INFECTION AND IMMUNITY, May 1987, p. 1132–1136
0019-9567/87/051132-05$02.00/0

Vol. 55, No. 5

Immune Response to the B Oligomer of Pertussis Toxin

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Received 3 October 1986/Accepted 28 January 1987

Immunization of mice with the B oligomer of pertussis toxin induced antibodies to the native toxin as measured by an enzyme-linked immunosorbent assay. These antibodies neutralized the ability of pertussis toxin to alter the morphology of Chinese hamster ovary cells. Furthermore, mice immunized with the B oligomer, when challenged with pertussis toxin, did not exhibit the leukocytosis normally associated with exposure to the toxin. These results demonstrate that the B oligomer, which does not contain the enzymatic activity of the holotoxin, can be used to induce a neutralizing antibody response and suggest that the B oligomer might be considered for use in acellular pertussis vaccines.

Pertussis is a human respiratory disease which has been effectively controlled through the use of vaccines (5, 24). The current vaccines consist of inactivated whole cells of *Bordetella pertussis*. The use of such vaccines has been associated with mild and occasionally serious side effects (1, 6). Efforts are therefore under way to develop less reactogenic vaccines containing purified protective antigens.

Pertussis toxin is one of the proteins which is included in some acellular pertussis vaccines since the toxin is thought to play a role in both pathogenesis and immunity (25, 27, 32). The toxin (also known as leukocytosis-promoting factor, histamine sensitization factor, islet-activating protein, and pertussigen) is composed of an enzymatically active A subunit and a B oligomer (composed of five polypeptide chains) which is responsible for binding of the toxin to the eucaryotic cell surface and introduction of the A subunit into the cell (30, 31). The A subunit catalyzes the ADP ribosylation of a family of GTP-binding regulatory proteins involved in hormonal signal transduction (8, 11, 12). Pertussis toxin is released from bacterial cells during infection and probably causes certain systemic effects of the disease (25, 32). In addition, the toxin may help to maintain infection of the respiratory tract by inhibiting migration of phagocytic cells to that site (17). Injection of mice with inactivated pertussis toxin or antibodies to the toxin has been shown to protect animals from subsequent challenge with *B. pertussis* (18, 19, 23, 27).

Since pertussis toxin has potent biological activity, it must be inactivated in a manner which retains its immunogenicity before it can be considered for inclusion in vaccines. One approach that has been used for detoxification of the molecule involves chemically modifying the protein with formaldehyde or glutaraldehyde (19, 23, 28). A second approach might be to isolate a part of the molecule which itself is nontoxic yet which would induce a neutralizing antibody response to the native toxin. Such an approach has the advantage that the molecule is not toxic and therefore need not be chemically modified, thus preserving epitopes on the molecule. Moreover, conversion to a toxic form is impossible, whereas chemically inactivated toxins can revert to toxic forms (14, 22). A candidate for such an immunogen is the B oligomer, which lacks the enzymatically active portion of pertussis toxin. The development of a mild procedure for dissociating the A subunit from the B oligomer and obtaining the B oligomer in high yields (4) has made such an approach possible. A similar approach has been used in the development of cholera vaccines in which the B subunit of cholera toxin was used (10). In this study we have examined the ability of the isolated B oligomer of pertussis toxin to induce a neutralizing antibody response in mice.

MATERIALS AND METHODS

Materials. Pertussis toxin was purchased from the Michigan Department of Public Health or prepared by the method of Sekura et al. (29). All toxin preparations were greater than 95% pure as assessed by sodium dodecyl sulfate (SDS) gel electrophoresis and were used interchangeably. Carboxymethyl Sepharose CL-6B and CNBr-activated Sepharose 4B were purchased from Pharmacia, Uppsala, Sweden. Fetuin (prepared by the Spiro method) was a product of GIBCO Laboratories, Grand Island, N.Y. Goat anti-mouse immunoglobulin G (whole molecule) alkaline phosphatase conjugate and phosphatase substrate (*p*-nitrophenyl phosphate) were from Sigma Chemical Co. St. Louis, Mo. BALB/c mice were purchased from the National Cancer Institute-Frederick Cancer Research Facility (Frederick, Md.). N:NIH(S) mice were obtained from Division of Research Resources of the National Institutes of Health (Bethesda, Md.).

CHO cell assay. CHO cells (American Type Culture Collection strain CCL 61) were prepared as previously described (9). Toxin or B oligomer was serially diluted (twofold) with medium (Ham F12; Flow Laboratories, Inc., McLean, Va.) containing 10% fetal calf serum in a 96-well microtiter plate to give various concentrations in 100 μl. CHO cells (1.0×10^4 in 100 μl of medium) were then added to each well. After incubation for 24 to 48 h in a CO_2 incubator at 37°C, the cells were examined under a microscope to determine the extent of clustering. The minimal concentration of toxin or subunit preparation which caused clustering of all cells is reported.

Antibody neutralization assays were performed in a manner similar to that previously described (7). Heat-treated (56°C, 30 min) serum from animals immunized with B oligomer or pertussis toxoid was serially diluted (twofold) with F12 medium containing 10% fetal calf serum and 50 μg of gentamicin per ml in a 96-well microtiter plate. The initial dilution was 1:10. Pertussis toxin (50 μl; 4 ng/ml) in medium

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was added to the diluted serum (50 μl). After mixing, the plates were incubated for 3 h in a CO_2 incubator at 37°C. CHO cells (2.0×10^4 in 100 μl of medium) prepared as described above were added to each well. After incubation at 37°C in a CO_2 incubator for 24 to 48 h, the plate was examined under a microscope to determine the ability of the serum to neutralize the clustering effect of pertussis toxin.

Preparation of the B oligomer. The B oligomer was separated from the A subunit by a modification of the precedure previously described (4). Holotoxin was incubated in 10 mM sodium phosphate buffer (pH 7.0) containing 3 M urea, 1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, and 100 μM ATP (buffer A) for 15 min. The solution, containing 1 mg of protein, was added to 0.75 ml of packed carboxymethyl Sepharose. The suspension was incubated at room temperature for 15 min with occasional stirring. At that time the suspension was poured into a small column. The A subunit did not bind to the carboxymethyl Sepharose and was eluted with an additional 0.75 ml of buffer A. The column was then washed with an additional six volumes of buffer A. The B oligomer was eluted from the column with 1.0 ml of 0.2 M potassium phosphate buffer (pH 7.5) containing 2 M urea.

The B oligomer was dialyzed overnight versus 10 mM sodium phosphate (pH 7.0) containing 3 M urea. The B oligomer in buffer A was then further purified by incubation with and elution from carboxymethyl Sepharose as described above.

Inactivation of pertussis toxin with glutaraldehyde. Pertussis toxin was inactivated with glutaraldehyde by a modification of the procedure previously described (19). The toxin (0.25 mg/ml in 50 mM potassium phosphate buffer [pH 7.5] containing 0.5 M urea) was incubated with glutaraldehyde (0.15%) for 2 h at room temperature. The reaction was stopped by addition of lysine (monohydrochloride form) to give a final concentration of 50 mM (no attempt was made to control pH). After further incubation for 2 h at room temperature, the mixture was extensively dialyzed against phosphate-buffered saline solution (PBS).

Immunization of mice. To each 3 ml of protein solution containing toxoid (600 μg/ml) or B oligomer (170 μg/ml), 1 ml of PBS containing 0.2% gelatin was added. These mixtures were dialyzed overnight versus PBS at 4°C to remove any excess urea or glutaraldehyde in the B oligomer or pertussis toxoid preparations, respectively.

A 1:1 ratio (on a weight basis) of $Al(OH)_3$ (Superfos a/s; Vedbaek, Denmark) to protein (preparation plus gelatin) was prepared. Preparations were incubated for 1 h at room temperature with occasional stirring. Five mice (BALB/c, female, 6 weeks old) were immunized with 0.5 ml of protein administered intraperitoneally containing 0.16 to 30 μg of pertussis toxoid or B oligomer as indicated in the tables. In addition, in one group, mice were injected with $Al(OH)_3$ alone (60 μg per mouse). Four weeks after injection, blood was collected from the tail vein of each mouse, and equal volumes of serum corresponding to each dose and preparation were pooled and stored at −20°C until assayed.

The data presented are representative of the results obtained in separate experiments.

Neutralization of the leukocytosis-promoting activity of pertussis toxin. Mice immunized with each preparation were challenged 37 days after immunization with pertussis toxin (500 ng/0.5 ml) administered intraperitoneally, with PBS-gelatin as the diluent. Five days after challenge, the leukocyte count per cubic millimeter of blood was measured.

Histamine-sensitizing activity. Histamine-sensitizing activity was measured essentially as described previously (27).

Protein determination. Protein was determined by the method of Bradford (2) with ovalbumin as the standard.

SDS-gel electrophoresis. SDS-gel electrophoresis was performed essentially as described by Laemmli (13).

ELISA. Mouse sera were tested for the presence of antibodies to pertussis toxin and B oligomer by the enzyme-linked immunosorbent assay (ELISA) method essentially as described by Manclark et al. (16). Microtiter plates were incubated with the appropriate solution (5 μg/ml). Nonspecific antibody adsorption was blocked by incubating the coated plates for 1 h with 5% fetal calf serum. Sera were diluted serially (threefold) with an initial dilution of 1:30. All incubations were performed at room temperature. Data were plotted as the $\log_{10}$ dilution versus optical density at 405 nm. ELISA antibody titers were calculated as the inverse of the antilog of the x intercept extrapolated from the linear portion of each curve.

Immunoblots. The absence of contaminating A subunit antibodies in anti-B oligomer serum was assessed by immunoblotting (3). The A subunit (32 μg) was run on an SDS-polyacrylamide gel. Proteins were electroblotted from the gel onto nitrocellulose paper for 1.5 h at 0.8 A in 25 mM Tris–192 mM glycine (pH 8.3) with 20% methanol at 5°C. Strips were cut from the nitrocellulose and blocked with 0.1% Brij 35 in Dulbecco PBS (B114). The sera were diluted 1:500 in B114 and incubated with individual nitrocellulose strips overnight at room temperature. The strips were washed three times with 15 ml of B114 and incubated with 5 ml each of a 1:500 dilution in B114 of horseradish peroxidase conjugated goat anti-mouse immunoglobulin G (Cooper Biomedical, Inc., West Chester, Pa.) for 6 h at room temperature. The strips were washed three times with B114 and then incubated with peroxidase substrate (60 mg of 4-chloronaphthol [Sigma Chemical Co., St. Louis, Mo.], 20 ml of methanol, 100 ml of deionized water, 75 μl of H_2O_2). The reaction was stopped by removing the substrate solution and adding 2 mM sodium azide.

RESULTS

Sera collected from mice immunized with B oligomer contained antibodies to both B oligomer and holotoxin as measured by the ELISA (Table 1). For comparison, the antibody titer of sera from mice injected with pertussis toxoid was also measured. The toxoid was prepared by modification of native toxin with glutaraldehyde. Sera from mice immunized with pertussis toxoid exhibited anti-holotoxin titers similar to those of sera from mice immunized with B oligomer (although only one immunization schedule was used) (Table 1).

The B oligomer preparation used for immunization appears to be free of the A subunit. SDS-polyacrylamide gel analysis of 50 μg of the preparation showed only the peptide chains which make up the B oligomer (Fig. 1). No A subunit was detected. In addition, the CHO cell assay was used to assess purity of the B oligomer. Picogram quantities of holotoxin will induce a clustered morphology of CHO cells (9); however, purified subunits are much less effective in altering the morphology of these cells (3a). Pertussis toxin at a concentration as low as 0.16 ng/ml clustered CHO cells. In contrast, the B oligomer preparation clustered CHO cells at 125 ng/ml, indicating that the B oligomer was at least 99.8% free of holotoxin. The B oligomer preparation did not exhibit significant leukocytosis-promoting activity or histamine-

TABLE 1. Antibody response to B oligomer or pertussis toxin as measured by ELISA

Immunogen (dose, μg)	ELISA antibody titer[a] to:	
	B oligomer	Holotoxin
$Al(OH)_3$	ND	ND
B oligomer		
30	14,025	4,599
6	2,075	2,571
1.2	917	674
0.24	ND	NA
Pertussis toxoid		
20	1,724	10,070
4	931	2,698
0.8	ND	389

[a] Values were calculated as described in Materials and Methods and represent the results obtained on pools of sera from five mice. Sera from uninjected mice did not exhibit a detectable titer to either B oligomer or pertussis toxin. ND, Not detectable; NA, not assayed.

sensitizing activity (Table 2). Serum from mice immunized with B oligomer was examined to ensure that antibodies to the A subunit of the toxin were not present. Immunoblot analysis of this serum shows that it did not contain antibodies to the A subunit (at least at the dilution tested) in contrast to serum from mice immunized with pertussis toxoid, which contained anti-A subunit antibodies (Fig. 2).

Antibodies raised against B oligomer were able to neutralize the action of pertussis toxin. Sera from mice immunized with the B oligomer neutralized the ability of the toxin to alter the morphology of CHO cells (Table 3). Neutralizing antibody titers were detected in sera from animals injected with as little as 6 μg of B oligomer. These results were similar to those obtained with sera from mice injected with pertussis toxoid.

Injection of mice with B oligomer protected the animals from at least one of the biological activities of pertussis toxin. Mice that had been injected with as little as 1.2 μg of B oligomer were protected from the leukocytosis-promoting activity of the toxin (Table 4). The protection afforded by the B oligomer was similar to that obtained after injection of inactivated pertussis toxoid.

DISCUSSION

The B oligomer is a potential candidate for a vaccine component, since it does not contain the ADP-ribosyltransferase activity of the holotoxin (30). Previous results indicate that the B oligomer, unlike pertussis toxin, does not enhance

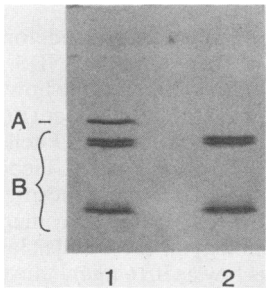

FIG. 1. SDS-gel electrophoresis of pertussis toxin and B oligomer preparations. Lanes: 1, pertussis toxin (20 μg); 2, B oligomer (50 μg). The positions of the polypeptide chains of the A subunit and B oligomer are indicated.

TABLE 2. Biological activities of the B oligomer and pertussis toxin

Prepn[a]	Leukocytosis-promoting activity (leukocyte count)[b]	Histamine-sensitizing activity (deaths/no. tested)[c]
Control (PBS–0.2% gelatin)	7,504 ± 536	1/10
B oligomer (0.5 μg)	7,388 ± 1,070	1/10
Pertussis toxin (0.5 μg)	31,100 ± 5,656	8/10

[a] Ten male N:NIH(S) mice (25 g) were each challenged intraperitoneally with the preparation indicated in a total volume of 0.5 ml of PBS–0.2% gelatin.
[b] Leukocytes per cubic millimeter of blood ± standard deviation measured 5 days after challenge.
[c] Six days after challenge, mice were each injected intraperitoneally with 1 mg of histamine base in 0.5 ml of PBS. Deaths due to histamine per total number of mice challenged with the preparation are reported.

insulin secretion (30). We found that the B oligomer did not exhibit the leukocytosis-promoting and histamine-sensitizing activities of pertussis toxin, findings which contrast with the conclusions of Nogimori et al. (21), who attributed these activities to the B-oligomer moiety. These workers found that acetamidinated pertussis toxin exhibited the ADP-ribosyltransferase activity of the native toxin and retained the ability to increase adenylate cyclase activity in C6 glioma cells. Acetamidinated pertussis toxin, however, was found to exhibit decreased leukocytosis-promoting and histamine-sensitizing activities. They concluded that these activities were due to the B oligomer. Our results could be reconciled with the conclusions of Nogimori et al. if the B oligomer were unstable in vivo and were degraded before it reached the target cells that are responsible for leukocytosis and histamine sensitization. Another possibility may be that the leukocytosis-promoting and histamine-sensitizing activities of pertussis toxin are indeed due to ADP ribosylation of eucaryotic regulatory proteins. The differences in the activities of the native and acetamidinated toxin that were previously reported (21) may be due to alteration in the ability of acetamidinated toxin to bind to cell types involved in leukocytosis and histamine sensitization. This explanation seems plausible, since Nogimori et al. (21) found that the ability of pertussis toxin to inhibit histamine release from rat

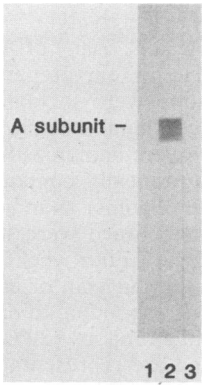

FIG. 2. Immunoblot analysis of sera from mice immunized with B oligomer or pertussis toxoid. The A subunit which had been transferred from an SDS-polyacrylamide gel to nitrocellulose strips was incubated with serum from mice immunized with either 20 μg of B oligomer (lane 1), 20 μg of pertussis toxoid (lane 2), or serum from nonimmunized mice (lane 3) as described in Materials and Methods. The position of the A subunit is indicated.

TABLE 3. Neutralization of the CHO cell clustering effect of pertussis toxin by sera from mice immunized with B oligomer or pertussis toxoid

Immunogen (dose, μg)	Neutralizing titer[a]
$Al(OH)_3$	<40
B oligomer	
30	160
6	80
1.2	<40
0.24	<40
Pertussis toxoid	
20	240
4	40
0.8	<40
0.16	<40

[a] Values are expressed as the reciprocal of the maximal dilution of serum (pooled from five mice) which neutralized CHO cell clustering. Assays were performed in duplicate, and the median of the two values is reported. Sera from uninjected mice did not exhibit a detectable neutralizing antibody titer. The minimal dilution tested was 1:40.

mast cells, an activity which required the ADP-ribosyltransferase activity of the toxin (20), was markedly attenuated by acetamidination. These workers attributed this result to alterations in the ability of the chemically modified toxin to bind to the target cell. Whatever the correct explanation may be for the lack of activity of the B oligomer in vivo, our data clearly indicate that the isolated B oligomer does not exhibit many of the biological activities of the holotoxin. The B oligomer, however, has been shown to display the ability to stimulate lymphocyte mitosis in vitro (31) at concentrations 2 to 4 orders of magnitude higher than those needed for other toxic acitivities of pertussis toxin. Further studies need to be conducted to determine whether this activity of the B oligomer might be observed in vivo.

The data presented in this study demonstrate that the B oligomer can induce an antibody response in mice which neutralizes the action of pertussis toxin. Serum from mice injected with the B oligomer contains antibodies which neutralize the ability of pertussis toxin to alter morphology of CHO cells. The clustered morphology of CHO cells induced by the toxin appears to be caused by the ADP-ribosyltransferase activity of the toxin (3a). Both the A subunit and the B oligomer are required for CHO cell clustering, since the B oligomer is needed for introduction of the enzymatically active A subunit into the CHO cells. Antibodies to the B oligomer may neutralize the activity of the holotoxin by preventing its binding to CHO cells.

TABLE 4. Effect of immunization of mice on the leukocytosis-promoting activity of pertussis toxin

Immunogen (dose, μg)	Leukocyte count[a]
None	24,330 ± 2,499 (5)
B oligomer	
30	4,876 ± 2,202 (4)
6	4,914 ± 2,205 (5)
1.2	5,547 ± 1,225 (4)
0.24	16,352 ± 6,065 (5)
Pertussis toxoid	
20	3,762 ± 1,064 (5)
4	7,050 ± 2,142 (5)
0.8	5,833 ± 1,733 (5)
0.16	10,668 ± 10,792 (5)

[a] Both nonimmunized and immunized mice were challenged with pertussis toxin as described under Materials and Methods. Five days later, leukocyte counts were measured. Values are expressed as leukocytes per cubic millimeter of blood ± standard deviation for (*n*) mice. Mice which were not challenged with pertussis toxin but injected with PBS alone had a leukocyte count of 4,495 ± 1,158 (four mice total).

In addition to inducing production of antibodies which neutralize action of the toxin in an in vitro assay, immunization with the B oligomer protects animals from the leukocytosis-promoting activity of pertussis toxin. Injection of nonimmunized mice with pertussis toxin resulted in about a fivefold increase in leukocyte count, whereas mice that had been injected with the B oligomer at doses of ≥1.2 μg were completely protected from this action of the toxin.

The B oligomer did not appear to be superior to inactivated pertussis toxin as an immunogen, as might be expected since inactivated pertussis toxin was chemically modified. Thus, the major advantage of using the B oligomer as a component in a vaccine would be the inability of this protein to revert to the enzymatically active form of the toxin, which has previously been reported with chemically modified toxins (14, 22).

Results from this study demonstrate that the B oligomer can produce a neutralizing antibody response in mice. The A subunit is also a potential vaccine candidate. Theoretically, the use of the A subunit might involve some risk, since this protein retains enzymatic activity, although the ability of the A subunit to enter a eucaryotic cell should be decreased in the absence of the B oligomer. Our preliminary attempts to use the A subunit as an immunogen have met with discouraging results. Not only have we had problems freeing A subunit preparations from contaminating B oligomer, but we have found that the A subunit appears to be a poor immunogen in mice (data not shown). Other workers have found that a monoclonal antibody to the A subunit neutralized many of the activities of the holotoxin, including its leukocytosis-promoting activity, whereas a monoclonal antibody to the B oligomer had no effect on these activities (26).

The B oligomer induces a good neutralizing antibody response to holotoxin. The production of sufficient quantities of B oligomer for use in a vaccine should not pose a problem. Preparations of pure B oligomer can be readily obtained with standard protein purification techniques. Contamination of the preparation with holotoxin can be monitored easily with the CHO cell assay, which can detect minute quantities of residual toxin. Recently, the genes encoding the pertussis toxin subunits were cloned (15). It is therefore conceivable that a *B. pertussis* strain which lacks the gene for the "toxic" A subunit may now be engineered using recombinant DNA techniques. Such an approach has the advantage that the B oligomer could be purified from these strains without the possibility of contamination with holotoxin.

ACKNOWLEDGMENTS

This work was supported in part by a fellowship from the Pan American Health Organization, Mexico (to J.L.A.). Additional aid was provided by a Participating Agency Support Agreement (BST-5947-P-HI-4265) between the United States Agency for International Development and the United States Public Health Service.

We thank Janice David for expert technical assistance and James Kenimer for assistance with immunoblot techniques.

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