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Contribution of the B Oligomer to the Protective Activity of Genetically Attenuated Pertussis Toxin

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An enzymatically deficient recombinant S1 subunit, in which Arg-9 was replaced by Lys, was combined with native B oligomer to form a mutant holotoxin molecule. This molecule exhibited decreased leukocytosis-promoting and histamine-sensitizing activities compared with those of the native toxin, supporting the view that the B oligomer is not responsible for these activities. The protective activity of this genetically attenuated pertussis toxin was compared with that of B oligomer alone. The mutant pertussis toxin and B oligomer were similarly capable of protecting mice against a respiratory infection with *Bordetella pertussis*, suggesting that the B oligomer makes a significant contribution to the protection afforded by the genetically attenuated holotoxin.

Adverse reactions associated with whole-cell pertussis vaccine have spurred development of improved vaccines consisting of antigens purified from *Bordetella pertussis*. Chemically inactivated pertussis toxin (PT) has been shown to be protective both in animal models and in humans (1, 20, 22); it is therefore a candidate for inclusion in this type of vaccine. PT is an exotoxin which interacts with many types of eucaryotic cells and impairs their signal transduction pathways by ADP-ribosylating a family of GTP-binding regulatory proteins (10, 14). PT resembles a number of other bacterial toxins in its A-B structural architecture (24). The A moiety, or S1 subunit, is an ADP-ribosyltransferase. The B moiety, a pentamer consisting of S2, S3, S4, and S5 subunits in a 1:1:2:1 ratio, is responsible for binding of the toxin to the eucaryotic cell surface. Among the multiple in vivo activities possessed by PT are its abilities to induce leukocytosis, alter glucose homeostasis, and sensitize mice to the lethal effects of histamine (26).

Since PT has potent biological activities, the molecule must be inactivated before it can be included in acellular vaccines. Identification of regions of the molecule which contribute to its protective capacity is therefore important in order to avoid destruction of these regions during the inactivation procedure. Investigators have postulated that the S1 subunit is the immunodominant portion of the PT molecule (4, 11). Indeed, monoclonal antibodies against the S1 subunit have been shown to neutralize toxic activities of the holotoxin and confer passive protection against *B. pertussis* infection in mice (21). Purified S1 subunit, however, neither elicited neutralizing antibodies to the toxin nor stimulated immunoprotection in animals challenged with PT (18). Therefore, association of the S1 subunit with the B oligomer may be necessary to stabilize the protective epitopes of the S1 subunit (6). The purified B oligomer has been demonstrated to induce neutralizing antibodies to the toxin in mice and to protect them from challenge with PT (2); it was recently shown to protect mice from the lethal effects of a respiratory infection with *B. pertussis* (23).

While both inactivated pertussis holotoxin and the isolated B oligomer can protect mice against an aerosol challenge with *B. pertussis*, the contribution of the B oligomer to the protection afforded by the holotoxin molecule has not been assessed. The recent availability of mutant holotoxin molecules which lack enzymatic activity yet which retain the structure of the native molecule (3) has permitted us to measure the contribution of the B oligomer to protection. We have assembled a holotoxin similar to the one previously described (3); the S1 subunit of this genetically attenuated holotoxin has a lysine residue in place of Arg-9, which results in the loss of 99.9% of the ADP-ribosyltransferase activity of the native S1 subunit (7). While its enzymatic activity is dramatically attenuated, the structure of the molecule is believed to be similar to that of the native protein, since this S1 mutant is capable of combining with the B oligomer to form a holotoxinlike molecule (3). In this study, we compared the ability of this genetically attenuated holotoxin with that of the B oligomer to protect mice from lethal aerosol challenge with *B. pertussis*.

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MATERIALS AND METHODS

Electrophoresis. Nondenaturing, nonreducing polyacrylamide gel electrophoresis (PAGE) was performed according to the method of Gabriel (9) with 7.5% polyacrylamide gels (pH 4); sodium dodecyl sulfate-PAGE in the presence of dithiothreitol (15% acrylamide) was performed essentially as described by Laemmli (16).

Protein determination. Protein concentration was determined by the method of Bradford (5), using ovalbumin as the standard.

Protein preparations. The recombinant analog form of the S1 subunit, with the Arg-9-to-Lys substitution (rS1/1-4), was purified as previously described (3). B oligomer was prepared as previously described (2) and was determined by the Chinese hamster ovary (CHO) cell assay (12) to be >99.8% free of PT. Pertussis holotoxin was inactivated with glutaraldehyde as previously described (2). Tetanus toxoid was a formaldehyde-inactivated preparation (Connaught Laboratories Inc., Swiftwater, Pa.).

Endotoxin testing. Endotoxin was measured by using a *Limulus* amebocyte lysate assay (13). The endotoxin content of the mutant S1 preparation was estimated to be 0.2 endotoxin units per μg of protein.

Reassociation. The in vitro assembly of the genetically attenuated holotoxin was performed essentially as described by Bartley et al. (3). Briefly, 3 μg of rS1/1-4 was mixed with 4.2 μg of B oligomer in a total volume of 150 μl of 50 mM potassium phosphate buffer, pH 7.5, containing 0.3 M NaCl and 2 M urea. The mixture was incubated at 37°C for 30 min and analyzed both by nondenaturing, nonreducing PAGE and by sodium dodecyl sulfate-PAGE in the presence of dithiothreitol. Densitometry of the resulting nondenaturing gels was performed with an LKB laser densitometer (2202 Ultrascan; Pharmacia LKB Biotechnology Inc., Piscataway, N.J.) attached to a recording integrator (LKB model 2220). The residual toxicity of the genetically attenuated holotoxin was quantified both in vitro and in vivo as CHO cell cytotoxicity and histamine-sensitizing (HS) activity in mice, respectively.

Leukocytosis-promoting (LP) and HS activity assays. NIH(S) mice (females, 14 to 16 g) were injected intraperitoneally with the indicated quantities of PT in 0.5 ml of phosphate-buffered saline (PBS) containing 0.2% gelatin. Five days later, mice were bled and the leukocyte count was determined in a Coulter Counter (model ZM; Coulter Electronics, Hialeah, Fla.). The following day, mice were challenged with histamine base (1 mg) and monitored for death as previously described (22).

Mouse aerosol challenge. The ability of preparations to protect neonatal mice (BALB/cAnNcR) against a lethal *B. pertussis* respiratory challenge was assessed as described by Shahin et al. (23). The highest dose of each antigen was prepared, with PBS containing 0.2% gelatin as diluent. The proteins (antigen plus gelatin) were adsorbed to an equivalent mass of Alhydrogel (Superfos a/s, Vedbaek, Denmark) for 1 h at room temperature and subsequently dialyzed overnight against PBS at 4°C to remove urea, inorganic salts, and inactivating agents. Lower doses of immunogen were prepared by dilution of this suspension with PBS containing 0.2% gelatin. Mice received two identical injections (0.1 ml intraperitoneally) on days 5 and 12 postpartum and were challenged 7 days after the last immunization. On days 13, 15, and 19 postinfection, 5 μl of blood was collected from the periorbital sinus of each mouse and leukocytes were counted in a Coulter Counter. Deaths were noted for 21 days after the challenge.

RESULTS

An enzymatically deficient holotoxin molecule was formed by the addition of a mutant S1 subunit (Arg-9 replaced by Lys) and B oligomer (3). When equimolar quantities of the mutant S1 subunit and the B oligomer were mixed, the yield of a genetically attenuated holotoxin was approximately 50% according to densitometric analysis of nondenaturing polyacrylamide gels (data not shown). Increasing the amount of S1 subunit used for reassociation resulted in an increase in the yield of genetically attenuated holotoxin. When a 2:1 molar ratio of S1 subunit to B oligomer was used, at least an 83% yield (based on B oligomer concentration) of the genetically attenuated holotoxin was obtained.

We found that this molecule was approximately 2,000 times less potent than PT in clustering CHO cells. While native PT caused detectable CHO cell clustering at concentrations of ≥0.16 ng/ml, attenuated PT clustered CHO cells only at concentrations of ≥350 ng/ml. Moreover, the genetically attenuated PT exhibited neither LP nor HS activity at the dose examined (Table 1).

TABLE 1. Biological activities of genetically attenuated holotoxin

Preparation and dose (ng)	LP activity (leukocyte count)[a]	HS activity (deaths/total)
Control (PBS–0.2% gelatin)	17,902 ± 4,520	0/10
Genetically attenuated holotoxin, 415	15,960 ± 3,884	0/10
Pertussis toxin		
125	19,898 ± 5,138	4/10
250	29,812 ± 6,018	6/10
500	59,810 ± 7,970	9/10

[a] Leukocytes per microliter of blood ± standard deviation (n = 5) measured 5 days after injection.

Neonatal mice were immunized with inactivated PT as well as individual moieties of the toxin and subsequently challenged with an aerosol of *B. pertussis* in order to examine the protective activities of each of these proteins. As shown in Table 2, immunization of mice with 8 μg of tetanus toxoid provided, as expected, no protection against death due to challenge with *B. pertussis*. In contrast, chemically inactivated PT (dose of 8 μg) completely protected mice from the lethal effects of bacterial challenge. The mutant S1 subunit (dose of 1 μg) provided only partial protection against the organism. Comparable doses of both the genetically attenuated holotoxin and the B oligomer completely protected the mice against the lethal effects of the organism (Table 2) and protected them against the leukocytosis caused by the bacterial infection (Fig. 1). The protection afforded by the B oligomer and the genetically attenuated holotoxin exhibited similar dependencies on dose.

DISCUSSION

The B oligomer of PT has previously been shown to protect mice from lethal challenge with *B. pertussis* (23). Thus, the B oligomer may be an important component of acellular pertussis vaccines either as an isolated moiety or as part of genetically attenuated holotoxin molecules. Because future vaccines will likely contain B oligomer that has not been chemically inactivated, careful analysis of the molecule

TABLE 2. Comparative protective efficacies of genetically attenuated holotoxin and B oligomer of PT after challenge of neonatal mice with *B. pertussis*

Antigen	Dose (μg)		No. of survivors/ no. tested	
	S1 mutant	B oligomer	Expt 1	Expt 2
Genetically attenuated holotoxin				
AB_H	1.0	1.4	9/10	4/6
AB_M	0.1	0.14	5/10	6/10
AB_L	0.02	0.03	1/9	3/10
B oligomer				
B_H		1.4	10/10	6/10
B_M		0.14	10/10	7/10
B_L		0.03	2/10	2/9
S1 mutant (A)	1.0		4/11	1/8
Tetanus toxoid (8 μg)			0/11	0/13
Pertussis toxoid (8 μg)			8/8	7/7

[a] The subscripts H, M, and L designate high, medium, and low doses, respectively.

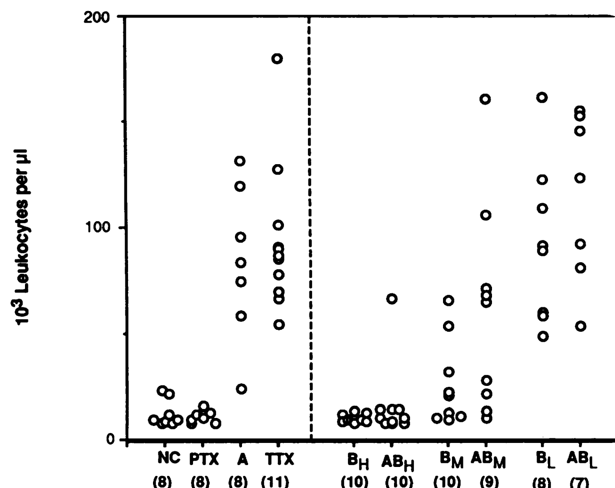

FIG. 1. Protection against leukocytosis after aerosol challenge of neonatal mice with *B. pertussis*. Mice were immunized with the preparations and doses described in Table 2. Fifteen days after challenge with *B. pertussis* as described in Materials and Methods, mice were bled and leukocyte counts were determined. Each circle represents the number of leukocytes per microliter of blood for a single mouse. The number of survivors for each treatment is shown in parentheses below each group. NC, not challenged; PTX, pertussis toxoid; A, S1 mutant; TTX, tetanus toxoid; B, B oligomer. The subscripts H, M, and L indicate, respectively, high, medium, and low doses.

is required to ascertain that none of the potent biological activities associated with PT are due to this component. Our results, which are consistent with previously published results (15, 17), suggest that the B oligomer as part of a genetically attenuated holotoxin exhibits neither the LP nor the HS activity of native toxin. Therefore, these activities are likely due to the ADP-ribosyltransferase activity of the toxin. These results are in contrast to the conclusions of Nogimori et al. (19), who attributed LP and HS activities to the B oligomer; these workers found that acetamidinated PT displayed decreased HS and LP activities yet exhibited the ADP-ribosyltransferase activity of the native toxin and retained the ability to increase adenylate cyclase activity in C6 glioma cells. Since neither isolated B oligomer (2) nor B oligomer that is part of a genetically attenuated holotoxin (15, 17; this study) exhibits LP and HS activities, it seems likely that the differences in the activities of the native and acetamidinated toxin that were previously reported (19) are due to alteration in the ability of acetamidinated toxin to bind to cell types involved in leukocytosis and histamine sensitization.

In this study, we found that the mutant S1 subunit only partially protected mice from an aerosol challenge with *B. pertussis*. These results are consistent with those of previous studies, which demonstrated that the native S1 subunit did not protect mice against the lethal effects of an intracerebral challenge with *B. pertussis* (18). In contrast to the tested dose of the mutant S1 subunit, comparable doses of B oligomer completely protected mice from aerosol challenge with *B. pertussis*. Moreover, the dose of B oligomer required for protection was similar to the dose of genetically attenuated holotoxin required. These findings suggest that the B oligomer contributes significantly to the protection afforded by the genetically attenuated holotoxin, at least in the animal model used in our study. Of course, caution must be exercised in extrapolating these results to protection of humans against disease.

Other investigators have previously suggested that the human humoral response to PT is directed for the most part to the S1 subunit (25). However, in those studies, the antibody contents of human sera were analyzed by immunoblot techniques. While S1 refolds to an enzymatically active conformation after exposure to the denaturing conditions used for immunoblot analysis (8), little is known concerning the ability of the conformational epitopes of the B subunits to re-form after subjection to such denaturing conditions. Therefore, an inability to detect antibodies to the B subunits of PT by immunoblot techniques must be interpreted with caution.

The B oligomer of PT plays a critical role in the action of the toxin by mediating binding of the toxin to eucaryotic cell receptors (24). Our results suggest that this moiety may also play a significant role in the protection afforded by genetically attenuated holotoxin molecules. Vaccines which contain genetically altered PT molecules having the Arg-9–Lys mutation as well as a Glu-129–Gly mutation are currently being tested in clinical studies (17). The B-oligomer component of these vaccines may be a critical constituent.

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