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Importance of an Immunodominant Surface-Exposed Loop on Outer Membrane Protein P2 of Nontypeable *Haemophilus influenzae*

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Nontypeable *Haemophilus influenzae* (NTHI) frequently causes recurrent infections of the respiratory tract in humans. Previous indirect evidence suggested that a strain-specific immune response occurs following infection and that this immune response is directed at an immunodominant epitope on the bacterial surface. To test this hypothesis, mice and rabbits were immunized with whole cells of a strain of NTHI and the antiserum was characterized to identify the antigens to which antibodies were directed. All animals made a prominent antibody response to the loop 5 region of the P2 molecule, which is the major outer membrane protein. Rabbit serum showed complement-dependent bactericidal activity. Adsorption of the immune serum with the loop 5 fusion peptide removed bactericidal activity and also abolished reactivity to P2 detected by an immunoblot assay, an enzyme-linked immunosorbent assay, and a radioimmunoprecipitation assay. These data indicate that immunization with whole cells of NTHI results in a prominent antibody response which is directed at epitopes on the loop 5 region of the P2 molecule. Thus, a strain-specific immune response to NTHI occurs as a result of the expression of an immunodominant epitope on the P2 molecule.

A hallmark of human infections caused by nontypeable strains of *Haemophilus influenzae* (NTHI) is the high rate of recurrences. Adults with chronic bronchitis experience recurrent episodes of lower respiratory tract infection caused by NTHI (16). A subset of children experience repeated episodes of otitis media (10). A prospective study by Faden et al. (4) showed that children develop a serum bactericidal antibody response to their infecting strain. Recurrent episodes of otitis media are caused by new strains of NTHI which are relatively resistant to serum killing in spite of persistent bactericidal antibody to the first strain (4). This observation suggests that a protective immune response to NTHI occurs following otitis media but that this immune response is specific for the infecting strain, leaving the host susceptible to infection by other strains of NTHI. Little is known about the mechanism by which a strain-specific antibody response occurs in nonencapsulated bacteria. The goal of the present study is to analyze that mechanism.

The outer membrane of NTHI contains six to eight major proteins (1, 15). The P2 protein is present in all strains and functions as a porin, allowing small hydrophilic molecules to traverse the outer membrane (21). P2 is the most abundant outer membrane protein, constituting approximately half the protein content of the outer membrane, and is an important target of the immune response to NTHI (12, 13). A topographical model of the P2 protein, as it is arranged in the outer membrane of the intact bacterial cell, has been determined. According to this model, P2 contains 16 transmembrane regions and 8 potentially surface-exposed loops (Fig. 1) (3, 17, 19). Analysis of sequences of P2 genes from 16 strains of NTHI indicates that the transmembrane regions are relatively conserved among strains while considerable heterogeneity exists in several of the loop regions of the molecule (2, 3, 17). Monoclonal antibody analysis of epitopes on P2 has revealed that many of the antibodies, which we have developed by immunizing mice with whole bacterial cells, recognize abundantly expressed epitopes on loop 5 of the P2 molecule (7, 23). These observations suggest that loop 5 is an immunodominant region of the molecule. We hypothesize that the expression of an immunodominant epitope by the bacterium induces a strain-specific host immune response. To test this hypothesis, mice and rabbits were challenged with whole bacterial cells of NTHI and the antigenic specificity of the antibody response was analyzed with particular emphasis on epitopes of the P2 molecule.

MATERIALS AND METHODS

Bacterial strain and growth conditions. NTHI 1479 was isolated from the sputum of a patient with chronic bronchitis in Buffalo, N.Y. This strain was cultured on chocolate agar at 37°C under 5% CO_2. For the radioimmunoprecipitation (RIP) assay, bacteria were grown in brain heart infusion medium supplemented with NAD (10 μg/ml) and hemin (10 μg/ml) at 37°C with vigorous shaking for 6 h.

Immunization of animals. Five rabbits were immunized intravenously with approximately 2×10^8 bacteria of strain 1479 on day 0 and with approximately 1×10^8 bacteria on day 26. Five mice were immunized intraperitoneally with approximately 7.5×10^7 bacteria of strain 1479 on day 0 and with approximately 5.0×10^7 bacteria on day 26. The bacteria were suspended in phosphate-buffered saline (PBS) and were administered without adjuvant. Antisera were collected on day 35.

Recombinant fusion proteins. Clones which contain the gene fragments of loop 1, 2, 3, 4, 6, or 7 were newly constructed in the pGEX-2T vector. Table 1 shows the amino acid numbers in each construct. Clones for loop 5 and loop 8 were made as described previously (7). Methods for construction and expression of the fusion proteins are described by Haase et al. (7). The plasmid insert of each clone was sequenced to confirm that the correct sequence was present in each of the constructs.

Immunoblot assay. Whole cells of strain 1479 were suspended in sample buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting as previously described (14). Prestained molecular mass standards (Bio-Rad) were included in a lane of each gel. After the gel was transferred to nitrocellulose, the blot was blocked with 3% skim milk in buffer A (0.01 M Tris, 0.15 M NaCl [pH 7.4]) and probed with an appropriate

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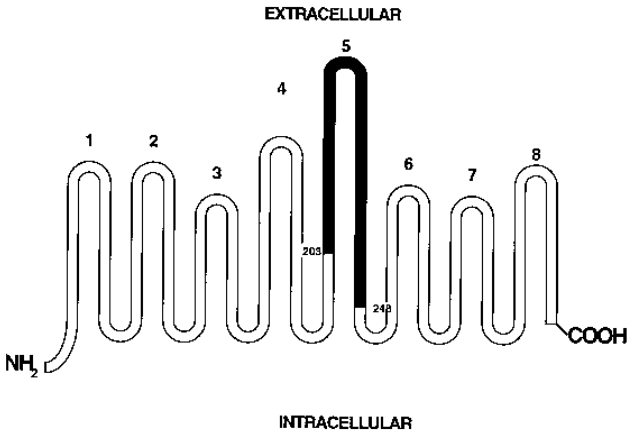

FIG. 1. Schematic diagram of the P2 molecule of NTHI 1479 depicting eight potentially surface-exposed loops. Each loop is numbered at the top. The region of loop 5 which is expressed as a fusion protein is noted, with amino acid numbers corresponding to those in the mature protein (17).

dilution of serum. Protein A-peroxidase (Zymed, San Francisco, Calif.) was used as a conjugate at a 1:3,000 dilution. Subsequently, the blot was developed with horseradish peroxidase color development solution containing 0.15% H_2O_2 (Bio-Rad Laboratories, Richmond, Calif.).

Preparation of LOS. Lipooligosaccharide (LOS) was prepared by proteinase K digestion by using a modification of the method of Hitchcock and Brown (9). Bacteria were harvested from one chocolate agar plate after overnight incubation and suspended in PBS to an optical density of 0.3 at 600 nm. The cells were centrifuged, suspended in 0.33 ml of lysing buffer (1 M Tris, 4% β-mercaptoethanol, 10% glycerol, 2% SDS [pH 6.8]), and incubated at 95°C for 1 h. A volume of 200 μl of proteinase K (1 mg/ml) in lysing buffer was added, and the suspension was incubated at 65°C for 1 h. Debris was removed by centrifugation at 11,000 × *g* for 15 min, and the supernatant was subjected to SDS-PAGE and immunoblot assay.

Adsorption of antisera. Aliquots of rabbit antiserum were adsorbed with purified loop 5 fusion peptide (from 800 ml of culture), purified loop 8 fusion peptide (negative control), and purified glutathione-*S*-transferase (GST) (negative control). Antiserum was heat inactivated (56°C for 30 min) prior to adsorption. Purification of the proteins was performed as described by Haase et al. (7). Glutathione-agarose beads coupled with GST fusion protein were mixed and incubated with antiserum at a dilution of 1:500 for 2 h at 4°C with continuous mixing. The mixture was centrifuged, and the supernatant was concentrated to a 1:10 dilution by difiltration and used as the adsorbed antiserum.

Enzyme-linked immunosorbent assay (ELISA). Purified P2 of strain 1479 was used to coat wells of a microtiter plate (14). P2 was diluted in coating buffer (0.1 M sodium carbonate, 0.1 M sodium bicarbonate [pH 9.6]) to 2 μg/ml, and 100 μl was added to each well. The plate was incubated at room temperature overnight. The wells were blocked for 1 h with PBS containing 3% goat serum. The plate was washed with PBS–0.05% Tween 20 five times. Adsorbed sera were diluted in assay diluent (PBS, 0.05% Tween 20, 3% goat serum), added to the wells, and incubated for 2 h at 37°C. The wells were washed, and protein A-horseradish peroxidase conjugate (Zymed) was applied to each well at a 1:3,000 dilution and incubated for 1 h at room temperature. Tetramethylbenzidine (Sigma)–0.015% hydrogen peroxide was used as the substrate for color development. The reaction was stopped by adding 4 N H_2SO_4, and the optical density at 450 nm was read.

RIP. Strain 1479 was grown in 3 ml of broth in the presence of 500 μCi of [^{3}H]leucine (New England Nuclear, Boston, Mass.) per ml. The bacteria were harvested at late logarithmic phase and divided into three portions. After four washes with PBS, each pellet was resuspended in 100-μl aliquots of antiserum adsorbed with GST, antiserum adsorbed with loop 5, and unadsorbed antiserum. The mixtures were incubated for 90 min at 4°C with inverting. After being washed twice with PBS, each sample was resuspended in 1 ml of solubilization buffer (0.01 M Tris, 0.15 M NaCl, 0.01 M EDTA, 1% Triton X-100, 0.2% [wt/vol] sodium deoxycholate, 0.1% [wt/vol] SDS [pH 8.0]). The samples were subjected to mixing for 60 min at 37°C. Solubilized mixtures were centrifuged at 16,000 × *g* for 30 min at room temperature to remove debris. To each supernatant, 300 μl of protein A-Sepharose (Pharmacia, Piscataway, N.J.) was added, and the mixture was incubated for 60 min at 4°C. The samples were washed three times with solubilization buffer and subjected to SDS-PAGE. Autoradiography was carried out by the method of Yohe et al. (24). The gel was treated in fixer (isopropanol-water-acetic acid [25:65:10]) for 30 min; this was followed by Amplyfy (Amersham Corp., Arlington Heights, Ill.) enhancement for 30 min with slow mixing. The gel was vacuum dried at 65°C for 1 h by using a gel dryer. XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) was hypersensitized with 7% hydrogen–93% nitrogen at 47°C for 18 h. The film was exposed to the gel for 3 days.

Bactericidal assays. Bactericidal assays were performed by a method which was modified slightly from a method used previously (7). Briefly, bacteria were grown to mid-logarithmic phase in broth, and then diluted to 5×10^4 CFU/ml in Gey's balanced salt solution containing 10% (wt/vol) bovine serum albumin and 2.5% (vol/vol) fetal bovine serum (dilution buffer). The reaction mixture was made of 25 μl of diluted bacteria, 22 μl of complement, serum at the appropriate dilution, and dilution buffer to make the total volume up to 250 μl. The sera were heat inactivated at 56°C for 30 min. The complement was prepared from normal human serum from which immunoglobulin G was depleted by adsorption over a protein G column. The complement was added last. The reaction mixture was incubated in a 37°C water bath with shaking. Duplicates of 25-μl aliquots from each reaction mixture were plated on chocolate agar at 0, 30, and 60 min. The plates were incubated at 37°C in 5% CO_2 overnight. Colonies were counted the next day.

TABLE 1. Amino acid numbers of fusion proteins of loops 1 through 8 of the P2 protein of NTHI 1479

Loop	Amino acids
1	22–43
2	69–85
3	115–133
4	155–175
5	203–248
6	262–277
7	303–315
8	331–364

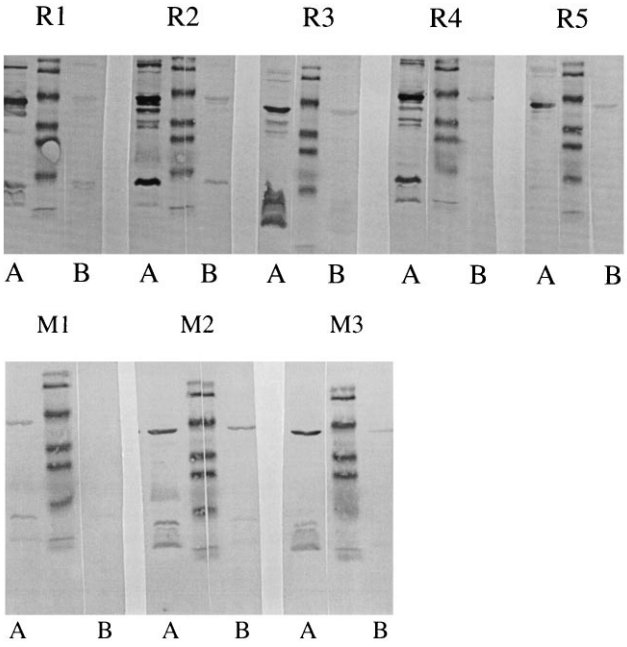

FIG. 2. Immunoblot assays with bacterial cell lysates of strain 1479. Panels marked R1 through R5 show sera from five rabbits immunized with whole bacterial cells. Panels marked M1 through M3 show sera from three of five mice immunized with whole bacteria. Lanes: A, 1:500 serum dilution; B, 1:5,000 serum dilution. The center lane of each panel contains prestained molecular mass standards with the following sizes from top to bottom in kilodaltons: 101, 83, 50.6, 35.5, 29.1, and 20.9.

RESULTS

Immunoblot assay of whole-cell lysates. Rabbit and mouse sera from animals challenged with whole bacterial cells were analyzed in an immunoblot assay with a whole bacterial cell lysate (Fig. 2). Each serum was tested at dilutions of 1:500 and 1:5,000. All animals made an antibody response to the P2

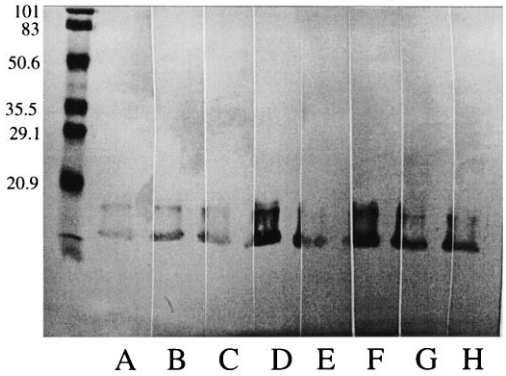

FIG. 3. Immunoblot assay. Lanes contain LOS prepared by proteinase K digestion of strain 1479. Lanes A through C were assayed individually with mouse sera, and lanes D through H were assayed with rabbit sera. All sera were tested at a dilution of 1:100. Molecular mass standards are noted on the left in kilodaltons.

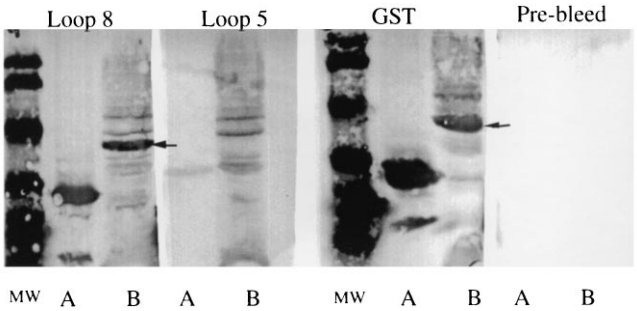

FIG. 5. Immunoblot assays of rabbit antiserum (1:2,000 dilution). Lanes A contain the loop 5 fusion protein of strain 1479, and lanes B contain whole bacterial cell lysates of strain 1479. The four panels were incubated with antiserum as follows: Loop 8, antiserum adsorbed with loop 8-GST fusion protein; Loop 5, antiserum adsorbed with the loop 5-GST fusion protein; GST, antiserum adsorbed with GST; Pre-bleed, serum obtained before immunization with strain 1479. The arrows identify outer membrane protein P2. Lanes MW contain molecular mass markers, which are as in Fig. 4.

protein. At the lower dilution, antibodies to other bands were observed. The band at approximately 16 kDa is outer membrane protein P6, proven by an immunoblot assay with purified P6 (data not shown). At the higher dilution, the most prominent band was the P2 protein. Three of the rabbit antisera were tested in an immunoblot assay with whole-cell lysates of seven other strains of NTHI. At a dilution of 1:5,000, the P2 band of strain 1479 was detected while the P2 bands of the other seven strains were not detected by the antisera (data not shown).

Some of the antisera contained antibodies to a broad band in the lower-molecular-weight range in immunoblot assays with whole bacterial cell lysates of strain 1479. To determine whether these were antibodies to LOS, LOS was purified by a method involving proteinase K. These preparations were tested in immunoblot assay at serum dilutions of 1:100. Figure 3 shows that the animals made antibodies to LOS and that these antibodies are detected at low dilutions of serum. Preimmune sera contained no antibodies to LOS.

Immunoblot assay with surface-exposed loops of P2. To analyze the epitopes of the P2 molecule to which antibodies were directed, the eight potentially surface-exposed loops of P2 of strain 1479 were individually expressed as fusion peptides with GST by using the pGEX2T plasmid vector (7, 18). The purified fusion proteins were analyzed by immunoblot assays against antisera. Figure 4 shows the results obtained with one of the mouse sera. A prominent antibody response exclusively to the loop 5 peptide is observed. To ensure that adequate transfer of the proteins had occurred, the immunoblot was stained with 0.2% amido black. The stain showed a band of the expected size (~28 kDa) in lanes 1 through 8.

All 10 sera of the animals challenged with whole bacterial cells were tested in immunoblot assays with fusion proteins corresponding to the eight loops of P2. All 10 sera yielded the identical result shown in Fig. 4; i.e., the sera reacted exclusively with the loop 5 fusion protein.

Adsorption of antisera with recombinant fusion proteins. Aliquots of rabbit serum were individually adsorbed with the loop 5 fusion protein to remove antibodies to epitopes expressed on the loop 5 peptide. The adsorbed serum was tested in an ELISA and an immunoblot assay along with aliquots of unadsorbed serum, serum adsorbed with the GST protein, and serum adsorbed with the loop 8 fusion peptide as controls. Figure 5 shows that adsorption with loop 5 specifically removed all reactivity for the loop 5 fusion peptide and for the P2 molecule in the immunoblot assay. Figure 6 shows that adsorp-

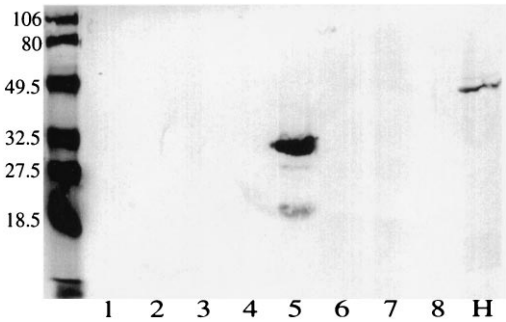

FIG. 4. Immunoblot assay of mouse antiserum (1:500 dilution). Lanes 1 through 8 contain fusion proteins of clones expressing peptides corresponding to loops 1 through 8 of the P2 protein of strain 1479. The peptides are part of fusion proteins with GST. Lane H contains a whole bacterial cell lysate of NTHI 1479. Molecular mass markers are noted on the left in kilodaltons.

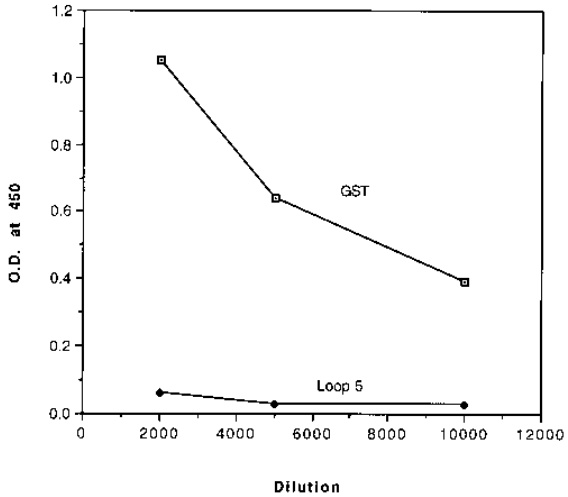

FIG. 6. Results of ELISA with adsorbed antiserum. The *y* axis represents optical density (O.D.) at 450 nm, and the *x* axis represents dilution. P2 purified under nondenaturing conditions was used as coating antigen. The curve marked GST depicts the results with serum adsorbed with GST, and the curve marked loop 5 depicts the results with serum adsorbed with loop 5-GST.

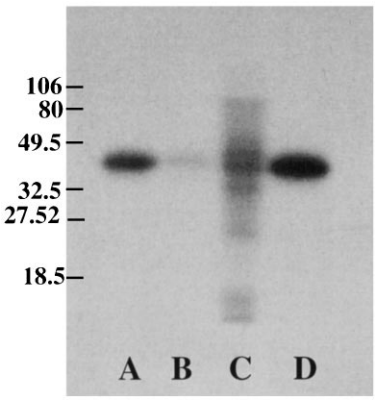

FIG. 7. Autoradiogram of radioimmunoprecipitation of cells of strain 1479 labeled with [^{3}H]leucine. Cells were incubated with aliquots of rabbit antiserum prepared as follows: lane A, antiserum adsorbed with GST; lane B, antiserum adsorbed with loop 5-GST fusion protein; lane D, unadsorbed antiserum. Lane C contains an aliquot of the [^{3}H]leucine-labeled cells. Molecular mass markers are noted on the left in kilodaltons.

tion of the sera with the loop 5 fusion peptide completely eliminated reactivity for the P2 protein in the ELISA. These results further support the conclusion that the most prominent antibody response to the P2 protein was to the loop 5 region.

Whole-cell RIP. To detect antibodies to conformational epitopes which may not be detected in the immunoblot assay, whole-cell RIP was used (8). Figure 7 is an autoradiograph which shows that whole-cell RIP detects a single band corresponding to P2, indicating that antibodies to surface structures were directed exclusively to P2 (by this method of detection) when animals were inoculated with whole bacterial cells. To assess whether whole-cell RIP detects antibodies to conformational epitopes which might not have been detected in the immunoblot assay, aliquots of sera were adsorbed with the loop 5 fusion peptide. We reasoned that if the loop 5 peptide adsorbed antibodies which were detected by RIP, one may conclude that the majority of antibodies in the serum were directed at epitopes on the loop 5 peptide. The adsorbed sera were subjected to RIP in parallel with aliquots of unadsorbed serum and serum adsorbed with GST as a control. Figure 7 shows that the loop 5 peptide adsorbed almost all the antibody reactivity seen by RIP. This experiment indicates that all of the detectable antibodies to P2 bind to epitopes which are present on the loop 5 peptide. These results indicate that when animals were challenged with whole bacterial cells, antibodies were made predominantly to epitopes on loop 5 of the P2 molecule.

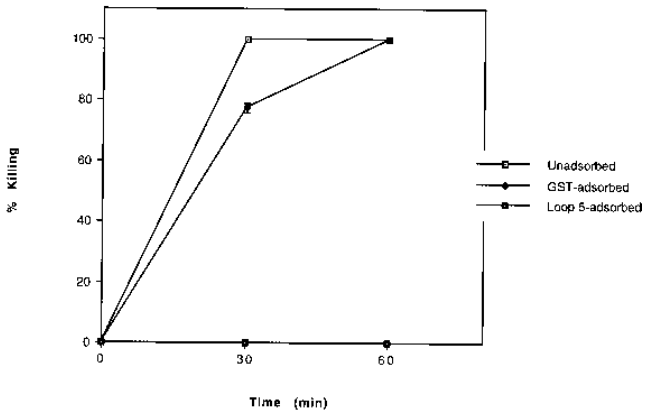

FIG. 8. Results of bactericidal assays. The *y* axis represents percent killing, and the *x* axis represents time in minutes. Each point represents the mean of duplicate values, and the confidence bars represent the ranges. The duplicate values were virtually identical in the points depicted without confidence bars.

Bactericidal assays. The bactericidal activity of the immune serum was evaluated. Serial dilutions of the serum were tested in bactericidal assays. The immune rabbit serum showed complement-dependent bactericidal activity at a concentration of 0.25% and higher. When the concentration of serum in the bactericidal reaction was lower than 0.25%, the serum showed less bactericidal activity. To more precisely characterize the epitopes to which bactericidal antibodies are directed, serum adsorbed with GST and serum adsorbed with the loop 5 fusion peptide were used in the assays. Figure 8 shows that the serum adsorbed with GST retained bactericidal activity while the serum adsorbed with loop 5 fusion peptide lost bactericidal activity at the same concentration (0.25%). Thus, adsorption with the loop 5 fusion peptide abolished the functional activity of the serum when the serum is tested near the end point of dilution of bactericidal activity. This result indicates that loop 5 contains not only immunodominant epitopes but also bactericidal epitopes.

DISCUSSION

The present study shows that immunization of two animal species with whole bacterial cells of NTHI produces antisera which contain a highly restricted population of antibodies. Challenging animals with whole bacterial cells resulted in a prominent antibody response directed at the P2 molecule, the major outer membrane protein on the bacterial surface. All or most of the antibodies to P2 bind a single peptide of 46 amino acids. This region of the P2 molecule is accessible on the surface of the bacterial cell (7). The high degree of surface accessibility of the loop 5 region of the P2 molecule is probably an important factor in the generation of the restricted population of antibodies following immunization with NTHI bacterial cells.

The P2 molecule consists of 16 amphiphilic beta strands and 8 potentially surface-exposed loops (3, 17, 19). While beta strands are embedded in the outer membrane, several loops are potentially surface exposed based on epitope-mapping and bactericidal assays (7, 23). Surface accessibility of epitopes on the bacterial surface is essential for antibodies to be protective. The surface-accessible regions of P2 show sequence heterogeneity among strains. The present study shows that the region of the P2 molecule which reveals the most heterogeneity is also immunodominant in animals.

Both primary structure and three-dimensional structure play important roles in the antigenicity of proteins (11). The immunoblot assay is effective in detecting epitopes defined by primary amino acid sequence. However, some epitopes are denatured when they are subjected to SDS-PAGE and immunoblot assay. Alternative methods are required to detect antibodies to conformational epitopes which are dependent on the secondary, tertiary, and quaternary structure of the protein. Two approaches were used to study the sera from animals immunized with whole bacterial cells for antibodies to conformational epitopes on P2 and other surface antigens.

In previous work, antibodies to conformational epitopes on P2 were detected by ELISA with whole P2 purified under nondenaturing conditions (13). This method detects antibodies which are not detectable in the immunoblot assay (13). The second approach used to detect antibodies to conformational epitopes was whole-cell RIP. The principle of the method is

that antibodies are allowed to bind to epitopes on intact, radiolabeled bacterial cells. This method is effective in identifying antibodies to linear and conformational epitopes on molecules which are present on the surface of intact bacterial cells (6, 8).

Faden et al. (4) showed that serum bactericidal antibodies are associated with strain-specific protection from otitis media. Van Alphen et al. (22) showed that strain-specific monoclonal antibodies exclusively manifested complement-dependent bactericidal activity among monoclonal antibodies raised to NTHI isolated from patients with chronic obstructive pulmonary disease. This suggests that antibodies to strain-specific epitopes are protective. In this study, we demonstrated that most of the functional antibodies were directed to loop 5, which contains strain-specific and immunodominant epitopes. The association of strain-specific antibodies with protection has significant implications in understanding how NTHI, as a species, uses this strategy to evade the host immune response.

The present study was performed with a single strain of NTHI. Indirect evidence suggests that other strains have immunodominant epitopes on P2 as well. Epitopes of monoclonal antibodies raised to whole cells of two other strains of NTHI were mapped to loop 5 of the immunizing strain (23). Furthermore, those monoclonal antibodies showed complement-dependent bactericidal activity, which demonstrated the surface exposure of the epitopes. Duim et al. showed that strains of NTHI from patients with chronic bronchitis underwent point mutations on the epitopes of surface-exposed loops (3). Mutation-prone epitopes were located predominantly on loop 6. They hypothesized that immune selective pressure is responsible for the accumulation of point mutations on surface-exposed, bactericidal, immunodominant epitopes. Different strains may express immunodominant epitopes on different loops. The immunodominant effect may be due to the position of epitopes in loop 5 and may be due to the amino acid content of loop 5. It is likely that both factors play a role. In view of the ability of the molecule to direct antibody formation to a single loop, the P2 molecule might be used as a vehicle to provoke an immune response to immunogenic peptides engineered into the loop 5 position.

The results of the present study directly demonstrate the presence of an immunodominant region on the P2 molecule which induces a strain-specific immune response. This concept is consistent with observations from several recent studies. Groeneveld et al. (5) immunized rabbits with whole cells of variant strains of NTHI which contained point mutations in the P2 gene in regions corresponding to surface-exposed loops. The sera contained bactericidal antibody which was specific for the variant strains and did not kill the parent strains, which differed by only 5 amino acids from the variant. Another study showed that monoclonal antibodies with bactericidal activity were directed at an immunodominant surface loop while antibodies which were directed at other regions of the molecule did not have bactericidal activity (22). Finally, opsonic antibodies were directed exclusively at strain specific epitopes on the surface-exposed loops of P2 (20). All of these observations are consistent with the P2 molecule containing an immunodominant, surface-exposed loop which induces a strain-specific immune response.

Our data indicate that challenging animals with whole cells of a strain of NTHI results in a highly defined immune response. It is tempting to speculate that a similar phenomenon occurs in humans, accounting for the observations of recurrent infections caused by NTHI. However, one must be cautious in view of the limitations of the animal model system. First, the immune responses of animals are different from those of humans due to the dissimilarity of B-cell repertoires, major histocompatibility complex restriction, and other differences. Second, *H. influenzae* is an exclusively human pathogen. Third, the difference in the route of antigen delivery in the present study and in the human situation is substantial. Animals which had not been previously exposed to NTHI were inoculated parenterally with a large number of bacteria. By contrast, patients with chronic obstructive pulmonary disease are intermittently colonized on their respiratory tract mucosa with multiple strains of NTHI over the years. While the present study does not define the immune response in humans, the results can be used as a guide to develop subsequent studies to test the hypothesis that a strain-specific immune response to P2 facilitates recurrent infections.

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REFERENCES

1. **Barenkamp, S. J., R. S. Munson, Jr., and D. M. Granoff.** 1982. Outer membrane protein and biotype analysis of pathogenic nontypable *Haemophilus influenzae*. Infect. Immun. **36:**535–540.
2. **Bell, J., S. Grass, D. Jeanteur, and R. S. Munson, Jr.** 1994. Diversity of the P2 protein among nontypeable *Haemophilus influenzae* isolates. Infect. Immun. **62:**2639–2643.
3. **Duim, B., J. Dankert, H. M. Jansen, and L. van Alphen.** 1993. Genetic analysis of the diversity in outer membrane protein P2 of non-encapsulated *Haemophilus influenzae*. Microb. Pathog. **14:**451–462.
4. **Faden, H., J. Bernstein, L. Brodsky, J. Stanievich, D. Krystofik, C. Shuff, J. J. Hong, and P. L. Ogra.** 1989. Otitis media in children. I. The systemic immune response to nontypable *Haemophilus influenzae*. J. Infect. Dis. **160:** 999–1004.
5. **Groeneveld, K., L. van Alphen, C. Voorter, P. P. Eijk, H. M. Jansen, and H. C. Zanen.** 1989. Antigenic drift of *Haemophilus influenzae* in patients with chronic obstructive pulmonary disease. Infect. Immun. **57:**3038–3044.
6. **Gulig, P. A., G. H. McCracken, Jr., C. F. Frisch, K. H. Johnston, and E. J. Hansen.** 1982. Antibody response of infants to cell surface-exposed outer membrane proteins of *Haemophilus influenzae* type b after systemic *Haemophilus* disease. Infect. Immun. **37:**82–88.
7. **Haase, E. M., K. Yi, G. D. Morse, and T. F. Murphy.** 1994. Mapping of bactericidal epitopes on the P2 porin protein of nontypeable *Haemophilus influenzae*. Infect. Immun. **62:**3712–3722.
8. **Hansen, E. J., C. F. Frisch, and K. H. Johnston.** 1981. Detection of antibody-accessible proteins on the cell surface of *Haemophilus influenzae* type b. Infect. Immun. **33:**950–953.
9. **Hitchcock, P. J., and T. M. Brown.** 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. **154:**269–277.
10. **Klein, J. O.** 1994. Otitis media. Clin. Infect. Dis. **19:**823–833.
11. **Lando, G., J. A. Berzofsky, and M. Reichlin.** 1982. Antigenic structure of sperm whale myoglobin. 1. Partition of specificities between antibodies reactive with peptides and native protein. J. Immunol. **129:**206–211.
12. **Munson, R. S., Jr., J. L. Shenep, S. J. Barenkamp, and D. M. Granoff.** 1983. Purification and comparison of outer membrane protein P2 from *Haemophilus influenzae* type b isolates. J. Clin. Invest. **72:**677–684.
13. **Murphy, T. F., and L. C. Bartos.** 1988. Human bactericidal antibody response to outer membrane protein P2 of nontypeable *Haemophilus influenzae*. Infect. Immun. **56:**2673–2679.
14. **Murphy, T. F., and L. C. Bartos.** 1988. Purification and analysis with monoclonal antibodies of P2, the major outer membrane protein of nontypable *Haemophilus influenzae*. Infect. Immun. **56:**1084–1089.
15. **Murphy, T. F., K. C. Dudas, J. M. Mylotte, and M. A. Apicella.** 1983. A subtyping system for nontypable *Haemophilus influenzae* based on outer-membrane proteins. J. Infect. Dis. **147:**838–846.
16. **Murphy, T. F., and S. Sethi.** 1992. Bacterial infection in chronic obstructive pulmonary disease. Am. Rev. Respir. Dis. **146:**1067–1083.
17. **Sikkema, D. J., and T. F. Murphy.** 1992. Molecular analysis of the P2 porin protein of nontypeable *Haemophilus influenzae*. Infect. Immun. **60:**5204–5211.
18. **Smith, D. B., and K. S. Johnson.** 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. Gene **67:**31–40.

19. **Srikumar, R., D. Dahan, M. F. Gras, M. J. H. Ratcliffe, L. van Alphen, and J. W. Coulton.** 1992. Antigenic sites on porin of *Haemophilus influenzae* type b: mapping with synthetic peptides and evaluation of structure predictions. J. Bacteriol. **174:**4007–4016.
20. **Troelstra, A., L. Vogel, L. van Alphen, P. Eijk, H. Jansen, and J. Dankert.** 1994. Opsonic antibodies to outer membrane protein P2 of nonencapsulated *Haemophilus influenzae* are strain specific. Infect. Immun. **62:**779–784.
21. **Vachon, V., D. N. Kristjanson, and J. W. Coulton.** 1988. Outer membrane porin protein of *Haemophilus influenzae* type b pore size and subunit structure. Can. J. Microbiol. **34:**134–140.
22. **van Alphen, L., P. Eijk, L. Geelen-van den Broek, and J. Dankert.** 1991. Immunochemical characterization of variable epitopes of outer membrane protein P2 of nontypeable *Haemophilus influenzae*. Infect. Immun. **59:**247–252.
23. **Yi, K., and T. F. Murphy.** 1994. Mapping of a strain-specific bactericidal epitope to the surface-exposed loop 5 on the P2 porin protein of nontypeable *Haemophilus influenzae*. Microb. Pathog. **17:**277–282.
24. **Yohe, H. C., C. S. Berenson, C. L. Cuny, and J. L. Ryan.** 1990. Altered B-lymphocyte membrane architecture indicated by ganglioside accessibility in C3H/HeJ mice. Infect. Immun. **58:**2888–2894.

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