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Conservation of Immune Responses to Proteins Isolated by Preparative Polyacrylamide Gel Electrophoresis from the Outer Membrane of Nontypeable *Haemophilus influenzae*

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Outer membrane proteins P2, P4, and P6 and two with molecular masses of 26 and 28 kDa have been purified from a strain of nontypeable *Haemophilus influenzae* by a preparative form of polyacrylamide gel electrophoresis (PAGE). Outer membrane protein P6, with a molecular mass of 16 kDa (determined by sodium dodecyl sulfate [SDS]-PAGE) was purified by both native PAGE and SDS-PAGE from three strains of nontypeable *H. influenzae* and one strain of type b *H. influenzae*. The same conditions were required for purification from each strain. The suitability of proteins isolated by these methods was assessed by studying the immune response of rats immunized with P6 in incomplete Freund's adjuvant into the Peyer's patches. P6 purified by either native PAGE or SDS-PAGE did not differ significantly from P6 purified by gel filtration and anion-exchange chromatography in the ability to enhance pulmonary clearance of live bacteria. This study also investigated the effects of SDS on P2 immunological responses in vivo and the effects of the reagents Zwittergent and sodium lauryl sarcosinate on outer membrane protein lymphocyte-proliferative responses in vitro. It was found that the presence of SDS in the immunization emulsion enhanced the antigen-specific cell-mediated response but suppressed the antigen-specific antibody responses. The presence of residual traces of Zwittergent in an outer membrane protein preparation inhibited antigen-specific cell-mediated proliferation, whereas extraction of outer membrane proteins with sodium lauryl sarcosinate did not inhibit antigen-specific proliferation. These results demonstrate that preparative PAGE is a suitable method for the purification of proteins from the outer membrane of *H. influenzae* required for investigation of their immunological significance as vaccine candidates and that traces of reagents used during protein purification may play an important role in determining the success of in vivo and in vitro studies.

Nontypeable *Haemophilus influenzae* (NTHI) organisms are unencapsulated gram-negative bacteria that are a common cause of respiratory tract infections, including pneumonia and sinusitis, as well as other invasive and noninvasive bacterial infections (13). The degree of strain variation demonstrated by the diversity in outer membrane protein (OMP) patterns for these bacteria assist their success in avoiding host defense mechanisms, particularly in patients with damaged airways or that are immunocompromised, despite their common existence as part of the nasopharyngeal microflora (3, 10, 17). The incidence of invasive disease due to *H. influenzae* in adults has been found to be almost equally proportioned between nontypeable strains and the encapsulated type b strains, with the rate of ampicillin resistance in *H. influenzae* isolates appearing to be on the increase (6). The design of effective vaccines against NTHI would significantly reduce the incidence of disease associated with this pathogen and alleviate the pressure for therapeutic drugs to treat antibiotic-resistant infections.

The protein composition of the outer membrane of NTHI is typical of gram-negative bacteria, that is, approximately 20 proteins of which 4 to 6 account for most of the protein content and the remainder exist as relatively minor proteins (11). Several OMPs have been studied to ascertain their suitability as components of a vaccine against NTHI; however, the OMP compositions in nontypeable strains show greater strain-to-strain variability than those in type b strains (1, 2, 10). The isolation of appropriate quantities of proteins from the outer membrane of NTHI that are conserved between strains is essential if the proteins are to be considered as vaccine candidates. One such protein is P6, a 16-kDa lipoprotein that contains an epitope that is a common determinant among all strains of *H. influenzae* (15) and that induces antibodies that have been shown to be bactericidal in vitro (16).

Purification of sufficient quantities of OMPs from *H. influenzae* can be difficult because of the number of lipoproteins in the outer membrane resulting in poor resolution with more traditional methods of column chromatography. To undertake immunization studies involving P6 and other OMPs from more than one strain of *H. influenzae*, a suitable method of purification was developed; this method involved the use of preparative electrophoresis that facilitated efficient production of suitable quantities of protein but was also reproducible between strains. This study has demonstrated that the proteins purified by both native electrophoresis and sodium dodecyl sulfate (SDS) preparative polyacrylamide electrophoresis methods initiated the same immune responses in immunized animals. The ability to use preparative SDS-polyacrylamide electrophoresis enabled more efficient purification of more than one protein from each OMP extraction.

The influence of some detergents used in protein purification on both in vivo and in vitro immune responses has also been investigated. The inclusion of SDS in the emulsion used to immunize rats with a purified protein via intestinal Peyer's patches differentially affected antigen-specific cell-mediated and antibody responses. In vitro cell culture assays to measure

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antigen-specific T-cell responses were affected by traces of Zwittergent used to extract OMPs but were not affected by traces of sodium lauryl sarcosinate. These findings demonstrate the significance of the capacity of reagents involved in protein purification to influence the immune responses.

MATERIALS AND METHODS

Bacterial strains. Four *H. influenzae* strains were isolated from sputa of patients with chronic bronchitis and characterized. These were two nontypeable strains of biotypes I and II (NTHI-I and NTHI-II), a biotype I capsule-deficient strain (type b capsule lost through in vitro passage; HI-CD), and a biotype II type b encapsulated strain (Hib-II).

OMP extraction. All strains were grown overnight at 37°C in 5% CO_2 on 100 plates of brain heart infusion agar supplemented with defibrinated horse blood (Hunter AntiSera, Callaghan, New South Wales [NSW], Australia) at a concentration of 50 ml/liter of agar. The bacteria were harvested by scraping the plates and washed twice by centrifugation at 10,000 × *g* for 10 min at 4°C. The crude outer membrane preparation was obtained by the method of Murphy and Bartos (14). Briefly, this involved resuspension of the washed pellet in 20 ml of 1 M sodium acetate–0.01 M β-mercaptoethanol, pH 4, which was then stirred at room temperature for 45 min before 80 ml of 5% (wt/vol) Zwittergent 3-14 (Calbiochem, Alexandria, NSW, Australia) in 0.5 M calcium chloride was added and the mixture was stirred for a further 90 min at room temperature. Ethanol was added to a final concentration of 20% (vol/vol), and the suspension was left overnight at 4°C before centrifugation at 17,000 × *g* for 10 min at 4°C. The supernatant was collected, and the ethanol concentration was adjusted to 80% (vol/vol). This suspension was left overnight at 4°C before centrifugation at 17,000 × *g* for 20 min at 4°C. The pellet was resuspended in a buffer containing 0.05% (wt/vol) Zwittergent 3-14, 0.05 M Tris, and 0.01 M EDTA, pH 8, and stirred at room temperature for 1 h before centrifugation at 12,000 × *g* for 10 min at 4°C. The supernatant was then dialyzed overnight against distilled H_2O at 4°C, frozen to −70°C, and then lyophilized. This method produced an OMP extract containing approximately 35 to 40 mg of protein.

Electrophoresis sample preparation. The dried bacterial extract was resuspended in a minimal amount of distilled H_2O (0.5 to 1 ml) and further dissolved in four times the volume of either SDS reducing buffer (62.5 mM Tris [pH 6.8], 10% [vol/vol] glycerol, 2% [wt/vol] SDS, 5% [vol/vol] β-mercaptoethanol, 1.2×10^{-3}% [wt/vol] bromophenol blue) or the same buffer without SDS for the native separation. The SDS preparation was incubated at 37°C for at least 30 min prior to being loaded onto the stacking gel. Tris and SDS were electrophoresis-grade reagents purchased from Bio-Rad Laboratories, Nth Ryde, NSW, Australia, and the others were laboratory-grade reagents.

Preparative SDS electrophoresis. An initial separation of the components of the outer membrane preparation was performed with an 80-ml 12% T-1.07% C acrylamide-BIS (*N,N'*-methylenebisacrylamide) separating gel prepared from dilution of a stock solution of 30% T-2.67% C acrylamide-BIS with 0.375 M Tris, pH 8.8, and polymerized with 0.025% (wt/vol) ammonium persulfate and 0.025% (vol/vol) *N,N,N',N'*-tetramethylethylenediamine (TEMED). The separating gel was degassed and prepared in a 37-mm (internal diameter [i.d.]) tube, overlaid with *tert*-amyl alcohol, and allowed to polymerize for 3 to 4 h according to the Bio-Rad model 491 Prep Cell instruction manual. The *tert*-amyl alcohol was removed, and the procedure was repeated with a 15-ml 4% T-0.36% C acrylamide-BIS stacking gel prepared by dilution of the stock monomer in 0.125 M Tris, pH 6.8, and polymerized with 0.05% (wt/vol) ammonium persulfate and 0.1% (vol/vol) TEMED. After 2 h the *tert*-amyl alcohol was removed from the stacking gel and replaced with distilled water, and the gel was allowed to fully polymerize overnight. All columns were prepared and polymerized in this way unless otherwise stated. The Prep Cell was set up for electrophoresis by using an electrode buffer containing 25 mM Tris, 0.2 M glycine, and 1% (wt/vol) SDS (pH 8.3) in the cathode and anode reservoirs and an elution buffer of 25 mM Tris (pH 7.5) (all buffer reagents were electrophoresis grade and were from Bio-Rad Laboratories), and after application of the sample to the top of the stacking gel, was run at maximum settings of 10 W (power) and 50 mA (current). Elution of fractions commenced when the bromophenol blue indicator band reached the base of the separating gel. Fractions with 6-ml volumes were collected with a peristaltic pump set at a flow rate of 1 ml/min and were monitored for protein content with a UV detector set at A_{280}. Fractions were analyzed for protein contents as described below.

Fractions containing P6 contaminated with lipooligosaccharide (LOS) and other proteins were pooled, lyophilized, and reconstituted in 1 to 2 ml of sample buffer as described above. The reconstituted material was loaded onto a 20-ml 16% T-1.42% C acrylamide-BIS separating gel with a 10-ml 4% T-0.36% C acrylamide-BIS stacking gel polymerized in a 28-mm (i.d.) column. Electrophoresis of the sample was performed with the same electrode and elution buffers as before and done at maximum settings of 10 W and 40 mA. Four-milliliter fractions were collected at a flow rate of 1 ml/min. These conditions allowed separation of P6 from the LOS and other contaminants.

Fractions that contained no evidence of contamination with other proteins were kept apart. For further purification of proteins ranging in molecular mass between 26 and 40 kDa, the proteins were pooled, lyophilized, and reconstituted in 2 to 3 ml of sample buffer as described above. The reconstituted material was loaded onto a 60-ml 14% T-1.25% C acrylamide-BIS separating gel with a 10-ml 4% T-0.36% C acrylamide-BIS stacking gel polymerized in a 37-mm (i.d.) column and run at maximum settings of 10 W and 45 mA by using the same electrode and elution buffers as described above. Four-milliliter fractions were collected at a flow rate of 1 ml/min. These conditions allowed the successful separation of proteins of approximately 26, 28, 30 (P4), and 39 (P2) kDa.

Proteins isolated under these conditions contained SDS, which was subsequently removed by a method described by Suzuki and Terada (22). Essentially, pooled fractions were concentrated by either lyophilization or centrifugation with Centristart tubes (Sartorius Laboratories, Chadstone, Victoria, Australia), potassium phosphate was added to a concentration of 20 mM, and the mixture was left on ice for 60 min. This precipitated the dodecyl sulfate, which was removed by centrifugation for 20 min at 10,000 rpm and 4°C with a Beckman Microfuge 12 centrifuge. The samples were desalted either by dialysis against distilled water or with a PD-10 desalting column containing Sephadex G-25M (Pharmacia, Nth Ryde, NSW, Australia). Aliquots of the purified proteins were stored at concentrations between 2 and 5 μg/μl in 10% (vol/vol) glycerol at −70°C. The presence of LOS was assessed by both silver staining of SDS-polyacrylamide gel electrophoresis (PAGE) gels and assaying with the E-TOXATE *Limulus* lysate test (Sigma, Castle Hill, NSW, Australia).

Preparative native electrophoresis. Native separation of P6 from the outer membrane extract was performed with a 30-ml 16% T-1.42% C acrylamide-BIS separating gel in a 28-mm

(i.d.) column prepared by dilution of a stock solution of 30% T-2.67% C acrylamide-BIS with 0.125 M Tris (pH 6.8) and polymerized with 0.025% (wt/vol) ammonium persulfate and 0.025% (vol/vol) TEMED, with a 5-ml 4% T-0.36% C acrylamide-BIS stacking gel prepared by the method used for SDS electrophoresis columns. The chambers were set up for electrophoresis by using 50 mM Tris–0.4 M glycine buffer (pH 8.3) in the cathode chamber (upper), 25 mM Tris–0.14 M glycine (pH 6.8) in the anode chamber (lower), and an elution buffer of 50 mM Tris (pH 7.5). After the sample was loaded on the top of the stacking gel, electrophoresis was carried out at maximum settings of 10 W and 40 mA for the first 3 h and then the current setting was increased to 45 mA. Fractions were collected in 4-ml volumes at a flow rate of 1 ml/min. Those fractions containing P6 were pooled and dialyzed prior to determination of protein concentration. Aliquots of the purified proteins were stored at concentrations between 2 and 5 μg/μl in 10% (vol/vol) glycerol at −70°C.

Fraction analysis. Fractions were concentrated to a volume of 200 μl by either centrifugation with Sartorius Centristart tubes (membrane exclusion limit, 10 kDa) or freezing to −70°C before lyophilization. Initially every fifth fraction was analyzed for protein composition by SDS-PAGE essentially as described by Laemmli (9). A 10-μl fraction sample was added to an equal volume of sample buffer containing SDS and β-mercaptoethanol and the mixture was boiled for 5 min. Electrophoresis was performed with a gel gradient of 10 to 15% by using the Pharmacia PhastSystem followed by silver staining with the PhastSystem staining unit. Low-molecular-mass standards (Pharmacia) were run on the same gels for determination of the molecular masses of the proteins.

Immunoblot. Proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose (0.2-μm pore size; Bio-Rad Laboratories) by a modification of the method of Towbin et al. (23) at a constant current of 0.8 mA/cm^2 for 55 min in buffer containing 25 mM Tris and 192 mM glycine, pH 8.8. The nitrocellulose was then soaked in TBS (20 mM Tris, 500 mM NaCl, pH 7.5) for 10 min prior to blocking in TBS containing 5% (wt/vol) skim milk and with gentle agitation for 30 min. The membrane was washed twice by 5 min (each time) of gentle agitation in TTBS (20 mM Tris, 500 mM NaCl, 0.05% Tween, pH 7.5). Rat serum was diluted 10-fold in TTBS–5% (wt/vol) skim milk powder, and the membrane was exposed to this for 90 min with gentle agitation. After two washings for 5 min each time with TTBS, the membrane was exposed to a 500-fold dilution of horseradish peroxidase-conjugated goat anti-rat immunoglobulin G (IgG) (Fc specific; Nordic Immunology, Tilberg, Netherlands) in TTBS–5% skim milk for 90 min with gentle agitation. The membrane was washed twice for 5 min each time in TTBS and were then subjected to a third 5-min wash in TBS, and the blots were developed in a solution of TBS containing 0.05% (wt/vol) 4-chloro-1-naphthol (Bio-Rad Laboratories), 16.7% (vol/vol) methanol, and 0.015% (vol/vol) H_2O_2. The reaction was stopped by washing three times in distilled water.

Protein concentration determination. The concentrations of protein were determined with the Pierce Micro BCA protein assay reagent and the Pierce albumin standard (Laboratory Supplies, Marrickville, NSW, Australia).

Immunization. Specific-pathogen-free DA male rats aged between 8 and 10 weeks were used. The procedure for immunization and bacterial challenge was performed by a modification of a previously established method (24). The animals were sedated with halothane to facilitate intravenous anesthetization via the tail vein with 3.6% (wt/vol) chloral hydrate in phosphate-buffered saline (PBS; Cytosystems Pty. Ltd., Castle Hill, NSW, Australia) at a dose of 0.25 g of chloral hydrate per kg of body weight. The small intestine was exposed through a mid-line abdominal incision, and the antigen was injected subserosal to each Peyer's patch with a 27-gauge needle. The immunization protein was prepared by emulsification of 200 μg of protein (P2 or P6) per ml in a 1:1 ratio of incomplete Freund's adjuvant and PBS, and a total inoculum of 10 μg of protein was administered to each animal. Nonimmune animals were sham immunized with an emulsion of PBS and incomplete Freund's adjuvant. The animals were sutured, allowed to recover, and kept under specific-pathogen-free conditions until pulmonary challenge with live bacteria 14 days postimmunization.

Bacterial challenge. Bacteria were prepared by overnight culture at 37°C in 5% CO_2 on brain heart infusion agar, scraped from the plates, and resuspended in PBS. The concentration of inoculum was estimated by optical density, read at 405 nm, and was confirmed by counting CFU of the overnight plating of serial dilutions of the inoculum.

On day 14 postimmunization the animals were sedated with halothane and a bolus inoculum of 5×10^8 CFU of live NTHI of the homologous strain in 50 μl of PBS was introduced into the lungs via an intratracheal cannula and dispersed with two 10-ml volumes of air. Animals were killed by an overdose of pentobarbital sodium (Nembutal) administered by intraperitoneal injection 4 h after lung inoculation. Bacterial clearance was assessed by CFU counts, which were estimated by plating of serial dilutions of the washings recovered from lung lavages with five 2-ml volumes of PBS through the trachea, which had been exposed through an incision in the neck. Serum was also collected from blood obtained by heart puncture following the pentobarbital sodium injection.

Antigen-specific ELISAs. Polysorb microtiter wells (Nunc, Roskilde, Denmark) were coated with 0.1 μg of purified P2 in 100 μl of coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) overnight at 4°C. The plates were washed five times in washing buffer (PBS containing 0.05% Tween 20). The wells were blocked with 100 μl of blocking buffer (5% skim milk in PBS–0.05% Tween 20) for 60 min at room temperature. Following washing of the plates five times, serum or bronchoalveolar lavage (BAL) samples serially diluted in blocking buffer were added to the wells and incubated at room temperature for 90 min. After removal of the samples by washing five times, 100 μl of horseradish peroxidase-conjugated goat anti-rat IgG (Fc specific; Nordic Immunology) diluted 1/2,000 in blocking buffer was added to the wells and incubated at room temperature for 90 min. Following washing, the wells were developed with the substrate tetramethylbenzidine (Fluka, Buchs, Switzerland) in phosphate citrate buffer, pH 5, containing H_2O_2, and the reaction was stopped with 0.5 M H_2SO_4. The plates were read at 405 nm on a Titertek Multiscan MCC/340 plate reader. The plate background was determined by coating rows with coating buffer alone and treating them the same as test wells. Between-plate variation was assessed by comparison of one immune and one control sample repeated for each plate. Mean enzyme-linked immunosorbent assay (ELISA) titers were calculated by multiplying the reciprocal of the serum or BAL dilution that gave an optical density reading between 0.4 and 0.9.

Antigen preparation for proliferation assay. P2 was purified by the method outlined above. OMP extracts were prepared by two methods. The Zwittergent-based extraction was the same as the method outlined above except the final OMP extract was dialyzed for 48 h against several changes of distilled H_2O. The sarcosyl-based extraction was essentially a method described by Klingman et al. (8). This involved washing in PBS the

bacteria grown overnight on 10 plates of brain heart infusion agar. The pellet was resuspended in 2 ml of 0.01 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.4, and sonicated on ice at 100 W for four 15-s periods. The lysate was centrifuged for 20 min at 10,000 × *g* and 4°C to remove unbroken cells and debris. The supernatant was centrifuged for 1 h at 100,000 × *g* and 4°C. The pellet was resuspended in 2 ml of 0.01 M HEPES, pH 7.4, containing 1% sodium lauryl sarcosinate and stirred at room temperature for 30 min before centrifugation for 1 h at 100,000 × *g* and 4°C. The pellet was resuspended in 0.2 ml of 0.01 M HEPES, pH 7.4.

Antigen-specific proliferation assay. The antigen-specific proliferation assay was performed essentially as described by Dunkley and Husband (5). Lymphocytes were obtained from the mesenteric lymph nodes (MLN) by removing the nodes and the surrounding fat and placing them into cold sterile buffer (PCM) prepared with PBS containing calcium and magnesium supplemented with 5% fetal calf serum (heat inactivated at 57°C for 30 min), 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 0.25 μg of amphotericin B (Fungizone) per ml. The MLN and fat were minced and pushed through a stainless steel sieve which was rinsed by passing PCM through the sieve to remove adhering cells. Cells were allowed to stand in a sterile centrifuge tube for 30 min to allow larger material to sediment. The supernatant was decanted to a new tube and washed twice by centrifugation at 1,000 rpm for 10 min in a Beckman CPR benchtop centrifuge. Viable cells were counted by trypan blue (0.1%) exclusion with a hemocytometer and resuspended in culture medium (Multicel RPMI 1640 [Cytosystem, Castle Hill, NSW, Australia] containing 0.01 M HEPES [pH 7.2], 5×10^{-5} M β-mercaptoethanol, 2 mM L-glutamine [ICN, Sydney, NSW, Australia], and 5% fetal calf serum and penicillin-streptomycin-amphotericin B [as described above]) to obtain a final concentration of 10^6 cells per ml. The antigen (OMP extract or P2) was suspended in culture medium in a 10-fold dilution series and sterile filtered. The cell suspension and antigen were added in triplicate to flat-bottomed multiwell microculture plates (Nunc) to obtain a final volume of 0.2 ml per well. Lymphocyte proliferation was estimated by [^{3}H]thymidine (Amersham Australia, Nth Ryde, NSW, Australia) incorporation for the last 8 h of a 4-day culture. Results were calculated by subtraction of background from the geometric means of triplicate wells and then the geometric mean ± the standard error for the entire treatment group.

Statistics. Statistical differences in pulmonary clearance between immunized and control groups were assessed by using a two-tailed Student *t* test.

RESULTS

OMP P6 was purified by both native PAGE and SDS-PAGE. The particular conditions used in the native PAGE were designed to elute only a 16-kDa protein (Fig. 1) known as P6, while the LOS and many other proteins in the extract were excluded by charge and molecular mass from the gel on the basis of analysis of the material that remained on the top of the gel (results not shown). The same electrophoresis conditions allowed isolation of P6 from all three strains of NTHI as well as the extract from the Hib strain. A yield of approximately 1 mg of P6 was obtained from the 35 to 40 mg of protein in the outer membrane extract. This method was designed to favor the migration of P6, and there was no recovery of other proteins in the extract. The yields of pure P6 by both methods were similar (Table 1); although the actual amount of P6 recovered in the SDS protocol was higher, a proportion remained slightly contaminated with LOS and has therefore not been taken into account in the final yield. The presence of LOS contamination was assessed by inspection of silver-stained SDS-PAGE gels, on which LOS stained a distinctive golden-brown color and was easily detected on the bases of both color and location on the gel. The amount of LOS in purified proteins as determined by a *Limulus* amoebocyte lysate assay was found to be equal to or less than 0.6 endotoxin units per mg of protein (data not shown) for both native and SDS-based protein preparations.

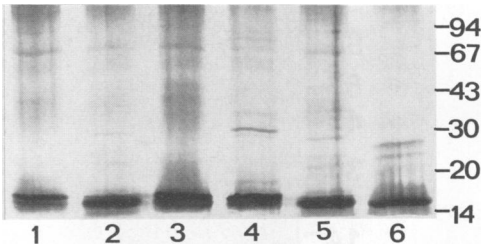

FIG. 1. SDS-PAGE analysis of P6 proteins purified in this study. Approximately 25 ng of each protein was run on a gel gradient of 10 to 15% polyacrylamide and silver stained. Lanes: 1 and 3, P6 HI-CD purified by SDS and native methods, respectively; 2 and 4, P6 NTHI-I purified by SDS and native methods, respectively; 5 and 6, P6 NTHI-II and P6 Hib-II, respectively, purified by native methods. Molecular mass standards (in kilodaltons) are shown on the right.

Several proteins have been purified by SDS-PAGE in which the OMP extract was incubated at 37°C rather than boiled in loading buffer containing SDS. The purified proteins include P6, a 16-kDa protein (Fig. 1); 26-, 28-, and 30-kDa (P4) proteins; and a protein of 39 kDa corresponding to the porin P2 (Fig. 2). To obtain these proteins the crude extract was originally applied to a 12% acrylamide-BIS column which crudely separated proteins into molecular mass ranges. Figure 3 shows the protein profile of every fifth 6-ml fraction eluted from the column at a rate of 1 ml/min. The fractions containing P6 with LOS contamination were pooled and applied to a 16% acrylamide-BIS column for further purification, which resulted in the protein shown in Fig. 1 and a total recovery of approximately 1 mg of pure P6 from 35 to 40 mg of protein in the original extract. Fractions that did not show contaminating bands were kept separate, while the remainder were pooled and further separated with a 14% acrylamide-BIS column, which resulted in the final purification of the other proteins (Fig. 2). The yields of these proteins are shown in Table 1 and are expressed as both a percent yield of the original extract and as milligram quantities.

OMP P6 was selected to assess the suitability of the purified proteins for in vivo studies. P6 preparations purified by both

TABLE 1. NTHI OMPs purified by preparative electrophoresis

Protein	Approximate yield (mg)	Yield (%)
Crude extract	35–40	100
P6 (16 kDa)	0.8–1	2.5–3
Native P6	0.9–1	2.8–3
26-kDa protein	1–1.2	3–3.5
28-kDa protein	0.35–0.4	1–1.2
P4 (30 kDa)	0.7–0.8	2
P2 (39 kDa)	4.4–5	12–13

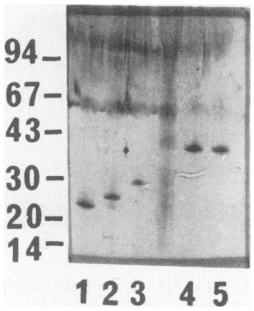

FIG. 2. SDS-PAGE analysis of proteins purified in this study. Samples (10 ng) of the proteins were run on a gel gradient of 10 to 15% polyacrylamide and silver stained. Lanes: 1, 26-kDa protein; 2, 28-kDa protein; 3, 30-kDa protein (P4); 4 and 5, 39-kDa protein (P2). Molecular mass standards (in kilodaltons) are shown on the left.

SDS-PAGE and native PAGE were used to immunize rats via Peyer's patch injections. The results of pulmonary challenge by live bacteria are shown in Table 2 and demonstrate that the immune responses to both preparations of P6 are capable of enhancing bacterial clearance from the rat lungs. The data for these P6 preparations were not significantly different from data previously reported by this laboratory (4) for animals immunized with P6 prepared by ion-exchange chromatography. Immunoblot analysis of the sera from both immunized groups show equivalent antibody recognition of each of the P6 preparations and no recognition by antibodies in the serum from nonimmunized rats (data not shown).

The influence of SDS on both humoral and cell-mediated immune responses was studied with P2 purified by the protocol described in this paper. The presence of 1% SDS in the immunization emulsion was found to have enhanced the P2-specific cell-mediated response (Table 3). Lymphocytes isolated from the MLN of rats immunized with P2 containing SDS demonstrated a greater P2-specific proliferative response than lymphocytes from the group immunized with P2 alone. The lymphocytes of both groups show significantly enhanced P2-specific proliferative responses compared with lymphocytes from the nonimmune rats. A study of levels of P2-specific antibody in serum and BAL indicate that the levels of IgG were highest in the group immunized with P2 alone (Table 3). However, the data show that the levels of P2-specific IgG were significantly reduced in the group immunized with P2 and SDS compared with the levels in the group immunized with P2 alone.

TABLE 2. Recovery of NTHI in BAL of rats following Peyer's patch immunization with different preparations of OMP P6

Protein	Purification method	No. of animals	CFU (log_{10}) in BAL[a]
None		12	6.16 ± 0.12
P6	SDS-PAGE	4	5.56 ± 0.09[b]
	Native PAGE	4	5.43 ± 0.19[b]
	Ion-exchange chromatography	6	5.10 ± 0.68[b]

[a] Values are means ± standard errors of the means.
[b] $P < 0.05$ compared with value for nonimmune rats.

The influence on in vitro cell cultures of traces of reagents that may remain in protein mixtures was observed in a comparison of antigen-specific cell-mediated responses to OMP extract preparations (Fig. 4). Cells isolated from the MLN and cultured in the presence of OMPs extracted by the Zwittergent-based procedure failed to respond to the OMPs even though the cultures remained viable, whereas OMPs extracted with sodium lauryl sarcosinate resulted in a preparation which did not adversely affect the antigen-specific assay and the lymphocytes were observed to proliferate in response to the presence of the antigen.

DISCUSSION

Several studies have proposed that a number of OMPs from NTHI may be suitable components of an effective vaccine against infections by this pathogen (12, 18, 19, 21). In investigations of the presentation of these proteins as purified antigens targeted to the common mucosal immune system, it has been important to find a suitable method of protein purification that is efficient and reproducible, gives a relatively high yield, and could also be used to isolate proteins from more than one strain. Previous attempts to purify OMPs from NTHI within this laboratory by ion-exchange and gel filtration chromatography resulted in considerable variability in the elution profiles and the purities of the proteins of interest (unpublished observations). This was most probably attributable to interference from and variations in the lipid content of the LOS and lipoproteins in the different outer membrane extract preparations.

PAGE is used to effectively separate analytical quantities of protein mixtures and to also remove traces of contaminating proteins before amino acid sequencing. The use of continuous elution electrophoresis to purify and extract larger quantities of a protein from crude preparations has been attempted with varying success in different laboratories over the last two

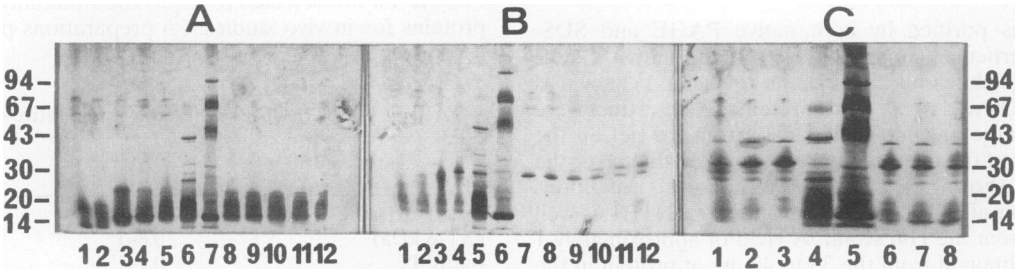

FIG. 3. SDS-PAGE analysis and silver staining of every fifth fraction following the dye front, eluted from one 12% acrylamide-BIS column used to perform the initial separation of proteins in the crude outer membrane extracts. Lanes 6, 5, and 4 on gels A, B, and C, respectively, are the crude outer membrane extract; lanes 7, 6, and 5 on gels A, B, and C, respectively, are molecular mass standards (in kilodaltons; shown on both the left and right sides).

TABLE 3. Effect of presence of SDS with P2 in the Peyer's patch inoculation on P2-specific lymphocyte proliferation of cells isolated from MLN and P2-specific levels of IgG

Rat group[a]	[^{3}H]thymidine incorporated (cpm)[b]	P2 ELISA titer of IgG in:	
		Serum	BAL
Nonimmune	3,740 ± 95	4.1	0.10
Immunized with 40 μg of P2	6,366 ± 958[c]	590[c]	4.98[c]
Immunized with 40 μg of P2 + 1% SDS	8,407 ± 1,239[c]	159[c]	1.18[c]

[a] n = 8 per group. Rats were immunized via Peyer's patches with protein emulsified in incomplete Freund's adjuvant on day 0, received an intratracheal boost of 10 μg of P2 in PBS on day 14 postinoculation, and were challenged with live bacteria on day 21 postinoculation. Rats in the nonimmune group were sham immunized and received a sham intratracheal boost of PBS on day 14.

[b] P2-specific lymphocyte proliferation was estimated by [^{3}H]thymidine incorporation. The concentration of P2 in culture was 10 μg/ml.

[c] $P < 0.05$ compared with value for nonimmune rats.

decades. Our interest in studies involving OMPs from strains of NTHI and Hib and the difficulties encountered with isolation methods involving various forms of column chromatography led us to investigate the potential of the Bio-Rad model 491 Prep Cell to purify proteins from NTHI.

Both native PAGE and SDS-PAGE have been used to purify proteins. This was done to ascertain differences between the two procedures with regard to the proteins, the yield, and the efficiency of purification. The particular conditions used in the native PAGE were designed to elute only a 16-kDa protein known as P6, while the LOS and many other proteins in the extract were excluded from the gel by charge and molecular mass. These conditions were successfully applied to all three strains of NTHI and one strain of Hib to isolate P6. The disadvantage of this method was that it was designed for one particular protein and there was no recovery of other proteins in the extract.

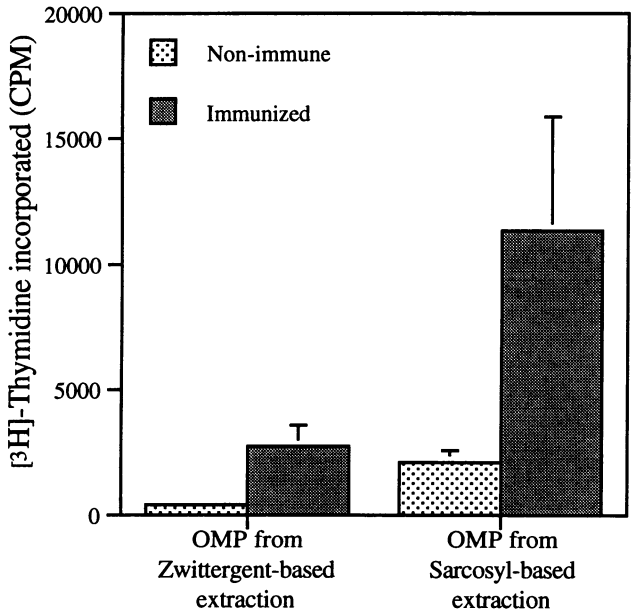

FIG. 4. Antigen-specific lymphocyte assay of cells isolated from the MLN of nonimmunized and immunized rats. Cells were cultured for 4 days in the presence of OMPs extracted by either a Zwittergent-based or sarcosyl-based procedure. The proliferative response was measured by incorporation of [^{3}H]thymidine on day 4. Values are the means ± the standard errors of the means for triplicate cultures and a minimum of four rats per group at a concentration of 1 μg of OMP per ml.

Several proteins have been purified by preparative SDS-PAGE in which the OMP extract was incubated at 37°C rather than boiled in the loading buffer containing SDS. The purified proteins include P6, a 16-kDa protein; 26-, 28-, and 30-kDa (P4) proteins; and a protein of 39 kDa corresponding to the porin P2. The success of purification of proteins from an extract was dependent upon the establishment of appropriate conditions. Selection of %T acrylamide-BIS was not the only criterion that required consideration when purifying the proteins. Column width and height were important and were chosen according to the amount of protein loaded onto the column and the number of components within the sample requiring separation. These factors, in combination with the %T acrylamide-BIS selected to suit the molecular mass differences of the proteins, resulted in good separation of the different components. The ease with which the columns can be prepared and designed to suit protein load and composition has made this method of purification suitable for the purification of *H. influenzae* OMPs. However, to be confident in the data from the immunological studies using proteins isolated by these methods, the proteins were assessed to determine whether the procedures induced any significant alterations in the epitopes required for immunological responses in vivo. Since the proteins purified are structural components and not enzymes or involved in any directly measurable reactions, assessment of significant alterations to structure can be difficult. Comparison between the immune responses to a protein purified by both PAGE methods and an immune response known to occur with the same protein purified by ion-exchange chromatography has been made.

P6 has been purified previously in our laboratory by ion-exchange chromatography and has been shown to induce enhanced bacterial clearance from rat lungs following bacterial challenge (4). It has also attracted the attention of researchers investigating the potential of OMPs as vaccine candidates against *H. influenzae* because of its apparent conservation of surface epitopes and bactericidal capabilities of antibodies in vitro (7, 16, 19, 20). The ability of the protein preparation to induce the appropriate immune responses following Peyer's patch immunization was assessed by measuring pulmonary clearance of live bacteria from rat lungs following challenge with live NTHI and immunoblot assessment of protein recognition by serum antibodies. The results demonstrate that both preparations of P6 are capable of affording protection by enhancing bacterial clearance from the rat lungs, and the data were not significantly different from data previously reported by this laboratory (4) for animals immunized with P6 prepared by ion-exchange chromatography.

The effects of leaving the SDS in the protein preparation on both humoral and cell-mediated immune responses induced by immunization with a purified protein were studied with P2 purified by the protocol described in this paper. A concentration of 1% SDS was chosen, as it was determined that this concentration would exceed the level of SDS in a protein preparation from which the SDS had not been removed and far exceed any traces of SDS that may remain in preparations with the SDS removed. The presence of SDS in the immunization emulsion was found to enhance the P2-specific cell-mediated response. This suggests that the presence of SDS either affected the presentation of antigen to the T cells by the antigen-presenting cells in the Peyer's patches or influenced the processing of the antigen by the T and B cells. However, a

study of levels of P2-specific antibody in serum and BAL indicated that the levels of IgG were higher in the group immunized with P2 alone compared with the group immunized with P2 containing SDS. Thus, the presence of SDS must have suppressed B-cell responses to immunization. These data demonstrate that the presence of a detergent such as SDS does not necessarily inhibit the total immune responses to immunization but can selectively affect different aspects of the processing of the antigens.

The influence of traces of reagents remaining in protein mixtures on in vitro cell cultures was observed in a comparison of antigen-specific cell-mediated responses to OMP extract preparations. Traces of the Zwittergent detergent that may remain in the OMP extract appear to inhibit antigen-specific proliferation of lymphocytes in vitro in cells isolated from the MLN of immunized rats, whereas cells cultured in the presence of sarcosinate-extracted OMPs responded to the presence of the antigen. These observations suggest that this may also be an important consideration in protein preparations intended for in vivo study.

This study has demonstrated that preparative native PAGE or SDS-PAGE can be used to purify proteins that are required for investigation of in vivo immune responses. The PAGE-purified P6 induced the same immune response as did the P6 purified by ion-exchange chromatography as demonstrated by the enhanced pulmonary clearance. As a result of this study, proteins from the outer membranes of strains of nontypeable *H. influenzae* are currently being isolated to assess in vivo and in vitro immune responses to these antigens to further the development of a suitable vaccine against this pathogen. The variations in the effects of SDS and residual traces of Zwittergent and sodium lauryl sarcosinate on immunological responses in vivo and in vitro demonstrate the need to assess the capacity of such agents to affect studies involving purified antigens. The influence of traces of agents used during protein purification may play an important role in determining the success of in vivo studies and is definitely relevant to the interpretation of data from in vitro experiments.

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