Effect of Systemic Immunization on Pulmonary Clearance of 
*Haemophilus influenzae* Type b

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The effect of systemic immunization on pulmonary clearance of *Haemophilus influenzae* type b (Hib) was studied in a mouse model system. Immunization of mice by intraperitoneal injection of viable Hib cells resulted in the appearance of Hib-directed antibodies in both serum and bronchoalveolar lavage fluid. The development of this Hib-directed antibody activity was associated with significant enhancement of early pulmonary clearance of Hib. Systemic immunization did not affect the recruitment of polymorphonuclear leukocytes to the alveoli, suggesting that the enhanced clearance of Hib observed in immunized animals was due to specific antibodies which promote either phagocytosis or extracellular killing of Hib. The spectrum of Hib-directed antibody specificities detected in sera from immunized animals was essentially identical to that detected in bronchoalveolar lavage fluids from these same animals. Similarly, intravenous administration of an immunoglobulin G monoclonal antibody specific for Hib lipopolysaccharide resulted in the subsequent appearance of this antibody in the alveolar spaces where it enhanced pulmonary clearance of Hib. This study shows that this mouse model system can be used to measure the effect of both active and passive immunization on the clearance of Hib from the lower respiratory tract.

The incidence of *Haemophilus influenzae* pneumonia appears to have increased in adults in the United States (1, 14, 21, 23, 35). In addition, recent studies suggest that pneumonia caused by this organism is an important disease in the pediatric population (2, 24). However, little is known about the mechanisms by which *H. influenzae* causes respiratory tract disease or about immunity to *Haemophilus pneumonia*. Studies performed with both mouse model systems and human serum samples have begun to address these issues. It has recently been shown that *H. influenzae* type b (Hib) initially multiplies when inoculated into murine lungs, suggesting that resident defenses are inadequate for pulmonary clearance of this organism (33). If large inocula are used, mice experience a uniformly fatal bacteremic infection (4). Also, it has been reported that deficiencies in serum opsonizing activity for *H. influenzae* may predispose persons to *Haemophilus pneumonia* (23). In addition, *H. influenzae*-directed immunoglobulin A (IgA) antibody in bronchopulmonary secretions may block bactericidal or opsonizing antibodies directed against this organism and thus facilitate the production of disease (22).

Previous studies have shown that antibody to Hib accelerates intravenous clearance of this organism (36). However, no studies have evaluated the effect of immunization against Hib on either pulmonary antibody levels or pulmonary bacterial clearance. The experiments described here show that intraperitoneal (systemic) immunization induces Hib-directed antibodies in serum and in alveolar spaces that are associated with the enhancement of early pulmonary clearance of Hib. Systemic immunization did not change the number of phagocytic cells in the lungs. The finding of enhanced pulmonary clearance without changes in phago-

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diluted in brain heart infusion broth containing Levinthal base (11), plated on chocolate agar, and incubated at 37°C for 24 h in a candle extinction jar. Cultures of each lung homogenate were expressed as CFU per lung. To compare test groups, the number of CFU per lung from each mouse at each time point was divided by the mean CFU in the lungs of all mice killed at 0 h for that experiment and then multiplied by 100. The resultant value was the percentage of viable bacteria that remained in the lung at each time point.

**BAL.** The recruitment of polymorphonuclear leukocytes (PMNs) to the alveolar spaces was assessed by bronchoalveolar lavage (BAL) by using a modification of a previously described procedure (26). Mice were killed by intraperitoneal injection of sodium pentobarbital (160 mg/kg), and the tracheae were exposed and cannulated with PE50 tubing (Clay Adams, Div. of Becton Dickinson and Co., Parsippany, N.J.). Iced heparinized saline was injected into the lungs and aspirated in 0.6-ml portions until a 3-ml volume was obtained. BAL fluid was collected on ice and subjected to centrifugation at 150 × g for 10 min at 4°C. The cell pellet was suspended in Hanks balanced salt solution without calcium or magnesium, and the total number of cells was determined with a Coulter Counter (model ZM; Coulter Electronics, Inc., Hialeah, Fla.). Differential cell counts of 200 cells each were made on cytocentruged Wright-stained preparations.

Collection of BAL fluids for antibody studies was performed as described above with the following modifications. Fetal calf serum was added to the BAL fluid immediately after lavage to a final concentration of 1% (vol/vol). This BAL fluid was then concentrated 25-fold in a Minicon macrosolute concentrator (Amicon Corp., Lexington, Mass.). All concentrated BAL fluids were stored at −70°C until used for further analysis.

**ELISA.** Serum and BAL fluid were assayed for the presence of antibodies directed against Hib outer membrane antigens as described by Robertson et al. (29). Serial dilutions of serum and concentrated BAL fluid used in the enzyme-linked immunosorbent assay (ELISA) system were made in PBS containing 1% (wt/vol) bovine serum albumin. Only ELISA optical density readings between 0.2 and 1.0 were used in the calculation of the mean ELISA titers. Mean ELISA titers were calculated by multiplying the optical density at 405 nm by the reciprocal of the dilution of serum or BAL fluid from which this reading was derived.

**Western blot analysis.** Antibodies in serum and BAL fluid directed against Hib outer membrane proteins and lipopolysaccharide (LPS) were detected by Western blot analysis. Outer membrane vesicles of Hib DL26 prepared as described previously (10) were used as the source of outer membrane proteins. Outer membrane vesicles (5 μg of protein per well) were solubilized (11), and the outer membrane proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 10% (wt/vol) polyacrylamide separating gels (11). LPS was purified from Hib DL26 by the method of Inzana (15). LPS (1 μg per well) was solubilized (11) and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a 10 to 20% (wt/vol) polyacrylamide gradient gel. Antigens were electrophoretically transferred to nitrocellulose membranes by the method of Towbin et al. (34). Serum (20 μl) or 150 μl of concentrated BAL fluid diluted in 10 ml of PBS containing 0.01% Tween 20 (PBS-Tween) was incubated with individual nitrocellulose strips to detect antibodies directed against outer membrane proteins. LPS-containing nitrocellulose strips were incubated in PBS containing 1% bovine serum albumin for 1 h before incubation with 20 μl of serum or 50 μl of BAL fluid diluted in PBS only. Washing of the nitrocellulose strips was performed with PBS-Tween (for outer membrane protein strips) or with PBS (for LPS strips). Detection of mouse antibodies bound to the Hib antigens on the nitrocellulose strips was performed as described previously (12).

**Intravenous immunization with monoclonal antibody.** A murine monoclonal antibody directed against Hib LPS (P. A. Gulig and E. J. Hannan, submitted for publication) was affinity purified with protein A-CL-4B Sepharose (Pharmacia, Piscataway, N.J.). This IgG3 monoclonal antibody is directed against the LPS of Hib DL26. A 200-μg portion of this purified antibody in 0.1 ml PBS was injected into the tail veins of mice. Blood for serum preparation was obtained by retroorbital puncture, and BAL fluids were collected both before and immediately after antibody injection and at 6 and 24 h postimmunization; four separate groups of mice were used for this purpose. Control mice were injected intravenously with 0.1 ml of PBS. Mice were challenged with Hib via an endobronchial catheter at 24 h postimmunization as described above.

**Statistical analysis.** Clearance data, the numbers of total phagocytic cells and PMNs, and Hib-directed antibody levels in immunized and control mice were analyzed using the Mann-Whitney test for nonparametric analysis (37). Probability values of less than 0.05 were considered significant.

**RESULTS**

**Pulmonary clearance of Hib.** The clearance of Hib from the lungs of immunized and control mice was assessed at 4, 6, and 24 h after intrabronchial inoculation of 10³ and 10⁴ CFU of Hib and is expressed as the percentage of Hib CFU relative to the number of CFU originally deposited in the lung (Fig. 1). At 4 h after inoculation with 10³ CFU of Hib (Fig. 1A), multiplication had occurred in the control animals (180 ± 26%), but clearance had occurred in the immunized animals (49 ± 6.3%) (P < 0.05). Multiplication continued to occur in the control animals at 6 h (290 ± 30%), whereas the immunized animals had completely cleared this inoculum by 6 h (P < 0.05). At 24 h both the immunized and the control animals had cleared the inoculum.

At 4 h after inoculation with a larger number of Hib (10⁴ CFU) (Fig. 1B), the bacteria had multiplied in the lungs of both control and immunized mice, but the immunized mice had a significantly smaller percentage of bacteria remaining than did the control mice (124 ± 11% versus 203 ± 21%; P < 0.05). At 6 h the immunized mice had begun to measurably clear Hib (17 ± 1.0%), whereas the bacterial burden remained large in the control mice (78 ± 9.0%; P < 0.05). At 24 h after challenge, the immunized mice had 8 ± 1% bacteria remaining, whereas the control mice had 32 ± 5.1% bacteria remaining (P < 0.05). Blood cultures were obtained from all mice at 4, 6, and 24 h and were negative at all time intervals in both immunized and control mice.

**Hib-directed antibody response after immunization.** Hib-directed serum antibodies were measured by an ELISA method before immunization and at 30, 45, and 60 days after the initial immunization (Table 1). A significant increase in Hib-specific antibody levels was noted at each time point after immunization. When compared with preimmunization levels, 12-, 140-, and 200-fold increases in Hib-specific serum antibody levels were noted at 30, 45, and 60 days postimmunization, respectively.

The level of Hib-directed antibodies in BAL fluid was measured in immunized and control mice on day 60 of the
imunization protocol (0 h) and at 4, 6, and 24 h after challenge with 10^5 Hib (Table 2). BAL fluid from immunized mice contained 240-fold more Hib-directed antibody than BAL fluid from control mice. The level of measurable Hib-directed antibody fell in BAL fluid from immunized animals at 4 h postchallenge to 37% of its original value and by 24 h postchallenge had risen to only half of the original level.

The specificities of the Hib-directed antibodies in immune serum and BAL fluid were determined by Western analysis. Both serum and BAL fluid contained antibodies directed against numerous different Hib outer membrane proteins and LPS (Fig. 2 and 3). A comparison of the antibody content of matched serum and BAL fluid from individual animals showed that the specificities of the antibodies present in serum were essentially identical to those of the antibodies present in the BAL fluid from each animal (Fig. 2 and 3).

**Passive intravenous immunization with monoclonal antibody.** To further define the role of circulating antibody in the augmentation of pulmonary clearance of Hib, we studied the appearance of Hib LPS-directed monoclonal antibody in BAL fluid after intravenous administration of this antibody (Fig. 4). Before administration of the monoclonal antibody, no Hib LPS-directed antibody activity could be detected in either serum or BAL fluid. Immediately after intravenous injection of this monoclonal antibody, Hib LPS-directed antibody was readily detectable in serum but not in BAL fluid. The level of LPS-directed antibody in BAL fluid rose gradually over 24 h, suggesting transudation of this IgG antibody from serum into the alveolar spaces.

Intravenous administration of this monoclonal antibody directed at LPS prepared from Hib DL26 also protected mice from subsequent pulmonary challenge with these bacteria. Passive immunization with this monoclonal antibody at 24 h before bacterial challenge significantly augmented pulmonary clearance of both 10^6 and 10^7 CFU of Hib DL26 (Table 3). A net increase in Hib CFU occurred by 6 h postinoculation in PBS-injected control animals, whereas clearance had occurred by this time in the monoclonal antibody-injected animals.

It was necessary to confirm that the enhanced pulmonary clearance ability exhibited by the passively immunized mice was due to the specific antigen-antibody interaction and did not result from some nonspecific effect of this monoclonal antibody. Mice immunized with this monoclonal antibody were challenged with a Hib strain (DL42) which bears LPS molecules not recognized by this monoclonal antibody. Passive immunization of mice with this monoclonal antibody did not enhance pulmonary clearance of Hib DL42 (Table 3). This result confirms the antigenic specificity of the enhanced pulmonary clearance of Hib DL26 provided by this monoclonal antibody.

**Recruitment of phagocytes to alveoli.** Total phagocytic cells and total PMNs in BAL fluid were determined in immunized and control mice before and at 4, 6, and 24 h after intrabronchial challenge with 10^5 and 10^6 CFU of Hib DL26 (Table 4). Before challenge there were no differences in total phagocytic cells between the immunized and control animals, and there were few PMNs found in BAL fluid from either group. By 4 h after challenge with 10^5 CFU of Hib, the total numbers of phagocytic cells and PMNs had increased in both the immunized and control animals compared with the values for animals at 0 h. At 6 and 24 h, approximately 50% of the cells in BAL fluid were PMNs. It is important that there were no differences in total phagocytic cells or PMNs at any time point when immunized animals were compared with control animals.

After inoculation of mice with 10^6 CFU of Hib, PMN recruitment was more rapid (Table 4). The total number of PMNs increased throughout the experimental period and accounted for approximately 75% of the cells recovered at 24 h. Again, there were no differences in the total number of phagocytic cells or PMNs between the immunized and control groups at any time point.

**DISCUSSION**

The resident defense system of the lower respiratory tract consists of alveolar macrophages (5, 7) and humoral substances including complement (8, 13, 32), surfactant (3, 16,
TABLE 2. Hib-directed antibody in BAL fluid before and after Hib challengea

<table>
<thead>
<tr>
<th>Mice</th>
<th>Hib-directed antibody (mean ELISA units) at*:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td>Control</td>
<td>0.52 ± 0.02</td>
</tr>
<tr>
<td>Immunized</td>
<td>1,190.00 ± 50.00</td>
</tr>
</tbody>
</table>

*a* Hib-directed antibodies were detected by ELISA with outer membrane vesicles as the antigen.

*b* Mean ELISA units were calculated as described in the text. This experiment was performed at 60 days postimmunization. BAL fluids were obtained from both immune and control mice immediately before challenge (0 h) and at 4, 6, and 24 h postchallenge.

20), and immunoglobulins (27, 28). Recent evidence (33) that Hib multiplies in the murine lung and resists clearance for at least 6 h prompted an analysis of the means by which this initial growth period of Hib in the lung could be inhibited or eradicated. The resident pulmonary surveillance and defense systems could theoretically be augmented in at least two ways. The first possibility involves phagocytic cell recruitment, and we have previously demonstrated (33) that the lung rapidly mounts a PMN response to augment resident alveolar macrophages after intrapulmonary inoculation of Hib. The second possibility for augmentation involves specific antibodies. It is possible that active systemic immunization might enhance pulmonary clearance of Hib by producing high levels of Hib-directed opsonic or bactericidal antibody in the alveolar spaces. In the present study, we augmented host defenses by systemic immunization and then determined the relationship between pulmonary clearance and the appearance of both specific antibodies and PMNs.

Systemic immunization clearly enhanced the pulmonary clearance of Hib. After a challenge of 10^7 CFU, net increases in the number of Hib organisms occurred only in the control animals at 4 and 6 h, whereas clearance of Hib occurred at all time intervals in the immunized animals. In contrast, after a challenge of 10^7 CFU, the immune animals retarded but did not prevent growth of this larger inoculum of Hib during the first 4 h postinoculation. Subsequently, clearance occurred more rapidly in the immunized mice than in the control animals. It also is of interest that the larger inoculum (10^7 CFU) was cleared more rapidly by the control mice than was the smaller inoculum (10^5 CFU). A similar pattern of increased pulmonary clearance in response to increased inoculum size has been observed to occur with Klebsiella pneumoniae (31). Regardless of the mechanism responsible for this paradoxical effect of inoculum size on pulmonary clearance, however, these results clearly indicate that systemic immunization enhanced lower respiratory tract defenses against Hib, resulting in enhanced pulmonary clearance of this pathogen.

Previous studies have suggested that immunization may enhance lung bactericidal activity by increasing the number of PMNs in the alveolar spaces (18, 19). In the present study no significant differences were found in the total number of phagocytic cells or PMNs at any time point when immunized animals were compared to control animals (Table 4). However, maximal enhancement of pulmonary clearance was noted when large numbers of PMNs were present in the alveoli (i.e., 6 h postchallenge).

The immunization regimen used in this study clearly resulted in the appearance of Hib-directed antibodies in serum. Additionally, Hib-directed antibodies were present in

![FIG. 2. Western blot analysis of Hib outer membrane protein-directed antibodies in serum and BAL fluid. Outer membrane vesicles were used as the source of outer membrane proteins, and Western blot analysis was performed as described in the text. The individual nitrocellulose strips were incubated with 20 μl of serum from control mice (lane a), 20 μl of serum from immune mouse number 1 (lane b), 150 μl of concentrated BAL fluid from immune mouse number 1 (lane c), 20 μl of serum from immune mouse number 2 (lane d), 150 μl of concentrated BAL fluid from immune mouse number 2 (lane e), 5 μl of serum from immune mouse number 3 (lane f), or 150 μl of concentrated BAL fluid from immune mouse number 3 (lane g).](http://iai.asm.org/)

![FIG. 3. Western blot analysis of Hib LPS-directed antibodies in serum and BAL fluid. Purified Hib DL26 LPS was used as the antigen. The sources of sera and BAL fluids are identical to those described in the legend to Fig. 2. The volume of serum used in this experiment was 20 μl, whereas the volume of concentrated BAL fluid used in this particular Western blot was reduced to 50 μl.](http://iai.asm.org/)
TABLE 3. Effect of intravenous administration of specific antibody on pulmonary clearance of Hib

<table>
<thead>
<tr>
<th>Hib strain and deposition (CFU)</th>
<th>Immunization agent</th>
<th>% Bacteria remaining (mean ± SEM) at 6 h postchallenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL26 (2.8 × 10⁴)³³</td>
<td>PBS</td>
<td>502 ± 41</td>
</tr>
<tr>
<td></td>
<td>MA</td>
<td>2 ± 0.05</td>
</tr>
<tr>
<td>DL26 (6.6 × 10⁴)³³</td>
<td>PBS</td>
<td>480 ± 45</td>
</tr>
<tr>
<td></td>
<td>MA</td>
<td>57 ± 5</td>
</tr>
<tr>
<td>DL42 (6.1 × 10⁴)³³</td>
<td>PBS</td>
<td>192 ± 27</td>
</tr>
<tr>
<td></td>
<td>MA</td>
<td>179 ± 14</td>
</tr>
</tbody>
</table>

³³ Purified monoclonal antibody (200 μg in 0.1 ml PBS) directed against LPS of Hib strain bearing DL26 was used for intravenous injection.
³⁴ Mice were injected with 0.1 ml of either monoclonal antibody (MA) or PBS.
³⁵ Each value represents the mean of six to eight animals.
³⁶ Hib strain bearing LPS recognized by the monoclonal antibody.
³⁷ Hib strain bearing LPS not recognized by the monoclonal antibody.

BAL fluids obtained before challenge with Hib. Although absolute amounts of Hib-directed antibody could not be determined, large differences in Hib-directed antibody levels were detectable between control and immunized animals, demonstrating that systemic immunization increased Hib-directed antibody titers both in serum and on alveolar surfaces. A small decrease in Hib-directed antibody activity in BAL fluid was noted after Hib challenge (Table 2), suggesting that some of this antibody may have bound to the bacteria or to inflammatory cells recruited in response to infection. These results, together with the data on phagocytic cell recruitment discussed above, suggest that the mechanism(s) by which systemic immunization enhances pulmonary clearance of Hib likely involves specific antibodies.

It is of interest that the specificities of antibodies in serum and BAL fluid for Hib outer membrane proteins were essentially identical (Fig. 2). Although these findings do not exclude the possibility that local synthesis of Hib-directed antibody occurred in the lung, we infer from these data that these Hib protein-directed antibodies in the alveolar spaces are derived at least in part from serum by transudation across alveolar capillary membranes.

Because antibodies in the BAL fluid were apparently derived in part from serum, we determined whether intravenous administration of an Hib-directed monoclonal antibody would result in the appearance of this antibody in the alveolar spaces. The absence of Hib-directed antibody on the alveolar surface immediately after intravenous injection of the monoclonal antibody and the subsequent and gradual appearance of this antibody in BAL fluid suggest that transudation of this IgG antibody into the alveolar spaces occurred with time (Fig. 4). More importantly, passive intravenous immunization with this LPS-specific antibody resulted in enhanced pulmonary clearance of Hib (Table 3).

The mechanism(s) of antibody enhancement of pulmonary clearance of Hib was not evaluated in this study. However, the fact that the recruitment of large numbers of PMNs to the alveoli coincided with increased clearance of Hib by immune animals suggests that antibody-mediated opsoniza-

TABLE 4. Recruitment of phagocytes to the alveoli after Hib challenge

<table>
<thead>
<tr>
<th>Group and hour after challenge</th>
<th>2.5 × 10⁷ CFU of Hib</th>
<th>3.5 × 10⁷ CFU of Hib</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total cells (10⁶ cells)</td>
<td>PMN (10⁶ cells)</td>
</tr>
<tr>
<td>0 h</td>
<td>Control</td>
<td>1.05 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>Immune</td>
<td>1.00 ± 0.08</td>
</tr>
<tr>
<td>4 h</td>
<td>Control</td>
<td>1.86 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>Immune</td>
<td>1.72 ± 0.08</td>
</tr>
<tr>
<td>6 h</td>
<td>Control</td>
<td>1.68 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>Immune</td>
<td>1.76 ± 0.08</td>
</tr>
<tr>
<td>24 h</td>
<td>Control</td>
<td>1.78 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>Immune</td>
<td>1.84 ± 0.09</td>
</tr>
</tbody>
</table>

³ The Hib challenge was administered to the lungs via an endobronchial catheter. BAL to collect cells was performed immediately before (0 h) and at 4, 6, and 24 h postchallenge.
tion is involved. Alternatively, Hib-directed antibody may augment extracellular bacterial killing by an interaction with the complement system (30). Because pulmonary clearance was enhanced in immunized animals without changes in phagocytic cell numbers relative to control animals, we suggest that specific antibody is important in early bacterial clearance and functions either by enhancing phagocytic efficiency or by augmenting extracellular killing of Hib.

This study also indicates that the animal model system used here is highly relevant in that it measures the effect of both active and passive immunization on the clearance of Hib from the lower respiratory tract, a common site of Haemophilus infections. Because the lower respiratory tract represents a unique milieu with regard to phagocytic cell populations (6) and levels of both immunoglobulins and complement (17, 25), it is essential to assay for the level of protection in the relevant organ system. It should be emphasized that our animal model system can be used to assess not only the induction of antibody synthesis but also whether these antibodies are delivered to the site of host-parasite interaction and whether they are functional in the lung (i.e., capable of enhancing pulmonary clearance of Hib).

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LITERATURE CITED


