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Enhanced Respiratory Clearance of Nontypeable *Haemophilus influenzae* following Mucosal Immunization with P6 in a Rat Model

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Nontypeable *Haemophilus influenzae* (NTHi) is a common cause of infection of the respiratory tract in children and adults. The search for an effective vaccine against this pathogen has focused on components of the outer membrane, and peptidoglycan-associated lipoprotein P6 is among the proposed candidates. This study investigated the immunogenicity of P6 in a rat respiratory model. P6 was purified from two strains of NTHi, one capsule-deficient strain and an *H. influenzae* type b strain, and assessed for clearance of both homologous and heterologous bacterial strains following mucosal immunization. A protective immune response was determined by enhancement of pulmonary clearance of live bacteria and an increased rate of recruitment of phagocytic cells to the lungs. This was most effective when Peyer's patch immunization was accompanied by an intratracheal (IT) boost. However, the rate of bacterial clearance varied between strains, which suggests some differences in anti-P6 immunological defenses recognizing the expression of the highly conserved P6 lipoprotein on the bacterial surface in some strains. P6-specific antibodies in both serum and bronchoalveolar lavage fluid were cross-reactive and did not differ significantly in strain specificity, demonstrating that difference in clearance was unlikely due to differences in P6-specific antibody levels. Serum homologous and heterologous P6-antibody was bactericidal against NTHi even when enhanced clearance had not been observed. Peyer's patch immunization induced P6-specific $CD4^+$ T-helper cell proliferation in lymphocytes isolated from the mesenteric lymph nodes. An IT boost increased the level of P6-specific antibodies in serum and bronchoalveolar lavage fluid, and P6-specific mesenteric node lymphocyte proliferation. Cells from rats immunized with P6 demonstrated proliferation following stimulation with P6 from nonhomologous strains; however, there was some variation in proliferative responses to P6 from different strains in lymphocytes isolated from animals immunized with killed bacteria. The increase in P6-specific antibodies and T-helper cell responses following an IT boost correlated with an increased rate of recruitment of phagocytic cells and enhanced bacterial clearance of both homologous and heterologous bacteria in the lungs. The data suggests that P6 has the potential to afford protection against pulmonary infection by NTHi following the induction of effective antigen-specific B- and T-cell responses in mucosal tissues.

Nontypeable *Haemophilus influenzae* (NTHi) is a common cause of otitis media (5), pneumonia (31), exacerbation of chronic bronchitis, and other invasive and noninvasive diseases in both children and adults (25). The search for an effective vaccine against this pathogen has focused primarily on components of the outer membrane of the bacteria. Studies of the outer membrane protein (OMP) composition of NTHi strains indicates greater strain-to-strain variability than in *H. influenzae* type b (Hib) (3, 4, 21). Several OMPs, including P2, P4, and P6, and some higher-molecular-weight proteins induce serum antibodies which have been shown to be bactericidal in vitro, although the biologic activity of these antibodies is often strain specific (2, 14, 27).

OMP P6 has been proposed as a likely candidate for an effective vaccine against NTHi infection. This protein appears to be highly conserved at both the amino acid and nucleotide levels in both Hib and NTHi strains of diverse geographic and clinical origins (33). P6 is a 16-kDa peptidoglycan-associated lipoprotein that is partly exposed on the bacterial surface (34) and makes up less than 5% of the total OMP content (24). It is still not conclusive that antibodies to P6 protect against NTHi infection in humans; however, there is evidence that a relationship exists. Antibody responses to P6 in children with otitis media suggest that P6-specific antibodies are commonly present in serum and the nasopharynx (45). In animal models, antibodies to P6 were found to protect infant rats from bacteremia following Hib challenge (24), whereas systemic immunization of chinchillas with P6 did not afford protection against NTHi challenge in a chinchilla otitis media model (15). In a rat model, Peyer's patch (PP) immunization with P6 was shown to enhance the rate of bacterial clearance of an NTHi pulmonary challenge (8).

Most of the research associated with the P6 protein has focused on detection of P6-specific antibodies in sera or mucosal secretions (17, 29, 45), screening of NTHi isolates with monoclonal antibodies to identify the degree of conservation of protein epitopes (28, 30, 33), and molecular characterization of the protein (32, 35, 46). To determine the effectiveness of immunization with P6, it is necessary to assess the degree of protection from infection and measure both humoral and cellular responses to the protein antigen that are responsible for

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this protection. Protective mechanisms include the recruitment of phagocytic cells and the existence of specific B- and T-cell responses, including the presence of antigen-specific antibodies. For example, the rate of recruitment of polymorphonuclear neutrophils (PMNs) into the lungs in response to a pathogen has been reported to be dependent upon the C5 component of complement, as well as other chemotaxins secreted in response to recognition of the presence of the pathogen (38). The degree of protection afforded by immunization with a protein antigen such as P6 is likely to depend upon the effectiveness of synergy between antigen-specific B- and T-cell responses in recognizing the pathogen at the site of infection and the induction of defenses required to influence the rate of recruitment of phagocytic cells, particularly PMNs, and enhance clearance of bacteria.

Since P6 has been proposed as an attractive candidate for a vaccine against NTHi, this study was undertaken to investigate in more detail the immunogenicity and protective activity of P6 by using a rat model that had been established previously in this laboratory (41). The data obtained indicate that the degree of protection assessed by determining bacterial clearance following PP immunization alone was dependent upon the *H. influenzae* strain, whereas PP immunization accompanied by a pulmonary boost not only enhanced the degree of clearance following challenge by the homologous strain but was also effective against challenges with different strains.

MATERIALS AND METHODS

Bacterial strains. Four *H. influenzae* strains were studied. These were two nontypeable strains of biotypes I and II (NTHi-I and NTHi-II, respectively), a biotype I capsule-deficient strain (HI-CD; type b capsule lost through in vitro passage), and a biotype II encapsulated Hib strain (Hib-II).

Purification of P6. P6 was purified from each of the four strains listed above as described by Kyd et al. (19). Briefly, each strain of bacteria was grown overnight at 37°C in 5% CO_2 on 100 plates of brain heart infusion agar supplemented with 50 ml of defibrinated horse blood per liter of agar (Hunter Anti-Sera, Callaghan, New South Wales [N.S.W.], Australia). The bacteria were harvested by scraping the plates and washed twice by centrifugation at 10,000 × *g* for 10 min each time at 4°C. A crude outer membrane preparation was obtained by the method of Murphy and Bartos (26) and as described by Kyd et al. (19). P6 was purified by using preparative polyacrylamide gel electrophoresis (PAGE) by either the sodium dodecyl sulfate (SDS) or the native method as previously described (19). Essentially, the OMP extract was lyophilized, resuspended in a minimal amount of distilled water, 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 of the electrophoresis column. Tris and SDS were electrophoresis grade reagents purchased from Bio-Rad Laboratories, Nth Ryde, N.S.W., Australia, and all others were laboratory grade reagents.

Preparative SDS-PAGE to purify P6 was performed with a Bio-Rad 491 Prep Cell with a 40-ml 16% T–1.42% C acrylamide–*N*,*N*-methylenebisacrylamide separating gel with a 10-ml 4% T–0.36% C acrylamide–*N*,*N*-methylenebisacrylamide stacking gel polymerized in a 28-mm (internal diameter) column as previously described (19). These conditions separated P6 from the lipooligosaccharide and other proteins. Fractions were concentrated by lyophilization and analyzed for protein content by analytical SDS-PAGE. P6 isolated under these conditions contained SDS, which was subsequently removed by a method described by Suzuki and Terada (37).

Some of the P6 used in these experiments was purified by native separation from the outer membrane extract with a 30-ml 16% T–1.42% C acrylamide–*N*,*N*-methylenebisacrylamide separating gel with a 5-ml 4% T–0.36% C acrylamide–*N*,*N*-methylenebisacrylamide stacking gel in a 28-mm (internal diameter) column, as previously described (19), with 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). Following electrophoresis, the fractions were concentrated by lyophilization and analyzed for protein by SDS-PAGE. Fractions containing P6 were pooled and dialyzed prior to protein concentration determination. The level of lipooligosaccharide present was assessed by both silver staining of SDS-PAGE minigels and assaying with the E-TOXATE *Limulus* lysate test (Sigma, Castle Hill, N.S.W., Australia) and found to be less than 0.6 μg of endotoxin per mg of protein by the E-TOXATE assay.

SDS-PAGE. SDS-PAGE was carried out essentially as described by Laemmli (20) to analyze fractions for the presence of P6. A 10-μl fraction sample was added to an equal volume of sample buffer containing SDS and β-mercaptoethanol and boiled for 5 min. Electrophoresis was performed with minigels with a 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 minigels for determination of the molecular masses of the proteins.

Protein concentration determination. Protein concentration was determined with the Pierce Micro BCA protein assay reagent and the Pierce albumin standard (Laboratory Supplies, Marrickville, N.S.W., Australia).

Immunization. The specific-pathogen-free (SPF) male DA rats used were between 8 and 10 weeks old and were maintained under SPF conditions until the start of the experiment. They were removed from behind the SPF barrier for PP immunization, an intratracheal (IT) boost (when required), and the final live bacterial challenge. At all other times, the animals remained in SPF cages under SPF conditions. The procedure for immunization and bacterial challenge was performed by a modification of a previously established method (41). The animals were sedated with halothane to facilitate intravenous anesthesia via the tail vein with 3.6% (wt/vol) chloral hydrate in phosphate-buffered saline (PBS; Cytosystems Pty. Ltd., Castle Hill, N.S.W., Australia) at a dosage of 0.25 g of chloral hydrate per kg of body weight. The small intestine was exposed through a midline abdominal incision, and the antigen was injected subserosally into each PP with a 27-gauge needle. The immunization protein was prepared by emulsification of 200 μg of protein per ml in a 1:1 mixture of incomplete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) and PBS, and a total inoculum of 10 μg of protein was administered to each animal. Two control groups of rats consisted of (i) a sham-immunized group receiving the same treatment as the P6-immunized rats but immunized with incomplete Freund's adjuvant and PBS only and (ii) a positive control group immunized with killed bacteria of the homologous NTHi strain. Bacteria were killed by suspension in 1% (wt/vol) paraformaldehyde in PBS and incubated at 37°C for 2 h. The bacteria were washed three times in PBS, killing was checked by plating onto agar and overnight culture, and the concentration was adjusted to an equivalent of 2×10^{10} bacteria per ml as estimated by determining the optical density at 405 nm. The killed bacterial suspension was prepared for immunization by emulsification in a 1:1 mixture of incomplete Freund's adjuvant and the suspension so that each animal received approximately 5×10^8 bacteria. Rats receiving only PP immunization received a pulmonary challenge of live bacteria 14 days postimmunization.

Rats receiving an IT boost were initially immunized as described above. On day 14 postimmunization via PP, they were sedated with halothane and 10 μg of P6 at a concentration of 200 μg/ml in PBS was introduced into the lungs via an IT cannula and dispersed with two 5-ml volumes of air. The nonimmune group received 50 μl of PBS, while the group immunized with killed bacteria received 50 μl of killed bacteria (count of 10^{10} bacteria per ml). Some treatment groups received a PP immunization with either P6 or killed bacteria followed by an IT boost on day 14 with killed bacteria or P6, respectively. In experiments involving an IT boost, killed NTHi-I bacteria or NTHi-I P6 was used for immunization. Animals were allowed to recover, kept under SPF conditions, and challenged with live bacteria 21 days after the first immunization.

Bacterial challenge. Bacteria were prepared by overnight culture as described above and resuspended in PBS. The concentration of the inoculum was estimated by measuring the optical density at 405 nm and confirmed by counting CFU of the overnight plating of serial dilutions of the inoculum.

On the day of pulmonary challenge with live bacteria, the animals were sedated with halothane and a bolus inoculum of 5×10^8 CFU of live *H. influenzae* in 50 μl of PBS was introduced into the lungs via an IT cannula and dispersed with two 5-ml volumes of air. Animals were killed by an overdose of pentobarbital sodium (Nembutal; Boehringer Ingelheim, Artarmon, N.S.W., Australia) administered by intraperitoneal injection 4 h after lung inoculation. Blood was collected by heart puncture, and aliquots of serum were stored at −20°C for antibody analysis. Lungs were subjected to lavage with five 2-ml volumes of PBS via the trachea, which had been exposed through an incision in the neck, and the pooled bronchoalveolar lavage (BAL) fluid was assessed for clearance by plating of serial dilutions of the washings for CFU determination. In some experiments, the lungs were removed following lavage with PBS. The lungs, trachea, and heart were excised intact; the heart and connective tissues were removed; and the lungs were placed in 10 ml of sterile PBS and homogenized (Ultra-Turrax T25 homogenizer [Janke and Kundel, IKA-Labortechnik] set at 9,500 rpm with no load). The lung homogenate was assessed for the presence of bacteria by plating serial dilutions (20 μl in a 10-fold series) onto supplemented brain heart infusion agar for CFU determination.

Cytospin and BAL fluid cell counts. Cytospin slides were prepared to determine percentages of PMNs, macrophages, and other cells present in the BAL fluid. A 100-μl aliquot of BAL fluid was spun for 10 min at 4.5 × *g* onto a microscope slide with a Cytospin apparatus (Shandon Inc., Pittsburgh, Pa.). The slides were fixed and stained in Diff Quick (Veterinary Medical Surgical Supply, Pty. Ltd., Maryville, N.S.W., Australia), and percentages were determined from three differential cell counts on each slide. Mean percentages ± the standard errors were calculated from the collected group data.

BAL fluid was centrifuged at 1,000 rpm for 10 min with a Beckman CPR bench top centrifuge. The supernatant was removed, and aliquots were stored at −20°C

for enzyme-linked immunosorbent assay (ELISA) analysis. The pellet was resuspended in a known volume of PBS, and the total number of cells present in the BAL fluid was determined with a hemocytometer and staining with methylene blue.

Antigen-specific ELISAs. Polysorb microtiter wells (Nunc, Roskilde, Denmark) were coated with 0.1 μg of purified P6 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. Plates were washed five times, and serum (1/25 to 1/200 for nonimmune serum; 1/100 to 1/3,200 for immune serum) or BAL fluid (1/2 to 1/16) samples were serially diluted in blocking buffer, added to the wells, and incubated at room temperature for 90 min. After removal of the samples by washing the wells five times, 100 μl of horseradish peroxidase-conjugated goat anti-rat immunoglobulin diluted in blocking buffer was added to the wells and incubated at room temperature for 90 min. Conjugated immunoglobulins used were immunoglobulin G (IgG) (1/2,000), IgA (1/1,000), and IgM (1/4,000) (Fc specific; Nordic Immunological Laboratories). The plates were washed five times, and the wells were developed with 100 μl of the substrate tetramethylbenzidine (Fluka, Buchs, Switzerland) in phosphate-citrate buffer (pH 5) containing 0.05% (vol/vol) H_2O_2. The reaction was stopped with 100 μl of 0.5 M H_2SO_4. Plates were read at 405 nm on a Titertek Multiscan MCC/340 plate reader. The plate background was determined by reading rows coated with coating buffer alone and treated the same as test wells. Between-plate variation was assessed by comparison of one immune sample and one control sample repeated on each plate. Mean ELISA titers were calculated by multiplying the reciprocal of the serum or BAL fluid dilution that gave an optical density reading of 0.4 to 0.9.

Antigen-specific lymphocyte assay. The antigen-specific lymphocyte assay was performed essentially as described by Dunkley and Husband (11) and reported by Kyd et al. (19). Briefly, lymphocytes obtained from the mesenteric lymph nodes (MLN) by passing tissue through a stainless steel sieve and washing it in cold, sterile PCM buffer 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. Viable cells were counted by trypan blue exclusion with a hemocytometer and resuspended in culture medium (Multicel RPMI 1640 [Cytosystem, Castle Hill, N.S.W., Australia] containing 0.01 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES, pH 7.2], 5×10^{-5} M β-mercaptoethanol, 2 mM L-glutamine [ICN, Sydney, N.S.W., Australia], 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 (P6) was suspended in culture medium in a 10-fold dilution series and sterilely filtered. The cell suspension and antigen were added in triplicate to flat-bottom multiwell microculture plates (Nunc) to give a final volume of 0.2 ml per well. Lymphocyte proliferation was estimated by determining [^{3}H]thymidine (Amersham Australia, Nth Ryde, N.S.W., Australia) incorporation for the last 8 h of a 4-day culture. Results were calculated by subtraction of the background radioactivity from the geometric means of triplicate wells and then from the geometric mean ± the standard error of the entire treatment group.

Subset depletion proliferation assay. Identification of the phenotype of the lymphocytes responsible for the antigen-specific proliferation was performed by subset depletion with a modification of the panning method of Wysocki and Sato (43) as described by Dunkley et al. (10). Essentially, lymphocytes isolated from the MLN were washed and counted as described above. Aliquots containing 3×10^7 cells were pelleted by centrifugation at $200 \times g$ and treated for 30 min at 4°C with monoclonal antibody W3/25 (recognizing predominantly CD4, helper-inducer phenotype; Serotec, Oxford, England), OX8 (recognizing predominantly CD8, cytotoxic-suppressor phenotype; Serotec), or OX33 (recognizing B cells; SeraLab, CSL, Melbourne, Victoria, Australia) diluted 1/50 in PCM. A fourth group of cells received no treatment with a monoclonal antibody. Cells were washed prior to incubation on panning dishes coated with rabbit anti-mouse IgG (Dako Immunoglobulin, Copenhagen, Denmark) diluted 1/100 in borate buffer (pH 8.7) for 90 min at 4°C with gentle swirling each 30 min. Nonadherent cells were transferred to a new panning dish, and the panning procedure was repeated. The nonadherent cells were recovered, counted, and resuspended in RPMI culture medium (as described above) to give a final concentration in culture of 10^6 cells per ml. Cell cultures were performed as described above except for the addition of mitomycin C-treated spleen lymphocytes as a source of antigen-presenting cells (at a concentration of 0.5×10^6 cells per ml in culture).

Flow cytometry analysis. Lymphocyte populations were assessed following subset depletion by staining with mouse monoclonal antibodies against rat lymphocyte subsets followed by fluorescein isothiocyanate-labelled anti-mouse antibody staining as described by Dunkley et al. (10). Briefly, 10^6 untreated or monoclonal antibody-treated cells (as described above) were pelleted and incubated with 30 μl of monoclonal antibody W3/25, OX8, or OX33 diluted 1/50 for 30 min at 4°C. Cells were washed three times, and the pellets were incubated for 30 min at 4°C with 30 μl of fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin (Cappel, West Chester, Pa.) diluted 1/50 in PCM containing 10% heat-inactivated normal rat serum. Cells were washed three times, resuspended in 0.5 ml of cold PCM, fixed by addition of 0.5 ml of 2% formaldehyde while vortexing, and analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif.).

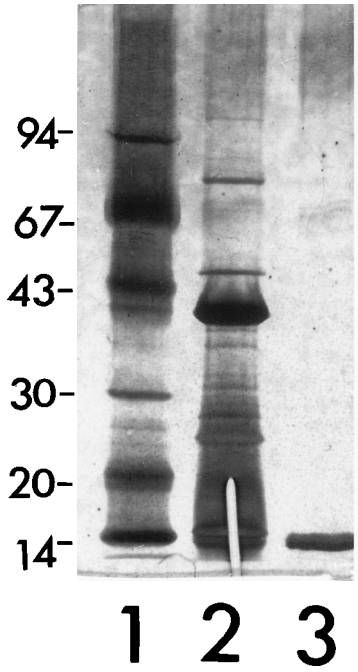

FIG. 1. SDS-PAGE analysis of the NTHi-I P6 used in this study. Samples were run on a 10 to 15% gradient polyacrylamide gel and silver stained. Lanes: 1, molecular mass standards (values on the left are in kilodaltons); 2, outer membrane extract of strain NTHi-I; 3, NTHi-I P6.

Bactericidal assays. Bactericidal assays were performed against NTHi-I live bacteria with sera from rats immunized via PP with P6 from the different strains, sera from rats immunized via PP and given an IT boost with NTHi-I-P6, and nonimmune sera from rats sham immunized with incomplete Freund's adjuvant and PBS. NTHi-I bacteria from an overnight culture were washed in PBS and resuspended in Hanks balanced salt solution to a density of 5×10^8 CFU/ml. A source of complement was prepared by incubating fresh rat serum with 10^{12} bacteria for 60 min on ice. The suspension was centrifuged at $20,000 \times g$ for 10 min, and the supernatant was resuspended in bacteria. The procedure was repeated, and the resulting supernatant was sterilely filtered and kept on ice for use on the same day. Absorbed serum used to remove NTHi-specific antibodies was prepared as described by Groeneveld et al. (16). The assays were set up in 96-well round-bottom microculture plates (Nunc) and incubated on a shaker at 37°C and 5% CO_2. Triplicate incubation mixtures consisted of 10 μl of a bacterial suspension, 10 μl of complement (control assays omitted complement), 10 μl of rat antiserum or 40 μl of absorbed rat antiserum, and Hanks balanced salt solution to give a final volume of 100 μl. Aliquots (20 μl each) were taken at 0-, 1-, and 2-h intervals, and CFU counts were determined by overnight culture of serial dilutions on supplemented brain heart infusion agar plates.

Statistical analysis. The data obtained were expressed as means ± the standard errors of the means. Pulmonary clearance data, total numbers of phagocytic cells, and differential cell counts in nonimmune and immunized rats were compared by one-way analysis of variance, followed by Tuckey's test for multiple-comparison analysis (Macintosh Systat). Antibody data were assessed for statistical significance of differences between groups by an unpaired *t* test, and lymphocyte proliferation was assessed by a fully factorial analysis of variance (Macintosh Systat). Linear correlation between two variables was determined with the Pearson correlation coefficient (Macintosh Systat).

RESULTS

Analysis of P6 preparation. P6 was isolated as described above from four strains of *H. influenzae*. These strains were two nontypeable strains of biotypes I and II, respectively (NTHi-I and NTHi-II), a capsule-deficient strain (HI-CD) that had lost the type b capsule through in vitro passage, and a biotype II type b strain (Hib-II). Figure 1 is a silver-stained SDS-polyacrylamide gel showing P6 isolated from strain NTHi-I and an OMP extract from the same strain. P6 has a

TABLE 1. Live bacteria recovered in BAL fluid from animals immunized via intestinal PP with 10 μg of P6 or killed bacteria and challenged on day 14 postimmunization with live bacteria from either the homologous *H. influenzae* strain or a different strain

Immunization[a]	Mean ± SE bacterial CFU (log_{10}) recovered in BAL after challenge with strain:			
	NTHi-I	NTHi-II	HI-CD	Hib-II
Nonimmune group	6.26 ± 0.04	6.17 ± 0.07	6.15 ± 0.20	7.22 ± 0.18
Killed bacteria of homologous strain	5.65 ± 0.26[b]	5.39 ± 0.16[b]	5.92 ± 0.14	7.13 ± 0.18
NTHi-I P6	6.29 ± 0.06		6.04 ± 0.45	
NTHi-II P6		6.19 ± 0.15		
HI-CD P6	6.46 ± 0.22		5.37 ± 0.37[b]	7.06 ± 0.17
Hib-II P6			5.21 ± 0.29[b]	7.20 ± 0.07

[a] There were four to seven rats per group.
[b] $P < 0.05$ compared with the nonimmune group for the homologous strain.

molecular mass of 16 kDa by SDS-PAGE analysis, and the procedure used to purify P6 enabled the separation of P6 from a 15-kDa protein (called PCP) described by Deich et al. (9a). Lipooligosaccharide contamination of protein preparations was assessed by both silver staining and the *Limulus* amoebocyte lysate assay and found to be less than 0.6 μg of endotoxin per mg of protein. This represents the detection limit of the assay.

Effect of immunization on pulmonary clearance. Table 1 shows the bacterial clearance obtained in rats immunized via intestinal PP with either 10 μg of purified P6 or paraformaldehyde-killed *H. influenzae* and challenged 14 days postimmunization with an inoculum of 5×10^8 CFU of live bacteria. A single immunization with killed bacteria resulted in a significant level of enhanced pulmonary clearance of the homologous strain of bacteria with only the two nontypeable strains. A single immunization with purified P6 significantly ($P < 0.05$) enhanced clearance of the capsule-deficient strain only when the immunizing antigen was from either the capsule-deficient strain or the Hib strain. Immunization with P6 purified from both of the nontypeable strains did not enhance clearance of either a homologous or a heterologous strain of *H. influenzae*, nor did Hib-II P6 clear bacteria of the Hib strain. These results suggest some differences in the susceptibility of the strains to killing following a single immunization with P6.

A previous study by Wallace et al. (41) had demonstrated a significant increase in the rate of bacterial clearance when immunization via PP was followed by a pulmonary boost at day 14 prior to a live bacterial challenge at day 21. To evaluate the influence of rats receiving a pulmonary boost 14 days after the initial immunization via PP, a study was undertaken which compared immunization with paraformaldehyde-killed bacteria with immunization with P6 from strain NTHi-I. In some of these experiments, lung homogenates were also used to measure bacterial numbers still associated with lung tissue following lavage. The results in Table 2 show a significant increase in the rate of clearance of bacteria from the lungs in the groups of rats receiving an IT boost of either 10 μg of P6 or killed bacteria following immunization via PP with NTHi-I P6. The degree of enhancement of bacterial clearance following an IT boost with P6 was not further increased by increasing the concentration of P6 from 10 to 40 μg in the initial PP immunization. Also, enhancement of clearance was not significantly altered by a P6 boost following immunization with killed bacteria compared with that in rats boosted with PBS. However, rats immunized and boosted with killed bacteria showed a significantly enhanced rate of clearance compared not only with that of the nonimmune group but also with that of rats receiving a boost with either PBS or P6. Lung homogenate results were closely related to BAL fluid clearance data, except for PP immunization with NTHi-I P6 with a PBS boost. The lung homogenates of this group appeared to have a reduction in bacteria remaining associated with the lung tissue; however, this difference was not statistically significant.

The results in Table 3 show the bacterial clearance in BAL fluids and lung homogenates from rats challenged with either another nontypeable strain or the capsule-deficient HI-CD strain following immunization and a boost with NTHi-I P6. Bacteria from either strain were significantly ($P < 0.001$) cleared following immunization with NTHi-I P6. The magni-

TABLE 2. NTHi-I recovery from BAL fluid and lung homogenates of rats immunized via intestinal PP and given an IT boost of either NTHi-I P6 or killed NTHi-I bacteria

PP immunization[a]	Sample	Log_{10} CFU of NTHi-I recovered in BAL or lung homogenate[b] (% clearance) after IT boost on day 14 with:		
		PBS or nothing	10 μg of NTHi-I P6	Killed NTHi-I
Nonimmune group	BAL fluid	6.37 ± 0.08[c]		
Nonimmune group	Lung homogenate	6.71 ± 0.05		
10 μg of NTHi-I P6	BAL fluid	6.36 ± 0.13	5.66 ± 0.11[d] (81)	5.69 ± 0.13[e] (79)
10 μg of NTHi-I P6	Lung homogenate	6.46 ± 0.09 (44)	6.12 ± 0.12[e] (75)	
40 μg of NTHi-I P6	BAL fluid		5.60 ± 0.06[d] (83)	
Killed NTHi-I	BAL fluid	5.78 ± 0.26[e] (74)	5.71 ± 0.20[e] (78)	5.04 ± 0.13[d] (95)
Killed NTHi-I	Lung homogenate			5.64 ± 0.20[d] (92)

[a] There were 5 to 11 rats per group.
[b] The values shown are means ± the standard errors of the means of (i) BAL fluid or (ii) lung homogenate of rats given a pulmonary challenge (for 4 h) with live NTHi-I on day 21 postimmunization via PP.
[c] Data for sham-immunized rats given an IT boost were combined with those for rats that received no boost (no significant difference).
[d] $P < 0.05$ compared with nonimmune rats.
[e] $P < 0.005$ compared with nonimmune rats.

TABLE 3. Pulmonary challenge with nonhomologous *H. influenzae* on day 21 postimmunization of rats given NTHi-I P6 via PP on day 1 and an IT booster dose of NTHi-I P6 on day 14

Rat group[a]	Sample	CFU (log_{10}) of *H. influenzae* recovered in BAL fluid or lung homogenate[b] (% clearance) after challenge with strain:	
		NTHi-II	HI-CD
Nonimmune	BAL fluid	6.23 ± 0.07	5.81 ± 0.07
Nonimmune	Lung homogenate	6.55 ± 0.23	6.23 ± 0.14
NTHi-I P6 immunized	BAL fluid	4.77 ± 0.17[c] (97)	4.08 ± 0.17[c] (98)
NTHi-I P6 immunized	Lung homogenate	5.16 ± 0.17[c] (96)	5.34 ± 0.09[c] (88)

[a] There were nine rats per group.
[b] The values shown are means ± the standard errors of the means of (i) BAL fluid or (ii) lung homogenate of rats given a pulmonary challenge (for 4 h) with live *H. influenzae* of the strain indicated on day 21 postimmunization via PP.
[c] $P < 0.001$ compared with the nonimmune group.

tude of clearance was also significantly increased ($P < 0.05$) compared with that observed for challenge with the homologous strain (Table 2), suggesting differences in *H. influenzae* strains with respect to P6-specific defense mechanisms.

Recruitment of phagocytic cells. BAL fluid was assessed for both the total number of cells and the differential cell populations (Table 4). The total number of cells recruited to the lungs significantly ($P < 0.001$) increased in immunized groups that demonstrated enhanced bacterial clearance. Although the total number of cells in the BAL fluid increased, the differential cell populations show that the percentages of PMNs and macrophages were not significantly different between nonimmune and immune groups. The Pearson correlation coefficient for a linear correlation between the log_{10} bacteria recovered in BAL fluid (CFU) and the total numbers of cells in BAL fluid was $r = 0.950$, indicating a high correlation between bacterial clearance and recruitment of phagocytic cells.

Anti-P6 antibody in serum and BAL fluid. Antibody responses following immunization with P6 and killed bacteria were assessed by ELISA. The data in Fig. 2 represent the cross-recognition of antibodies to NTHi-I P6 in serum and BAL fluid from groups of rats following PP immunization with each of the four P6 preparations. The data show a significant ($P < 0.005$) increase in anti-P6 IgG and IgA in each of the four immunized groups compared with the nonimmune rats. Nonimmune rats did have a low anti-P6 antibody titer, which may be attributed to cross-reaction with antibodies to normal microflora, i.e., *Escherichia coli*, which has a peptidoglycan-associated lipoprotein with a high degree of sequence homology with *H. influenzae* P6 (46). The levels of anti-NTHi-I P6 antibody did not significantly differ in the serum or BAL fluid of rats immunized with P6 from the different strains, thus supporting the evidence of a high degree of sequence homology and antibody cross-recognition. The effect of an IT boost on the level of antibody to NTHi-I P6 showed a significant increase ($P < 0.005$) of IgG, IgA, and IgM in the serum and of IgG and IgA in the BAL fluid of groups immunized with both pure antigen and killed bacteria (Table 5). Increasing the concentration of P6 from 10 to 40 μg in the PP immunization did not significantly further increase the final antibody levels in the serum; however, there was a further increase in IgG and IgA in the BAL fluid, although this increase did not correspond to increased bacterial clearance in this group. In rats immunized with killed bacteria by PP alone, there was a slight elevation of antibody to P6; however, this increase over the nonimmune group antibody level was not statistically significant. An IT boost with killed bacteria did significantly ($P < 0.025$) elevate the P6-specific antibody in this group to a level comparable to that of the group immunized with 10 μg of P6 by PP alone.

Bactericidal activity of anti-P6 antibody to NTHi-I. Serum from rats immunized by PP with P6 from each of the four strains was assessed for bactericidal activity by using a concentration of 10% serum in each assay. The rates of killing of live NTHi-I bacteria were compared in sera from rats immunized by PP with P6 from each strain, sera from rats immunized by PP and given an IT boost with NTHi-I P6, and sera from nonimmune, sham-treated rats. The bactericidal activity of anti-P6 antibody was more effective in the group immunized with P6 from the homologous strain than in the groups immunized with P6 from nonhomologous strains (Fig. 3); however, significant bactericidal activity was shown by antibody from the nonhomologous strains compared with nonimmune serum. Antibody to bacterial surface determinants of P6 was removed by absorption of serum with NTHi-I bacteria. Absorbed serum failed to demonstrate bactericidal activity. The effect of higher anti-P6 antibody levels in the serum of rats that had received an IT boost (Table 5) corresponded to an increase in the bactericidal activity of serum from this group (Fig. 3).

Antigen-specific lymphocyte proliferation. Lymphocytes from the MLN of groups of rats immunized with P6 or either NTHi-I, HI-CD, or Hib-II killed bacteria were cultured with the P6 antigen from each of these three strains to assess levels of antigen-specific responses and cross-strain responses. Significant antigen-specific proliferation of all three strains immu-

TABLE 4. Phagocytic cell counts in the BAL fluid 4 h post-pulmonary challenge with live nontypeable *H. influenzae*

Immunization	Mean differential cell count (%) ± SE			Total no. of cells in BAL fluid (10^6)
	PMNs	Macrophages	Other cells	
Nonimmune group	94.7 ± 1.2	3.0 ± 0.8	1.7 ± 0.8	13.6 ± 1.9
10 μg of P6	94.8 ± 0.8	4.2 ± 0.7	1.1 ± 0.3	17.7 ± 2.3
10 μg of P6 + IT boost	95.4 ± 0.5	1.6 ± 0.3	2.9 ± 0.3	27.6 ± 2.5[a]
40 μg of P6 + IT boost	97.6 ± 0.4	1.9 ± 0.3	0.7 ± 0.3	24.1 ± 1.3[a]

[a] $P < 0.001$ compared with the nonimmune group.

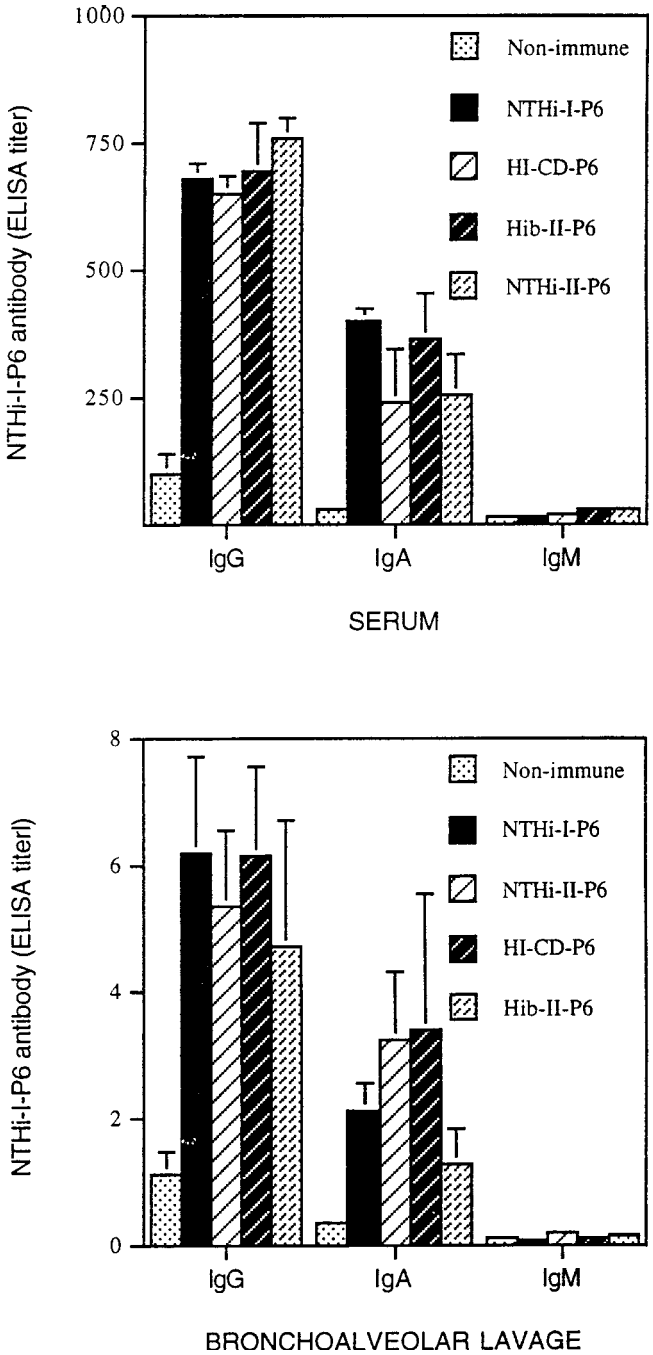

FIG. 2. Anti-NTHi-I P6-specific IgG, IgA, and IgM antibodies in serum and BAL fluid of nonimmune and immune rats 14 days after PP immunization. Immune serum and BAL fluid were from rats immunized with either NTHi-I P6, NTHi-II P6, HI-CD P6, or Hib-II P6 (as indicated) and assessed for antibody recognition of NTHi-I P6 by ELISA. Each value represents the mean ± the standard error of the mean for samples from four rats in each group and was calculated by multiplication of the reciprocal of the dilution that gave an optical density of 0.4 to 0.9.

nized with P6 was observed in response to both homologous and heterologous P6 (Fig. 4A to I). Lymphocytes from rats immunized with NTHi-I P6 showed significant ($P < 0.05$) proliferation in response to P6 from strains NTHi-I (Fig. 4A), HI-CD (Fig. 4B), and Hib-II (Fig. 4C). Likewise, rats immunized with HI-CD P6 (Fig. 4D to F) and Hib-II-P6 (Fig. 4G to I) significantly responded to P6 from strains NTHi-I, HI-CD, and Hib-II. P6-specific proliferation further increased in the group that received an IT boost compared with the group immunized by PP alone (Fig. 4A).

In rats immunized with killed NTHi-I, there was a significant P6-specific proliferative response to NTHi-I P6 which was similar in magnitude to the response of cells isolated from rats immunized with 10 μg of P6 (Fig. 4A). The proliferative response to HI-CD P6 in cells from rats immunized with strain NTHi-I killed bacteria was not significantly different from that of the nonimmune group, demonstrating poor recognition by these cells (Fig. 4B). Rats immunized with killed HI-CD bacteria, however, showed significant ($P < 0.05$) P6-specific proliferation in response to P6 from both strains HI-CD and NTHi-I (Fig. 4D to E). These results are in contrast to the data for the nontypeable *H. influenzae* strain. Cells from rats immunized with killed Hib-II bacteria significantly responded to both HI-CD P6 and Hib-II P6, but the response to NTHi-I P6 was only slightly greater than that observed for the nonimmune group and the difference was not statistically significant (Fig. 4G to I).

Determination of the identity of the antigen-specific lymphocytes responding to P6 was performed with a subset depletion assay. Essentially, antigen-specific proliferative responses were measured in lymphocyte cultures from which a particular subset of lymphocytes had been removed by panning. The effectiveness of the depletion of a population of lymphocytes was assessed by FACScan analysis of fluorescein isothiocyanate-labelled cells. The results of this analysis showed effective depletion of the subset of cells in a population count of 5,000 cells (<2% $OX8^+$, <1% $OX33^+$, or <4% $W3/25^+$ remaining) in populations depleted of each of these subsets but a normal distribution of 17% $OX8^+$, 21% $OX33^+$, and 62% $W3/25^+$ where cells had been panned in the absence of a depletion monoclonal antibody. P6-specific proliferation was observed in cultures from which B cells or $CD8^+$ T cells had been removed, but in cultures where $CD4^+$ T cells had been depleted there was no P6-specific proliferation (Fig. 5). Therefore, it appears that the antigen-specific proliferation measured in the mixed lymphocyte cultures was due to cells with a $CD4^+$ T-helper phenotype.

DISCUSSION

In the search for an effective vaccine against NTHi infection, the focus has been on identification of suitable bacterial surface antigens. To facilitate this search, the main aim has been to identify suitable candidates by correlation of conservation of protein structure with the biologic activity of antibodies as demonstrated by in vitro bactericidal assays. Among the proteins that have been identified as potential NTHi vaccine candidates is peptidoglycan-associated lipoprotein P6. In the present study, we undertook to extend the knowledge of the potential of this antigen by investigation of the antigen-specific immune responses and the corresponding degree of protection as assessed by bacterial clearance following induction of immunity at mucosal surfaces. P6 has been reported to be highly conserved in both Hib and nontypeable *H. influenzae* strains (13, 28, 33, 34), and so a comparative analysis of the immune responses to immunization with P6 and a challenge with live bacteria from two nontypeable strains, one capsule-deficient strain, and a Hib strain was done.

NTHi is an opportunistic pathogen commonly causing localized infections in the respiratory tract, but it may also invade beyond the respiratory mucosa. Thus, an effective vaccine must be capable of inducing effective protection at the respiratory

TABLE 5. Comparison of NTHi-I P6-specific antibodies in serum and BAL fluid following immunization via PP with or without an IT boost with either purified protein or killed bacteria

Treatment	Mean titer of antibody to NTHi-I P6 (ELISA titer[a]) ± SEM in					
	Serum			BAL fluid		
	IgG	IgA	IgM	IgG	IgA	IgM
Nonimmune group	203 ± 63	24 ± 5	ND[b]	0.6 ± 0.1	0.28 ± 0.08	ND
10 μg of P6 (PP)[c]	1,020 ± 105[d]	387 ± 40[d]	12 ± 4	7.4 ± 0.7[d]	2.3 ± 0.5[d]	ND
10 μg of P6 (PP + IT)[e]	5,281 ± 1,088[d]	1,752 ± 405[d]	63 ± 16[f]	13.1 ± 2.8[d]	11.3 ± 1.6[d]	ND
40 μg of P6 (PP + IT)	4,061 ± 1,243[d]	1,598 ± 362[d]	61 ± 9[f]	19.4 ± 6.2[d]	17.2 ± 4[d]	ND
Killed bacteria (PP)	352 ± 98	36 ± 5	ND	4.0 ± 0.8[d]	0.7 ± 0.1[f]	ND
Killed bacteria (PP + IT)	1,717 ± 539[f]	405 ± 98[d]	ND	7.5 ± 0.8[d]	6.8 ± 1.1[d]	ND

[a] Antibody titers were calculated as described in Materials and Methods.
[b] ND, undetectable levels.
[c] PP represents immunization via PP only.
[d] $P < 0.005$ compared with the nonimmune group.
[e] PP + IT represents immunization via PP followed by an IT boost on day 14. The amount of purified P6 or killed bacteria administered in the IT inoculum was 10 μg or 5×10^8, respectively.
[f] $P < 0.025$ compared with the nonimmune group.

mucosa. Systemic routes of immunization result in effective cell-mediated immunity and antibodies in systemic tissues but are essentially limited in the ability to induce effective mucosal immunity (22, 44). Presentation of antigen to cells in the intestinal PP induces secretory antigen-specific IgA^+ B cells and $CD4^+$ T-helper cells and leads to the dissemination of these to other mucosal tissues, such as the respiratory tract, via the common mucosal immune system for subsequent antigen-specific responses (6, 23). A recent study by Green and coworkers (15) investigated the protective capacity of immunization with P6 in a chinchilla otitis media model. The failure of immunization with this protein to afford significant protection may be associated with the route of immunization. The effectiveness of immunization via intestinal PP in enhancing clearance of live bacteria following pulmonary challenge has been reported previously by workers in this laboratory for both *H. influenzae* and *Pseudomonas aeruginosa* (7, 41) and is analogous to that of immunization by oral delivery of vaccine antigens (12).

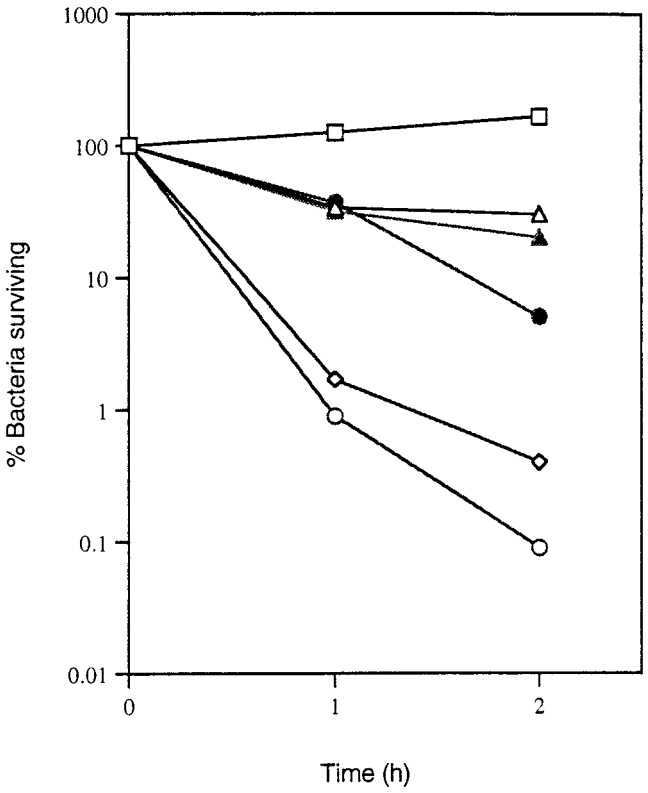

FIG. 3. Bactericidal activity of serum from P6-immunized and nonimmune rats to bacteria from strain NTHi-I. Serum samples were from nonimmune rats (□), rats immunized via intestinal PP with NTHi-I P6 (◇), NTHi-II P6 (△), HI-CD P6 (▲), or Hib-II P6 (●), and rats immunized via intestinal PP and given an IT boost with NTHi-I P6 (○). The values shown are percentages of bacteria surviving from the means of triplicate cultures at 0, 1, and 2 h with 10% serum in the presence of complement.

The data presented in this study suggest that there is some variation in response between strains following immunization with the P6 antigen. PP immunization with P6 from the Hib and capsule-deficient strains enhanced clearance after a challenge with the capsule-deficient strain, indicating a role for the capsule in protecting the P6 epitopes. PP immunization with P6 from the NTHi-I and NTHi-II strains did not result in clearance of the challenge bacteria of any of the four strains. The effectiveness of the pulmonary boost with P6 enhanced the protective capacity of antigen-specific responses such that clearance was observed after challenges with both NTHi strains and the capsule-deficient strain; however, there was still some difference in the degrees of clearance observed with the different strains. These results suggest differences between strains in responding to P6-specific immunity, whether as a result of the way the protein is expressed on the bacterial surface, such as alteration of the percentage of protein content, or of the degree of exposure of surface epitopes. However, the successful enhancement of clearance of the *H. influenzae* strains investigated indicates that immunization with NTHi-I P6 was effective in clearing both the nontypeable and capsule-deficient strains.

Clearance of most bacteria from the lungs involves recruitment of phagocytic cells in response to the presence of a foreign particle and to chemotaxins produced at the site of infection (36, 40). The role of pulmonary alveolar macrophages is to protect the lungs against inhaled infectious microorganisms and to act as a secretory organ capable of augmenting the antimicrobial activities of other types of leukocytes through their ability to both produce and secrete cytokines but also by acting as antigen-presenting cells for induction of antigen-specific responses following immunization (36). The rate of recruitment of PMNs to the lungs is important to the early clearance of an NTHi infection (38, 41). The data for recruit-

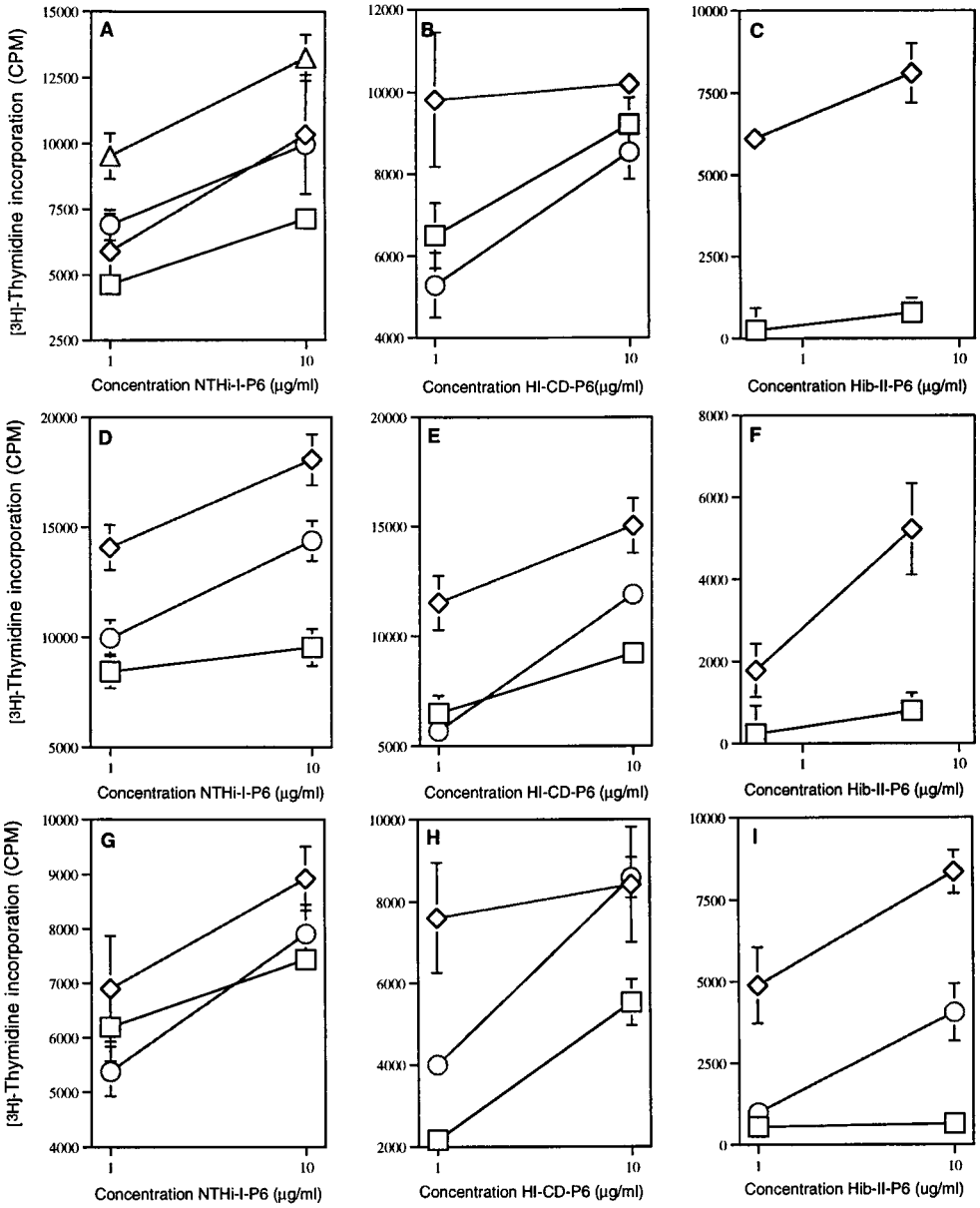

FIG. 4. P6-specific proliferation of lymphocytes isolated from the MLN of groups of immune and nonimmune rats. Rats were immunized with killed bacteria or P6 from strain NTHi-I (A, B, and C), HI-CD (D, E, and F), or Hib-II (G, H, and I) and cultured with either NTHi-I P6 (A, D, and G), HI-CD P6 (B, E, and H), or Hib-II P6 (C, F, and I). MLN were removed from nonimmune rats (□), rats immunized with P6 (◇), and rats immunized with killed bacteria (○) 14 days postimmunization via intestinal PP. MLN from rats immunized via PP and given an IT boost with P6 (△) were collected 21 days postimmunization via PP. The values shown are means ± the standard errors of the means of triplicate cultures for each of four rats per group at two concentrations.

ment of PMNs and the correlation with enhancement of clearance found in this study are in agreement with these previous findings. In the rat model, the percentage ratio of PMNs to macrophages in BAL fluid does not significantly differ between challenge groups at 4 h postinoculation with live bacteria; however, the total number of cells recruited to the lungs at this time is significantly increased by immunization and correlates with clearance. Effective recruitment of PMNs in animals immunized with NTHi-I P6 for clearance of challenge NTHi bacteria occurred when PP immunization was followed by a pulmonary boost. The importance of a pulmonary boost with antigen in affording protection from challenge may be due to a requirement to enhance the local antigen-specific immune responses to overcome some inhibitory mechanism inherent in NTHi. Recently, Cundell and coworkers (9) have demonstrated the inhibitory activity of low-molecular-weight *N*-acetyl-D-glucosamine-containing glycopeptides from NTHi on neutrophil chemotaxis and have suggested that these compounds may contribute to the persistence of this bacterium in vivo.

The importance of antigen-specific antibodies in protection against NTHi infection is still not well defined. A recent study by Yamanaka and Faden (45) found that antibody to P6 in both otitis media-prone and normal children was common and present in the middle ear spaces and while the levels of antibody in the middle ear fluid and the nasopharynx did show some differences between the two groups, only the level of

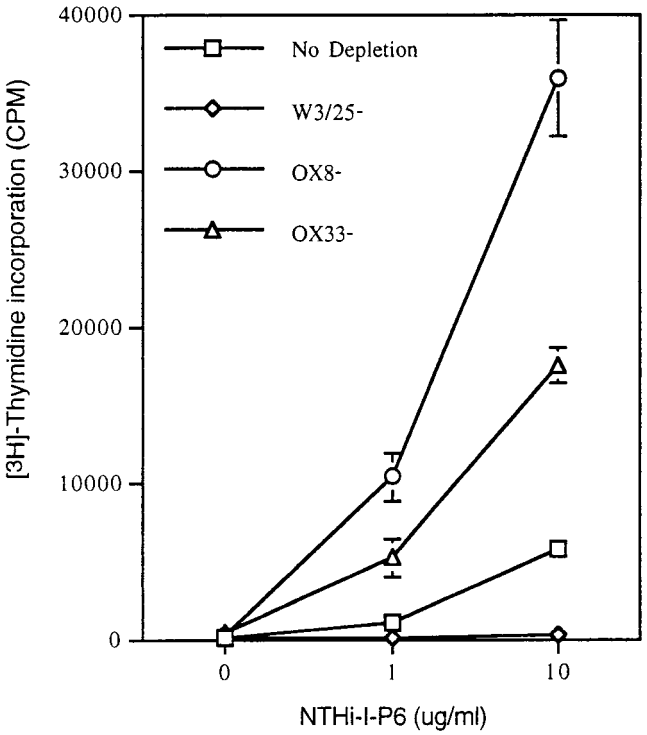

FIG. 5. P6-specific lymphocyte proliferation following depletion of lymphocyte subsets with monoclonal antibodies. The values shown are means ± the standard errors of the means for triplicate cultures of lymphocytes isolated from the MLN of four rats per group immunized with NTHi-I P6 following depletion of the $CD4^+$ helper phenotype with W3/25 (◇), the $CD8^+$ cytotoxic phenotype with OX8 (○), and B cells with OX33 (△).

secretory IgA was significantly elevated in the middle ear fluid of the noninfected children. Mucosal antibodies have been shown to inhibit microbial adherence, and in this respect, IgA and secretory IgA are more effective than IgG of the same specificity. A number of studies have shown IgA to be capable of potentiating the function of the innate antibacterial factors present in external secretions and interacting with mucosal phagocytic cells and lymphocytes (23). Antigen-specific IgG antibody is important for opsonophagocytosis (40), although a correlation between the presence of opsonizing antibodies and protection in vivo has not been clearly determined as an essential mechanism of effective immunity (38, 39, 42). Immunization with P6 induced significant levels of IgG and IgA, which were increased by an IT boost. Anti-P6 antibody in both serum and BAL fluid was shown to be highly cross-reactive and did not differ significantly for P6 from any of the four strains studied. Hence, the variation in clearance observed following a single immunization could not be attributed to significant changes in cross-reacting antibodies.

The suitability of P6 as a vaccine component has been demonstrated by studies showing that P6 was a target of human bactericidal antibody in vitro (14, 29) for both Hib and nontypeable strains. The bactericidal assays performed in the present study have shown that the serum from rats immunized with P6 was bactericidal, with the percentage of killing being greater with serum from the homologous strain and greatest with serum from animals that had received an IT boost. Immunoblot analysis (data not shown) detected the presence of anti-P6 antibodies as the only difference between P6-immunized and nonimmune sera. The bactericidal activity of anti-P6 antibody in serum does not correspond to clearance, since PP immunization without an IT boost failed to enhance bacterial clearance, indicating a lack of correlation between the in vitro bactericidal assay and in vivo protection.

PP immunization induced a P6-specific $CD4^+$ T-helper cell response in the lymphocytes isolated from the MLN. The response of these lymphocytes was not specific to P6 from the homologous strain, again demonstrating the high degree of cross-reaction with this antigen; however, there was some variation in P6-specific responses to cells isolated from animals that had been immunized with killed bacteria. This supports the clearance data indicating some variation in the surface recognition of this protein between strains or mechanisms integral to the bacterial strain that influence the immune response. The antigen-specific lymphocytes in the MLN can migrate from the inductive site in the small intestine to the effector sites of other mucosal surfaces, including the lungs (18). The enhanced proliferation in the MLN following an IT boost with P6 suggests that the antigen expanded the population of antigen-specific lymphocytes in the bronchus-associated lymphoid tissue either by restimulated lymphocytes in the lungs recirculating to the MLN or by antigen reaching the MLN. Since the IT boost was effective with a relatively small soluble protein without an adjuvant, it is likely that the activation of T cells followed presentation and recognition of peptide determinants on the surface of immune B cells present in the lungs (1). This may also be important in the failure of animals immunized with killed bacteria and boosted with P6 to clear live bacteria to the same extent as did animals given killed bacteria in both inoculations. PP immunization with killed bacteria failed to induce significant levels of P6 antibodies, and hence, there would be very low levels of P6-specific B cells in the lungs when these rats received an IT boost with P6.

These studies have attempted to assess the potential of OMP P6 as a candidate for an effective vaccine against nontypeable *H. influenzae* infection. The presence of significant levels of P6 antibody that were also bactericidal did not correlate with clearance in vivo. Essential for successful induction of clearance of NTHi challenge bacteria was the ability to induce effective mucosal antigen-specific responses in vivo leading to recruitment of phagocytic cells to the lungs. The data indicate that this highly conserved protein has the potential to afford early clearance of challenge bacteria by both homologous and heterologous strains of live *H. influenzae* but only in accompaniment with antigen-specific B- and T-cell responses in mucosal tissues.

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