 (6/6)	15 (6/6)	<0.001
	R2	+	Eag	860	350 (6/6)	16 (6/6)	<0.001
	R3	+	Eag	860	1,210 (6/6) [1]	5 (5/6)	<0.001
	R4	+	Eag	860	1,350 (6/6)	2 (3/6)	<0.001
2	R1	−	Eag	900	3 (4/6)	0.6 (1/6)	NS[c]
	R1	+	Eag	900	874 (6/6)	21 (5/6)	<0.01
3	R4	+	Eag	330	2,990 (7/7) [1]	0.7 (1/7)	<0.005[d]
	R4	+	H113	230	880 (7/7) [1]	0 (0/7)	<0.005[d]
	R4	+	1079	220	416 (7/7)	0.8 (1/7)	<0.005[d]
4	R4	+	Eag	280	46,400 (5/5) [2]	0.8 (1/5)	<0.001
	R4	+	1054	300	930 (5/5) [1]	0 (0/5)	<0.025[d]

[a] Absorption of serum was performed as follows. Expt. 1, alum-capsule-LPS, 0°C for 90 min; expt. 2, alum-capsule-LPS, room temperature for 30 min and then 0°C for 90 min; expt. 3 and 4, Sepharose-capsule-LPS.

[b] Numbers in parentheses indicate no. of bacteremic rats/no. tested. Numbers in brackets indicate the number of rats dead by 72 h after challenge.

[c] NS, Not significant.

[d] Chi-squared analysis for significant difference between no. bacteremic and no. not bacteremic; all other analyses by *t* test for significance of extent of bacteremia.

of strains with a 47K protein *a* was unexpected. With the immunoblot assay (15) I found that only strains with the L-form of protein *a* absorbed antibodies to protein *a* from rabbit antiserum to strain Eag (Loeb and Woodin, in press). Similarly, whereas strain Eag absorbed antibodies from the antiserum to purified protein *a*, strain H113 did not. However, since only positive results are meaningful in the immunoblot method, these results do not imply a conflict. A model that explains the data is that protein *a* has at least two surface epitopes, designated h and l. The h epitope induces protective antibodies and is present in both H and L strains but does not survive the immunoblotting procedure, possibly because it is a conformational rather than a linear epitope; the l epitope is present only on L strains and does survive immunoblotting.

In addition to protecting infant rats from disease, antibodies to protein *a* were bactericidal in vitro when tested against strain Eag. This is in contrast to results obtained in another laboratory, where only unencapsulated strains were killed (29). These differences may be due to variations in strains, in the specificity of the antibodies, or in the complement source. In this system, the complement source and concentration were critical.

The results described above indicate the need for future experiments. The absolute amount of antibodies to protein *a* in the protective sera, the isotype distribution of protective antibodies, and whether humans would respond in the same manner as rabbits have yet to be determined. In addition, although antibodies to protein *a* predominate in the postimmune sera, as assayed by immunoblots (Fig. 3), antibodies to other membrane components, albeit at very much lower titers, are also present in these sera. These were manifested by performing the immunoblot assay under conditions of high sensitivity. The specificity and origin of these antibodies on the immunoblots are unknown; they may be specific for protein *a* but are reacting with multimers of protein *a* or with degradation products of this protein; they may be specific for protein impurities present in the protein *a* inoculum; or they may represent antibodies that were induced nonspecifically during the immune response to protein *a*, either by B cells that were in the neighborhood of anti-protein *a*-specific B cells during T cell activation or possibly because protein *a*, like some other outer membrane proteins, is a mitogen (4). Until further experiments are performed, their role in protection will not be clear. It may be that these small background levels of heterologous antibodies to outer membrane proteins are an unavoidable consequence of experiments done with polyclonal sera.

In conclusion, I have succeeded in purifying protein *a* from one strain of *H. influenzae* type b. Rabbit antibodies to this protein were bactericidal for the encapsulated parental strain, and they protected infant rats from infection by heterologous as well as homologous strains. This is highly encouraging for consideration of protein *a* as a vaccine candidate.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI-14411 from the National Institute of Allergy and Infectious Disease.

I thank Jurg Rosenbusch for a constant supply of detergent and for advice on purifying outer membrane proteins, Mano Kuratana for performing the bactericidal assays, and Porter Anderson for his encouragement, his help with immunological assays, and a critical review of the manuscript.

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