Surface-Exposed and Antigenically Conserved Determinants of Outer Membrane Proteins of *Branhamella catarrhalis*

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The outer membrane proteins (OMPs) of *Branhamella catarrhalis* were studied in an effort to identify surface-exposed determinants that are conserved among strains of the bacterium. Aliquots of polyclonal antiserum were absorbed individually by strains of *B. catarrhalis*. The absorbed antisera were tested in contrast with unabsorbed antiserum in an immunoblot assay against OMPs of the homologous strain. The absence of a band recognized by antibodies in the absorbed antiserum compared with the unabsorbed antiserum indicated that surface-exposed determinants of the absorbing strain cross-react with determinants on the homologous strain. Two antisera were absorbed individually by 20 strains of *B. catarrhalis*, and the absorbed sera were studied in this way in immunoblot assays. OMP E (molecular weight, ca. 56,000) expresses surface-exposed determinants that are shared among 17 of the 20 strains studied. Antibodies to OMP G (molecular weight, 28,000) were absorbed from both antisera by 14 of the 20 strains. These studies demonstrate that OMP E and OMP G express determinants that are exposed on the surface of the intact bacterium. Furthermore, these determinants are antigenically conserved among a majority of strains of *B. catarrhalis*. On the basis of these observations, OMPs E and G should be considered when bacterial antigens are evaluated as potential vaccine candidates.

The role of *Branhamella catarrhalis* as a human pathogen has become increasingly apparent in the last decade. The organism, previously regarded as one of the nonpathogenic neisseriae, is being isolated more frequently from patients in specific clinical settings, particularly otitis media and chronic pulmonary disease (3, 5, 17, 19, 20, 22). *B. catarrhalis* is the third most common cause of bacterial otitis media after *Streptococcus pneumoniae* and nontypable *Haemophilus influenzae*, as shown by cultures of middle-ear fluid (3, 6, 9, 12, 13, 22). A variety of studies involving cultures and studies of the immune response show that *B. catarrhalis* causes lower respiratory tract infection in patients with chronic pulmonary disease (1, 4, 7, 17, 18, 21).

Recent studies indicate that the outer membrane proteins (OMPs) of 50 strains of *B. catarrhalis* from diverse geographic and clinical sources are similar in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), in contrast to other nonenteric gram-negative bacteria (2). These OMPs range in molecular mass from ca. 98,000 to 21,000 daltons and are designated OMP A through OMP H (2). The antigenic characteristics of these proteins have not been described. The current study is designed to promote an understanding of the antigenic characteristics of these OMPs. This will allow us to determine whether the molecular mass similarities among OMPs of strains of *B. catarrhalis* are paralleled by antigenic conservation of OMPs. Identifying antigenically conserved, surface-exposed determinants of OMPs represents an initial step in studying potential vaccine candidates.

**MATERIALS AND METHODS**

Zwittergent extract of OMPs. The outer membranes of strains ATCC 25240 and 8184 were obtained from Zwittergent extracts. Thirty chocolate agar plates were used. Bacteria were scraped from the plates into 25 ml of PBS (0.01 M sodium phosphate, 0.15 M sodium chloride [pH 7.2]) and harvested by centrifugation at 12,000 × g for 20 min at 4°C. The bacteria were suspended in 10 ml of 1 M sodium acetate-0.001 M β-mercaptoethanol (pH 4.0). A 90-ml volume of a solution containing 5% Zwittergent Z 3-14 (Calbiochem-Behring, La Jolla, Calif.) and 0.5 M CaCl₂ was added, and the suspension was mixed for 1 h at room temperature. Nucleic acids were precipitated by the addition of 25 ml of cold ethanol and subsequent centrifugation at 17,000 × g for 10 min at 4°C. The resulting proteins were precipitated by the addition of 375 ml of cold ethanol and collected by centrifugation at 17,000 × g for 20 min at 4°C. The pellets were allowed to dry and were then suspended in 10 ml of buffer Z (0.05% Zwittergent, 0.05 M Tris, 0.01 M EDTA [pH 8.0]) and mixed for 1 h at room temperature. OMPs are present in the buffer Z soluble fraction after centrifugation of this material at 12,000 × g for 10 min at 4°C. To assess for cytoplasmic membrane contamination, we assayed these samples for the presence of succinic dehydrogenase activity.

**Development of antisera.** Two antisera (anti-25240 and anti-8184) were obtained by intravenous immunization of rabbits with whole bacteria. To remove background reactivity in immunoblot assays, we subjected the sera to affinity chromatography on a column of Sepharose-4B, containing covalently bound protein A, prior to all absorption experiments.

**Absorption of antisera.** Antisera were absorbed by the method of Loeb and Woodin (10, 11). Mid-logarithmic-phase cells were suspended in 0.8 ml of PCM (PBS containing 0.5 mM MgCl₂ and 0.15 mM CaCl₂) and put on ice. An equal volume of a 1:50 dilution of protein A-purified antiserum in PCM was added to the cell suspension, and the mixture was kept on ice for 30 min with intermittent shaking. Absorbed antiserum was then obtained by centrifugation.

**Stability of bacteria.** To determine the stability of *B. catarrhalis* strains during absorption conditions, we grew 70

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ml of strain 8184 to mid-logarithmic phase in double-strength medium B4, a defined medium for *B. catarra*halis* (8). To this medium, 12.5 μCi of L-[4,5-3H]leucine per ml (63 Ci/mmol; Amersham Corp. Arlington Heights, Ill.) was added at the time of inoculation. The cells were collected and washed as described above, and absorptions were carried out separately with diluted antiserum (anti-8184) and with PCM only. After the cells were centrifuged, the amount of radioactivity released during absorption was determined. The released radioactivity was size fractionated into high- and low-molecular-weight material on a 2-ml Bio-Gel P2 column (Bio-Rad Laboratories Richmond, Calif.).

**SDS-PAGE and immunoblot.** OMP preparations were subjected to SDS-PAGE and immunoblot assays by previously described methods (15). The nitrocellulose was then cut into individual strips, blocked for 1 h in 0.1% PBS-Tween, and incubated with shaking in a 1:100 dilution of the appropriate absorbed or unabsorbed antiserum for 2 h. After being washed with 0.1% PBS-Tween, the strips were incubated in a 1:3,000 dilution of protein A-peroxidase conjugate in 0.1% PBS-Tween for 1 h, washed again, and developed in a solution of horseradish peroxidase color development solution (0.15% H2O2; Bio-Rad) for 45 min. The intensities of the antibody reaction to individual components on the strips with unabsorbed and absorbed antisera were compared. A decrease in intensity of a particular band demonstrated that the absorbing strain bound antibodies to that particular outer membrane component.

**RESULTS**

**Outer membrane preparations.** To identify the OMPs against which antibodies are directed, it was necessary to subject purified outer membranes of *B. catarra*halis to SDS-PAGE and immunoblot assays. To accomplish this, a method involving extraction of outer membrane with the nonionic detergent Zwittergent was adapted for *B. catarra*halis from methods developed for isolating OMPs of *H. influenzae* (14). The resulting preparations were tested to determine whether they represented purified outer membrane samples. Comparison of Zwittergent-extracted outer membrane with that isolated by isopycnic centrifugation showed that the preparations were identical (16). All eight of the previously identified OMPs (OMPs A through H) of *B. catarra*halis were present in the Zwittergent-extracted outer membrane (2, 16). The preparations were assayed for the presence of succinic dehydrogenase, a marker for cytoplasmic membrane. The Zwittergent-extracted outer membrane of strain 8184 contained no detectable succinic dehydrogenase activity, whereas a simultaneously assayed whole-organism lysate hydrolyzed 4.2 μmol of succinate per min per mg of protein. Similar results were obtained with Zwittergent extracts of two other strains. These assays indicated that the Zwittergent-extracted outer membrane preparations were free of contamination by cytoplasmic membrane.

**Stability of bacteria.** Experiments were performed to determine whether the outer membranes of bacterial cells remained intact during incubation in buffer and antiserum. To accomplish this, bacterial cells were radiolabeled. Initially, strains of *B. catarra*halis were grown in Mueller-Hinton broth with [3H]leucine added to the media. However, the bacteria did not incorporate the radiolabel under these conditions. Therefore, cells were grown in the defined media described by Juni et al. (8), with [3H]leucine added to the media. Cells incorporated ample radiolabel under these conditions.

Radiolabeled logarithmic-phase cells of strain 8184 were incubated in PCM and in a 1:100 dilution of anti-8184 rabbit antiserum under conditions identical to those used for the absorption studies. The amount of radiolabel released from the bacteria in these experiments was 0.57% in PCM and 0.47% in rabbit antiserum; the percentages represent the fraction of radiolabel released into the supernatant relative to the radiolabel that remained in the bacterial cells in the pellet after centrifugation. These results indicate that there is minimal release of radiolabel from the bacterial cells during absorption experiments.

To determine whether the small amount of radiolabel released from the bacterial cells represents fragments of outer membrane, we subjected the supernatants to gel filtration chromatography by using a Bio-Gel P2 column. Fragments of outer membrane will elute at the void volume of the column, whereas free leucine will elute in later fractions. The small amount of [3H]leucine released into the supernatant during absorption was low-molecular-weight material. When free [3H]leucine was chromatographed over the same column, the radiolabel eluted in the same fractions as the radiolabel that was in the supernatant after absorptions.

Taken together, these experiments indicate that the bacterial cells remain intact under conditions of absorption. The cells do not release outer membrane into the supernatant.

**Identification of cross-reactive surface epitopes on OMPs.** (i) **Anti-8184.** Rabbit antiserum raised to intravenously administered whole cells of strain 8184 was studied in an immunoblot assay with Zwittergent-extracted outer membranes of the homologous strain. Antibodies to OMPs C, D, E, and G were detected (Fig. 1, lane a). It is interesting that OMP G appears as a doublet in immunoblot assays of strain 8184. This antiserum was absorbed individually by logarithmic-phase cells of 20 different strains, and the absorbed sera were similarly assayed by immunoblot. Figure 1 shows the results with six strains. Unabsorbed serum is assayed in lane a. Lanes b through g show that absorption by each of six heterologous strains did not remove antibody to OMPs C, D, and E. However, four of the six strains completely removed antibodies to OMP G (lanes b, c, d, and f). This observation indicates that these four strains share epitopes on OMP G, that these epitopes are expressed on the surface of the intact bacterium, and that the surface-exposed determinants are antigenically conserved among strains.
TABLE 1. Absorption of antibody to OMP G by whole bacteria

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>City of origin</th>
<th>Absorption of antibody to OMP G:</th>
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<tbody>
<tr>
<td>25240</td>
<td>ATCC*</td>
<td></td>
<td>Anti-8184 Anti-25240</td>
</tr>
<tr>
<td>8184</td>
<td>Middle ear</td>
<td>Buffalo, N.Y.</td>
<td>+</td>
</tr>
<tr>
<td>8185</td>
<td>Nasopharynx</td>
<td>Buffalo, N.Y.</td>
<td>+</td>
</tr>
<tr>
<td>4223</td>
<td>Middle ear</td>
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<td>-</td>
</tr>
<tr>
<td>3614</td>
<td>Middle ear</td>
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<td>+</td>
</tr>
<tr>
<td>112</td>
<td>Middle ear</td>
<td>Buffalo, N.Y.</td>
<td>-</td>
</tr>
<tr>
<td>135</td>
<td>Middle ear</td>
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<td>-</td>
</tr>
<tr>
<td>4361</td>
<td>Sputum</td>
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<td>+</td>
</tr>
<tr>
<td>4608</td>
<td>Sputum</td>
<td>Buffalo, N.Y.</td>
<td>+</td>
</tr>
<tr>
<td>3583</td>
<td>Nasopharynx</td>
<td>Buffalo, N.Y.</td>
<td>+</td>
</tr>
<tr>
<td>555</td>
<td>Middle ear</td>
<td>Buffalo, N.Y.</td>
<td>+</td>
</tr>
<tr>
<td>4629</td>
<td>Adenoid</td>
<td>Buffalo, N.Y.</td>
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</tr>
<tr>
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<td>Mountain Home, Tenn.</td>
<td>+</td>
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<tr>
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<td>-</td>
</tr>
<tr>
<td>56</td>
<td>Sputum</td>
<td>Mountain Home, Tenn.</td>
<td>+</td>
</tr>
<tr>
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<td>Houston, Tex.</td>
<td>+</td>
</tr>
<tr>
<td>M7</td>
<td>Sputum</td>
<td>Houston, Tex.</td>
<td>+</td>
</tr>
<tr>
<td>M8</td>
<td>Sputum</td>
<td>Houston, Tex.</td>
<td>+</td>
</tr>
</tbody>
</table>

* ATCC, American Type Culture Collection, Rockville, Md.

Table 1 summarizes the results of these experiments with all 20 strains with regard to determinants on OMP G. Fourteen strains absorbed antibody to OMP G from anti-8184, indicating that antigenically conserved determinants of OMP G are expressed on the bacterial surface of these strains.

(ii) Anti-25240. Antiserum to strain 25240 was studied. When it was subjected to an immunoblot assay with the outer membrane of the homologous strain, antibodies to OMPs B, C, D, E, and G were detected (Fig. 2, lane a). In addition, antibodies to three other bands were detected; these include a high-molecular-weight band that migrates above OMP A on the gel and two additional bands that migrate above and below OMP G. Finally, a prominent antibody response to lipooligosaccharide occurred at the bottom of the immunoblot.

Figure 2 shows the results of absorption of aliquots of anti-25240 antiserum individually by six heterologous strains. Four of the six strains completely removed antibody to OMP G (lanes b, c, d, and e). Aliquots of the antiserum were absorbed individually by 20 strains, and the absorbed antisera were assayed in an immunoblot to outer membranes of strain 25240 in the same way. The results with regard to OMP G are shown in Table 1. Fifteen strains absorbed antibody to OMP G from anti-25240. Fourteen of these were the same strains that absorbed antibody to OMP G from anti-8184. Only one strain, a middle-ear isolate from Buffalo (strain 135), demonstrated different results with the two antisera with regard to OMP G. This strain absorbed antibody to OMP G from anti-25240 but not from anti-8184.

The results of absorption experiments with anti-25240 were analyzed with respect to OMP E. Of the 20 strains, 17 completely or nearly completely absorbed antibody to OMP E from anti-25240. Figure 2 shows the results for 6 of the 20 strains studied. Lanes c, d, e, and g show that absorption of aliquots of anti-25240 by each of these strains removed the antibody to OMP E. These experiments indicate that OMP E contains antigenically conserved determinants that are expressed on the bacterial surface.

FIG. 2. Immunoblot assay in which OMPs of strain 25240 (all lanes) were tested with antiserum to 25240. Lane a contains unab sorbed antiserum. Lanes b through g represent anti-25240 antiserum absorbed individually by the following strains: M2 (lane b), M8 (lane c), 8185 (lane d), 3583 (lane e), 4608 (lane f), and 135 (lane g). OMP designations are on the left, and molecular size standards (in kilodaltons) are on the right.

DISCUSSION

The emergence of *B. catarrhalis* as an important human pathogen in the setting of otitis media and chronic lung disease has stimulated investigation of the surface antigens of the organism and the immune response to infection. In previous work, we studied the OMPs of 50 strains of *B. catarrhalis* and identified eight OMPs, which have been designated OMPs A through H (2). A somewhat surprising observation was that all strains, including those from diverse sources, showed strikingly similar OMP patterns by SDS-PAGE. The goal of the present study was to begin to analyze the antigenic characteristics of OMPs from strains of *B. catarrhalis*.

The immunoblot method of Loeb (10) was used to identify surface-exposed epitopes on OMPs and to assess the degree of antigenic conservation of these epitopes among 20 strains of *B. catarrhalis*. This immunoblot method requires that the surface-exposed determinants retain their native conformation on nitrocellulose. In addition, for a positive result to be obtained, the majority of antibody to an antigen must be directed at the surface-exposed portion of the molecule. If these two conditions are present, a positive result with the immunoblot method is highly reliable.

Since the immunoblot method involves subjecting purified outer membranes to SDS-PAGE and immunoblot assays, a method for purifying the outer membrane of *B. catarrhalis* was developed. In previous studies the outer membrane has been purified by sucrose density centrifugation, and two convenient methods for purifying outer membrane were identified (16). These include collection of outer membrane...
vesicles from broth culture supernatants and EDTA- and heat-treated cells. Although these methods yield uncontaminated outer membrane preparations, they are not optimal for immunoblot assays, because it is difficult to apply a predictable quantity to each lane of an SDS-gel. This is probably a result of the relative insolubility of these vesicles. Therefore, we developed and tested an alternative method.

The method involves extraction of outer membranes with the nonionic detergent Zwittergent. The procedure is identical to the initial stages of the purification of the porin protein from H. influenzae (14). The Zwittergent extracts of B. catarrhalis contain all eight of the previously identified OMPs (2, 16). In addition, these extracts contain no detectable succinic dehydrogenase activity, indicating that they are free of cytoplasmic membrane contamination. Outer membranes isolated in this way yield OMPs that are soluble in detergent and result in a highly predictable preparation for immunoblot assays. All of the immunoblots in the present study use Zwittergent extracts as the outer membrane preparation.

It is important to consider the differences in the two antisera used in this study when interpreting the results of the absorption experiments, because no single antiserum is likely to be appropriate for studying all outer membrane antigens by the immunoblot method (11). The inability to demonstrate surface-exposed determinants by using this absorption-immunoblot method does not exclude the possibility that such determinants are present. For a positive result for a particular antigen to be obtained, the majority of antibody to that antigen in the antiserum being absorbed must be directed against surface-exposed epitopes. Therefore, this study does not prove that OMPs C and D lack surface-exposed epitopes, despite the inability of these proteins to remove antibodies from the antiserum.

The absorption experiments in this study demonstrated that two of the major OMPs (E and G) of B. catarrhalis express epitopes on the surface of the intact bacterium. The data further show that the surface-exposed epitopes are antigenically conserved among a majority of 20 strains of diverse clinical and geographic origin.

One goal of studies of OMPs of B. catarrhalis is identification of potential vaccine candidates. An optimal vaccine antigen should (i) be expressed on the bacterial surface, (ii) induce protective antibody, and (iii) be antigenically conserved among the strains of the bacterial species. Since OMPs E and G fulfill the first and third criteria, they deserve further study as potential vaccine candidates. Future studies should be undertaken to determine whether antibodies to these proteins are protective.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**


