Mechanism of Pertussis Toxin B Oligomer-Mediated Protection against *Bordetella pertussis* Respiratory Infection

ROBERTA D. SHAHIN,1,* MAARTEN H. WITVLIET,2 AND CHARLES R. MANCLARK1

Laboratory of Pertussis, Center for Biologies Evaluation and Research, Food and Drug Administration, Bethesda, Maryland 20892,1 and Laboratory of Bacterial Vaccines, National Institute for Public Health and Environmental Protection, Bilthoven, The Netherlands2

Received 19 June 1990/Accepted 24 September 1990

Immunization with the B oligomer of pertussis toxin protected neonatal mice from a lethal respiratory challenge with *Bordetella pertussis*. All mice immunized with 8 μg of B oligomer survived aerosol challenge and had peripheral leukocyte counts and weight gains similar to those of mice immunized with pertussis toxoid before challenge and to those of control mice that were not challenged. Unprotected mice challenged with an aerosol of *B. pertussis* had an increase in peripheral leukocyte count, failed to gain weight, and died within 21 days of challenge. Protection appeared to be dose dependent, since a dose of 1 μg of B oligomer per mouse prevented death in 100% of the mice challenged with *B. pertussis*, whereas 0.4 μg of B oligomer protected 50% of the challenged mice. Mice immunized with the B oligomer had increases in immunoglobulin G (IgG) anti-B oligomer in sera and in IgG and IgA anti-B oligomer in bronchoalveolar lavage fluids 1 to 3 weeks after respiratory challenge. Specific anti-B oligomer antibodies could not be detected in unimmunized, infected mice at the same time after challenge. Intravenous administration of the monoclonal antibody 170C4, which binds to the S3 subunit of the B oligomer, protected neonatal mice from *B. pertussis* respiratory challenge, while administration of an IgG1 anti-tetanus toxin monoclonal antibody, 18.1.7, was not protective. We conclude that anti-B-oligomer-mediated neutralization of pertussis toxin is one mechanism of protection in the mouse model of *B. pertussis* aerosol challenge.

*Bordetella pertussis*, the etiologic agent of pertussis (whooping cough), is a gram-negative rod that was first isolated by Bordet and Gengou in 1906 (4). Pertussis, which may be characterized by episodes of paroxysmal coughing, can be fatal, especially in unimmunized infants (7). Mass immunization of children in the United States with an inactivated whole-cell pertussis vaccine has resulted in a decreased incidence of disease (6). However, the association of common local and systemic reactions, such as pain and swelling at the injection site and fever, with use of the whole-cell pertussis vaccine (9) has led to an effort to develop an effective acellular vaccine against *B. pertussis* with fewer side effects. This has led to the purification and analysis of components of *B. pertussis* as protective antigens. Of these, the most widely studied has been pertussis toxin.

Biological activities mediated by pertussis toxin include leukocytosis, inhibition of leukocyte chemotaxis, histamine sensitization, and insulin secretion (18). Pertussis toxin is an ADP-ribosyltransferase, with the classic A-B composition of many bacterial toxins (27). The A promoter (also known as S1) ADP-ribosylates a guanine nucleotide binding protein of eucaryotic cells, thus interfering with signal transduction (12). The B oligomer, composed of five subunits (S2, S3, S5, and two molecules of S4), is responsible for the binding of the toxin to eucaryotic membranes (28).

The B oligomer of pertussis toxin dissociates from the A promoter in the presence of ATP and detergent and can be purified by ion-exchange chromatography (2, 5). Arciniega and coworkers have demonstrated that purified B oligomer elicits antibodies in mice which can neutralize pertussis toxin-induced clustering of Chinese hamster ovary cells in vitro and that mice immunized with B oligomer are refractory to pertussis toxin-induced leukocytosis in vivo (2). Because certain parameters of pertussis in humans, such as leukocytosis, specific attachment of bacteria to the ciliated epithelium of the respiratory tract, and an increased severity of disease in the neonate, are also observed in mice challenged with an aerosol of *B. pertussis* (22, 23), we have evaluated the B oligomer as a protective antigen in a murine model of *B. pertussis* respiratory infection (23). In preliminary experiments (26), we have demonstrated that immunization with a fixed dose of B oligomer protects neonatal mice from leukocytosis, weight loss, and death 17 days after *B. pertussis* aerosol challenge. In this paper, we analyze B oligomer-induced protective immunity and define one mechanism of protection conferred by the B oligomer.

**MATERIALS AND METHODS**

Mice. Newborn specific-pathogen-free BALB/c AnNcr mice were obtained, with mothers, on day 3 postpartum from the Animal Production Program, Division of Cancer Treatment, National Cancer Institute, Frederick, Md. Mice were maintained in microisolators under specific-pathogen-free conditions and fed food and water ad libitum.

Antigens. Pertussis toxin was purchased from the Michigan Department of Public Health and was demonstrated to be pure by sodium dodecyl sulfate gel electrophoresis (15). This preparation contained 1.5 ng of endotoxin per μg of protein by Limulus amoebocyte lysate assay (8). Pertussis holotoxin was inactivated with 0.15% glutaraldehyde as previously described (2).

The B oligomer of pertussis toxin was purified by incubating holotoxin in ATP and detergent, followed by ion-exchange chromatography, as previously described (2). B oligomer preparations as well as pertussis toxoid preparations contained less than 0.3 ng of active pertussis toxin per...
\(\mu g\) of B oligomer or pertussis toxoid, as determined by an assay that measures pertussis toxin-induced clustering of Chinese hamster ovary cells (11). Some preparations of purified B oligomer were kindly provided by Juan Arciniega, Food and Drug Administration, Bethesda, Md.

Pertussis toxoid, B oligomer, and tetanus toxoid (Connaught Inc., Swiftwater, Pa.) were diluted in phosphate-buffered saline at a concentration of approximately \(10^4\) CFU per ml of inoculum. The challenge inoculum was administered to mice as an aerosol by using a standard nebulizer (Fisons Corp., Bedford, Mass.) within a biosafety level 3 glove box (Blickman Co., Weehawken, N.J.), as previously described (23). Mice were removed from the chamber 1 h after termination of the aerosol challenge, at which point viable B. pertussis cells cannot be recovered from the surface of the animals or the chamber (23). Two mice were sacrificed upon removal from the chamber in order to determine the number of viable B. pertussis cells in the lungs. All animals tested had approximately \(10^4\) CFU in their lungs 1 h after aerosol challenge. At different times after infection, the mice were weighed, any deaths were noted, and 5 \(\mu l\) of blood was collected from the peribronchial sinus. The number of leukocytes (WBC) per \(\mu l\) of blood was determined in a model ZM Coulter Counter (Coulter Electronics, Hialeah, Fla.).

**Aerosol challenge.** A 21-h culture of B. pertussis 18323 grown on Bordet-Gengou agar was suspended in sterile phosphate-buffered saline at a concentration of approximately \(10^4\) CFU per ml of inoculum. The challenge inoculum was administered to mice as an aerosol by using a standard nebulizer (Fisons Corp., Bedford, Mass.) within a biosafety level 3 glove box (Blickman Co., Weehawken, N.J.), as previously described (23). Mice were removed from the chamber 1 h after termination of the aerosol challenge, at which point viable B. pertussis cells cannot be recovered from the surface of the animals or the chamber (23). Two mice were sacrificed upon removal from the chamber in order to determine the number of viable B. pertussis cells in the lungs. All animals tested had approximately \(10^4\) CFU in their lungs 1 h after aerosol challenge. At different times after infection, the mice were weighed, any deaths were noted, and 5 \(\mu l\) of blood was collected from the peribronchial sinus. The number of leukocytes (WBC) per \(\mu l\) of blood was determined in a model ZM Coulter Counter (Coulter Electronics, Hialeah, Fla.).

**Analysis of respiratory and serum immunoglobulin.** Mice anesthetized with 2,2,2-tribromoethanol (Alirdich Chemical Co., Milwaukee, Wis.) were bled from the brachial arteries, and their tracheas were cannulated with a piece of PE-50 polyethylene tubing (Clay Adams, Parsippany, N.J.). Sterile phosphate-buffered saline (0.3 ml) was gently instilled into the lungs and withdrawn three times. The bronchoalveolar lavage fluid was centrifuged, and the supernatant was removed and frozen at \(-20^\circ C\) prior to analysis.

Sera and bronchoalveolar lavage fluids were analyzed for specific antibodies by an enzyme-linked immunosorbent assay (17). Microtiter plates (Immunolon I; Dynatech Laboratories, Chantilly, Va.) were coated with 5 \(\mu g\) of either B oligomer or pertussis toxoid per \(\mu l\) overnight, washed, and incubated with dilutions of mouse serum or bronchoalveolar lavage fluid for 3 h. After being washed, plates were incubated overnight with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G or IgA (Southern Biotechnology, Inc., Birmingham, Ala.). The isotype specificities and sensitivities of the alkaline phosphatase conjugates were established by using a panel of purified monoclonal antibodies generously provided by John Cebra, University of Pennsylvania, Philadelphia. The plates were read 30 min after addition of Sigma 104 phosphatase substrate (Sigma Chemical Co., St. Louis, Mo.) by using a Bio-Tek EL 312 reader (Bioket Instruments, Winooski, Vt.). Anti-B oligomer titers are expressed as the reciprocal of the endpoint dilution, calculated by extrapolation to zero from the linear part of the titration curve.

**Monoclonal antibodies.** Protein A–Sepharose-purified 18.1.7, an IgG1 anti-tetanus toxoid monoclonal antibody (13), was a generous gift of Jane Halpern, Laboratory of Bacterial Toxins, Food and Drug Administration, Bethesda, Md. 170C4, an IgG1 monoclonal antibody that specifically binds to the S3 subunit of pertussis toxin (30), was a generous gift of Jan Poolman, Rijksinstituut, Bilthoven, The Netherlands. 170C4 was purified from ascites fluid by ammonium sulfate precipitation followed by ion-exchange chromatography on DEAE-Sepharose. The IgG1 antibody was eluted with a gradient of 0 to 0.2 M KCl in 0.01 M Tris buffer, pH 8.0 (16). The purity of fractions constituting the first eluted peak was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

**Chinese hamster ovary (CHO) cell assay.** Serial dilutions of pertussis toxoid or B oligomer in 0.1 ml of Ham’s medium were incubated with an equal volume of \(10^7\) CHO cells (ATCC CCL61) in Ham’s F12 medium (Flow Laboratories, Inc., McLean, Va.) containing 10% fetal calf serum (HyClone Laboratories, Logan, Utah) prepared as previously described (11). The cells were incubated at 37°C in 7% CO\(_2\) for 24 to 48 h and then were examined with an inverted microscope. The highest dilution of toxoid or B oligomer to cause complete clustering of all cells in a well was scored.

Inhibition of pertussis toxin-induced CHO cell clustering by dilutions of purified monoclonal antibody was done as previously described (10). The highest dilution of monoclonal antibody that inhibited clustering of all cells in a well is reported.

**RESULTS**

**Active immunization with B oligomer protects against respiratory B. pertussis infection.** Electrophoretic analysis, using 15% acrylamide-sodium dodecyl sulfate gels, demonstrated that the preparations of B oligomer used in these experiments contained all subunits of pertussis toxin except for S1, the ADP-ribose transferase (data not shown). All of the mice immunized with 4 \(\mu g\) of B oligomer survived lethal B. pertussis respiratory challenge, as did positive controls immunized with 8 \(\mu g\) of pertussis toxoid (Fig. 1), confirming our previous observation of protection with 8 \(\mu g\) of B oligomer (26). Mice immunized with 4 \(\mu g\) of B oligomer prior to aerosol challenge had a WBC count and a weight gain over a time course of 20 days postinfection that were similar to those of uninfected control mice and mice immunized with 8 \(\mu g\) of pertussis toxoid (Fig. 1). In contrast, mice immunized with 8 \(\mu g\) of tetanus toxoid, as a negative control, exhibited a pronounced increase in peripheral WBC count and failed to gain weight, and all of those mice died within 21 days after respiratory challenge (Fig. 1).

Immunization with B oligomer protected neonates in a dose-dependent manner. Figure 2 shows leukocytosis evaluated on day 16 postinfection, the peak day of leukocytosis. On this day, the mean peripheral WBC count was 15,000 WBC per \(\mu l\) (range, 12,000 to 21,000) for mice that had received 4 \(\mu g\) of B oligomer and 30,000 WBC per \(\mu l\) (range, 8,000 to 106,000) for mice that had received 1 \(\mu g\) of B oligomer, while mice that had received 0.4 \(\mu g\) of B oligomer exhibited a mean count of 83,000 WBC per \(\mu l\) (range, 26,000 to 236,000). Although the highest mean WBC count per \(\mu l\) was observed day 16 postinfection, some individual mice varied in their day of peak leukocytosis. During the entire 21-day period of observation following aerosol challenge, some mice exhibited an increase in WBC counts that subsequently returned to baseline, and these mice survived, while the remaining mice exhibited an increase in WBC that remained high until the mice died. Following infection, 90% of the mice receiving 4 \(\mu g\) of B oligomer and 100% of the mice receiving 1 \(\mu g\) of B oligomer survived, while only 41% of the mice receiving 0.4 \(\mu g\) of B oligomer survived (Fig. 2).
Induction of serum and respiratory antibody. No detectable antibody to either B oligomer or pertussis holotoxin was observed at the time of aerosol challenge in the sera or bronchoalveolar lavage fluids of neonatal mice immunized with 8 μg of B oligomer (Table 1). However, increased titers of specific antibody to B oligomer were detected in the sera and bronchoalveolar lavage fluids of immunized mice 1, 2, and 3 weeks after aerosol infection. IgG anti-B oligomer was detected in the sera, while both IgG and IgA antibodies to B oligomer were detected in bronchoalveolar lavage fluids of immunized mice after challenge. Specific antibodies to B oligomer could not be detected in the sera or bronchoalveolar lavage fluids of unimmunized adult mice 1 or 2 weeks after aerosol challenge (Table 1). Adult mice were used as the controls in the latter experiment, as unimmunized neonates die within 14 to 21 days after aerosol challenge.

Protection mediated by passive antibody. The mouse monoclonal antibody 170C4 is an IgG1 antibody that binds to the S3 subunit of the pertussis toxin B oligomer (30). Less than 250 ng of purified 170C4 per ml inhibited the clustering of CHO cells induced by 0.5 ng of pertussis toxin per ml, while 250 μg of 18.1.7, an IgG1 monoclonal anti-tetanus toxoid antibody, per ml failed to inhibit clustering (Table 2).

Since transudation of IgG1 into murine lungs 24 h after intravenous injection has been well documented (25, 29), we wished to determine whether passively administered 170C4 could protect young mice against lethal B. pertussis respiratory challenge. Sixteen- to 18-day-old BALB/c mice were injected intravenously with 250 μg of 170C4 24 h prior to aerosol challenge with 10⁶ CFU of B. pertussis 18323 per ml. As a negative control, a second group of mice were injected with 250 μg of 18.1.7 (IgG1 anti-tetanus toxoid) prior to challenge. Mice receiving 170C4 had no increase in WBC count and did not die (Table 3). In contrast, mice receiving 18.1.7 exhibited severe leukocytosis and failed to gain weight, and all of these mice died by day 19, as did unprotected controls.

DISCUSSION

We have demonstrated that active immunization of neonatal mice with the purified B oligomer of pertussis toxin protects against lethal B. pertussis respiratory challenge (26; this study). Mice immunized with 4 μg of B oligomer exhibited no increase in WBC count, gained weight, and did not die over a 21-day period following B. pertussis aerosol infection (Fig. 1). However, unprotected mice immunized with tetanus toxoid exhibited an elevated peripheral WBC count, apparent 1 week after infection, that increased in
TABLE 1. Specific anti-B oligomer titers in sera and bronchoalveolar lavage fluids after immunization and challenge

<table>
<thead>
<tr>
<th>Group and time of immunization</th>
<th>Serum⁵</th>
<th>Lavage fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total anti-B oligomer</td>
<td>A&lt;sub&gt;αβ&lt;/sub&gt;⁶</td>
</tr>
<tr>
<td></td>
<td>IgM</td>
<td>IgG</td>
</tr>
<tr>
<td>Unimmunized and uninfected⁴</td>
<td>&lt;40</td>
<td>0</td>
</tr>
<tr>
<td>Immunized and infected⁴</td>
<td>&lt;40</td>
<td>0.01</td>
</tr>
<tr>
<td>Day of challenge</td>
<td>480</td>
<td>0.01</td>
</tr>
<tr>
<td>Wk 1 postinfection</td>
<td>960</td>
<td>0.01</td>
</tr>
<tr>
<td>Wk 2 postinfection</td>
<td>2,564</td>
<td>0.02</td>
</tr>
</tbody>
</table>

| Unimmunized and infected⁴     | <40 | ND | ND | ND | <2 | ND | ND | ND |
| Wk 1 postinfection            | <40 | ND | ND | ND | <2 | ND | ND | ND |
| Wk 2 postinfection            | <40 | ND | ND | ND | <2 | ND | ND | ND |

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Leukocytosis⁷</th>
<th>% Survivors⁸</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-B oligomer³ (10)</td>
<td>15.6 ± 2.7</td>
<td>100</td>
</tr>
<tr>
<td>Anti-tetanus toxoid³ (10)</td>
<td>154.6 ± 95.8</td>
<td>0</td>
</tr>
<tr>
<td>Infected control (8)</td>
<td>142.1 ± 33.8</td>
<td>0</td>
</tr>
<tr>
<td>Uninfected control (9)</td>
<td>12.1 ± 3.0</td>
<td>100</td>
</tr>
</tbody>
</table>

⁴ Mice were immunized with 8 µg of B oligomer on days 5 and 12 postpartum and challenged at 19 days of age.
⁵ ND, Not done.
⁶ Endpoint dilution of total immunoglobulin, as described in Materials and Methods.
⁷ Determined at a 1:100 dilution of serum and a 1:4 dilution of lavage fluid.
⁸ 19-day-old mice.
⁹ 8-week-old mice.

magnitude until the mice died 2 to 3 weeks after B. pertussis aerosol challenge (Fig. 1).

B oligomer appeared to protect in a dose-dependent fashion, as only 41% of the animals receiving 0.4 µg of B oligomer survived respiratory B. pertussis challenge, compared with 90 to 100% survival observed in mice receiving 1 and 4 µg of B oligomer.

Little or no antibody to B oligomer was detected in either the sera or the bronchoalveolar lavage fluids of neonatal mice at the time of aerosol challenge. However, increasing amounts of serum IgG anti-B oligomer and IgG as well as IgA anti-B oligomer in the bronchoalveolar lavage fluids were detected 1 to 3 weeks after aerosol challenge. The IgG antibody detected 1 to 2 weeks after challenge in immunized neonates was not elicited solely by aerosol challenge, since anti-B oligomer was not detected in the sera or bronchoalveolar lavage fluids of unimmunized adults 1 to 2 weeks after aerosol challenge. Halpern and colleagues have observed that adult BALB/c mice given a respiratory challenge of B. pertussis do not make detectable serum anti-pertussis toxin antibodies until at least 4 weeks postchallenge and that a specific primary antibody response to challenge in neonates is very low or undetectable (S. Halpern, personal communication). IgA antibody was detected in bronchoalveolar lavage fluids but not in the sera of immunized mice 3 weeks after aerosol challenge. This observation suggests that some of the antibody detected in the lungs resulted from the local production of antibody in the respiratory mucosa. We have previously demonstrated that parenteral immunization of neonates with pertussis toxinoid disseminates a primed population of memory B lymphocytes to the lungs in the absence of high levels of circulating anti-pertussis toxin (26).

TABLE 2. Neutralization of pertussis toxin by monoclonal anti-B oligomer

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Conc of IgG (µg/ml)</th>
<th>CHO cell neutralization titer⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>170C4</td>
<td>S3 of B oligomer</td>
<td>500</td>
<td>2.048</td>
</tr>
<tr>
<td>18.1.7</td>
<td>Tetanus toxin</td>
<td>500</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

⁴ Endpoint titer, as described in Materials and Methods.
against respiratory infection with *B. pertussis*. Sato et al. have reported that a monoclonal antibody that binds to both the S2 and the S3 subunits of B oligomer protects 7-day-old DDY mice when it is administered intraperitoneally 2 h before *B. pertussis* aerosol challenge (20). This monoclonal antibody also inhibited the pertussis toxin-induced clustering of CHO cells, leukocytosis, and hemagglutination. These protection data indicate that antibodies to B oligomer that can neutralize pertussis toxin are sufficient to protect neonatal mice against lethal respiratory *B. pertussis* infection.

Monoclonal antibodies to S1, the ADP-ribosyltransferase, have previously been shown to neutralize pertussis toxin-induced CHO cell aggregation in vitro (14, 21, 24) and leukocytosis in vivo (1, 19, 24) as well as to protect neonatal mice from lethal *B. pertussis* aerosol challenge. These anti-S1 monoclonal antibodies appear to react with a single immunodominant conformational epitope of S1 that is necessary for the enzymatic activity of the molecule as well as for eliciting neutralizing antibody (3). We show here that a neutralizing antibody to the S3 subunit of B oligomer that does not cross-react with S1 is sufficient to protect neonatal mice against lethal *B. pertussis* aerosol challenge. This monoclonal anti-S3 antibody also inhibits pertussis toxin-induced hemagglutination, suggesting that anti-B oligomer may protect mice by interfering with the B oligomer-mediated binding of pertussis toxin to the eucaryotic cell membrane. The active and passive protection data presented here suggest that elicitation of antibodies that interfere with B oligomer-mediated binding to the eucaryotic membrane, in addition to elicitation of antibodies that neutralize the ADP-ribosylation capacity of S1, may provide optimal protection against symptoms of disease mediated by pertussis toxin. Further analysis of the mechanisms of protection mediated by purified antigens of *B. pertussis* will aid in the construction of new vaccines that are safe and yet optimally effective.

ACKNOWLEDGMENTS

We thank Drusilla Burns, Sally Hausman, and Juan Arciniega for helpful discussions and Mayumi Endoh for expert technical assistance.

This work was supported in part by participating agency support agreement BST-5947-P-HI-4265 between the U.S. Agency for International Development and the U.S. Public Health Service and by grant 28-1431 from the Dutch Praeventiefonds.

LITERATURE CITED


