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Properties of Pertussis Toxin B Oligomer Assembled In Vitro from Recombinant Polypeptides Produced by *Escherichia coli*

W. NEAL BURNETTE,[1]* JUAN L. ARCINIEGA,[2] VERNON L. MAR,[1] AND DRUSILLA L. BURNS[2]

Amgen Inc., Amgen Center, Thousand Oaks, California 91320-1789,[1] and Center for Biologics Evaluation and Research, U.S. Food and Drug Administration, Bethesda, Maryland 20892[2]

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The subunits that make up the pentameric B oligomer of pertussis toxin (S2, S3, S4, and S5) were individually synthesized as recombinant polypeptides in *Escherichia coli*, isolated as insoluble inclusion bodies, and assembled into a multimeric form in vitro by spontaneous association following treatment with a chaotropic agent, reduction, and reoxidation. The recombinant B multimer, purified by fetuin-Sepharose affinity chromatography, contained all four of the individual subunits and possessed the mitogenic and hemagglutinating activities characteristic of the native B oligomer. Immunization of mice with the recombinant B oligomer elicited antibodies that neutralized pertussis toxin in vitro and, moreover, provided protection in vivo against the leukocytosis-promoting activity of the toxin. These results demonstrate the potential for assembly of complex multimeric proteins from recombinant DNA-derived polypeptides and provide a novel means for production of an acellular pertussis vaccine component.

The demand for pertussis vaccines with decreased adverse reactions has stimulated research to define the immunogenic constituents of the causative agent, *Bordetella pertussis*. An important component of new acellular vaccines is a detoxified form of pertussis toxin (PT), a heterohexameric protein with an A-B toxin structure (33). The A (active) moiety, or S1 subunit, is an ADP-ribosyltransferase (15, 16, 33) that interferes with hormonal signal transduction in mammalian cells (7) and is responsible for the cytopathic effects of the toxin (2, 5). The B (binding) oligomer contains subunits S2, S3, S4, and S5 in a molar ratio of 1:1:2:1, respectively (15). Subunit S4 forms dimers with both S2 and S3; these dimers are apparently joined into a pentameric structure by subunit S5 (33). The cell receptor recognition qualities reside in the B oligomer, which interacts with complex glycoconjugates of mammalian cells (9, 29, 35, 37).

As a chemical toxoid, the holotoxin elicits immunoprotection in mice against intracerebral challenge with *B. pertussis* (21, 27) and has been demonstrated to be efficacious for prevention of disease in human clinical studies (1, 6, 22, 23, 32). It was recently demonstrated that the B oligomer alone may be sufficient to provoke protective immune responses (2, 31) and that it confers protection in mice equivalent to that of a genetically attenuated holotoxin molecule (3). While biochemically purified B oligomer that is 99.8% free of the S1 subunit can be obtained (2), the possibility remains that the residual holotoxin actually elicits the immunoprotection or enhances the immunogenicity of the B oligomer through an adjuvant effect (18). In this study, we assembled the B oligomer in vitro from *Escherichia coli*-produced recombinant subunits and evaluated its potential to elicit a toxin-neutralizing immune response in experimental animals. In so doing, we hoped to provide an alternative approach for production of this oligomeric protein as a vaccine component, eliminating not only active holotoxin but also other toxic constituents of the *B. pertussis* bacterium.

* Corresponding author.

MATERIALS AND METHODS

Native B oligomer. B oligomer was prepared from native PT as previously described (2) or, where indicated, purchased from List Biologicals (Campbell, Calif.).

Recombinant PT subunits and B oligomer assembly. The individual B oligomer subunits were produced in recombinant *E. coli* as previously described (12). B oligomer was assembled in vitro from these recombinant protein subunits by the following procedure. Inclusion body preparations containing each of the subunits were individually analyzed for protein content by using the Bio-Rad protein assay (8). The relative amount of specific subunit protein in each preparation was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (17), followed by quantitative densitometric scanning of gels stained with Coomassie brilliant blue R-250. In a typical preparation, inclusion bodies containing S2 (13.8 mg [wet weight] of protein, of which 4.92% was S2), S3 (30.9 mg [wet weight], of which 9.74% was S3), S4 (118 mg [wet weight], of which 8.74% was S4), and S5 (31.3 mg [wet weight], of which 15.3% was S5) were combined and the admixture was solubilized with gentle stirring overnight at room temperature in a total volume of 100 ml of 25 mM Tris-HCl (pH 8.5) containing 6 M guanidinium hydrochloride (GuHCl) and 10 mM dithiothreitol. The mixture was then centrifuged (18,000 rpm, Beckman JA20 rotor, 30 min, 4°C) to remove insoluble materials. Dithiothreitol was removed from the supernatant fraction (85 ml) by chromatography on a column (5 by 25 cm) of GH25 (Amicon) in GuHCl-Tris buffer free of dithiothreitol. The column eluant was monitored by an in-line UV spectrophotometer (Pharmacia), and the densitometric peak at 280 nm was collected. Cu_2SO_4 was added to the peak chromatographic fraction (162 ml) to give a final concentration of 50 μM. The sample was stirred overnight in an air atmosphere at room temperature and then dialyzed extensively against 100 mM potassium phosphate buffer (pH 7.4) containing 2 M urea. The insoluble precipitate was removed by centrifugation (as described above), and the supernatant (372 ml) was retained. A portion (300 ml) of this supernatant fraction was dialyzed overnight against phosphate-buffered saline (PBS). Fetuin-coupled Sepharose

4B was prepared from purified fetuin (Spiro method; GIBCO) as previously described (30). The dialyzed sample (300 ml) was applied to a column (1.5 by 5 cm) of the affinity resin at room temperature. The column runthrough was reapplied to the resin, and the resin was washed sequentially with 50 ml of PBS and then with 50 ml of 50 mM Tris-HCl (pH 7.5) containing 1 M NaCl. The assembled B oligomer binding to the resin was eluted with 50 mM Tris-HCl (pH 7.5) containing 4 M $MgCl_2$ (30). From this particular preparation, a peak fraction containing 639 μg of purified B oligomer was obtained; dilute side fractions were discarded. Prior to testing of the mitogenic activity of the preparation, the sample was dialyzed against PBS containing 2 M urea. Purity and composition of the recombinant multimer were evaluated by SDS-PAGE (17).

Analysis of mitogenic activity. The ability of recombinant B oligomer to stimulate lymphocyte mitogenesis was assessed by the procedure described by Burns et al. (13). Briefly, twofold serial dilutions of native B oligomer (2) and recombinant B oligomer were made in RPMI 1640 medium containing 10% fetal bovine serum and 50 μg of gentamicin per ml. Mouse splenic lymphocytes were added to the B oligomer preparations and incubated for 48 h, and [^{3}H]thymidine was then added. After further incubation for 24 h, incorporation of [^{3}H]thymidine was measured.

Evaluation of antibody responses. Preparations of recombinant and native B oligomer were diluted with PBS containing 0.02% gelatin, adsorbed to a mass of $Al(OH)_3$ (Alhydrogel; Superfos a/s, Vedbaek, Denmark) equivalent to that of the total protein (antigen plus gelatin) for 1 h at room temperature, and subsequently dialyzed overnight against PBS at 4°C to remove urea and salts. Groups of five female BALB/c mice (14 to 16 g) were each injected intraperitoneally (i.p.) with 6 μg of the indicated antigen. Twenty-nine days later, the animals were bled and sera were prepared. On the following day, the mice were each again immunized i.p. with 2 μg of the indicated antigen. Fourteen days later, the mice were again bled and sera were prepared. Enzyme-linked immunosorbent assays (ELISAs) (2) were performed in microtiter plates coated with either PT (4 μg/ml) or native B oligomer (3 μg/ml). The ability of sera to neutralize PT-induced clustering of Chinese hamster ovary (CHO) cells was measured as previously described (2). Assays were performed in duplicate; the minimum dilution tested was 1:20.

Toxin challenge of immunized mice. Recombinant and native B oligomer preparations were diluted in PBS containing 0.02% gelatin, adsorbed for 1 h at room temperature to a weight equivalent of $Al(OH)_3$, and subsequently dialyzed overnight at 4°C against PBS. Five mice were each injected i.p. with preparations of either recombinant or native B oligomer at a dose of 8, 1, or 0.1 μg in a total volume of 0.5 ml of PBS. Each mouse was challenged i.p. with 500 ng of PT at 1 month following immunization. As controls, one set of mice mock immunized with PBS was challenged with PT. The mice were bled 4 days after challenge, and leukocyte counts were made with a ZM Coulter Counter (Coulter Electronics, Hialeah, Fla.).

RESULTS

We have previously described the production of the individual B oligomer subunits in recombinant *E. coli* (12). In this system, each of the subunit polypeptides is synthesized with an initiating methionine residue substituting for its native signal peptide sequence; the heterologous amino-terminal methionine residues are cleaved from the recombinant proteins, except in the case of subunit S4, by endogenous methionyl aminopeptidase to yield mature subunit polypeptides. For in vitro assembly of B oligomer from these subunits, each recombinant polypeptide was isolated as insoluble inclusion bodies following lysis of the bacteria. Individual inclusion preparations were combined, solubilized in 6 M GuHCl under reducing conditions, and reoxidized, and spontaneous association of the subunits was facilitated by dialysis against 2 M urea.

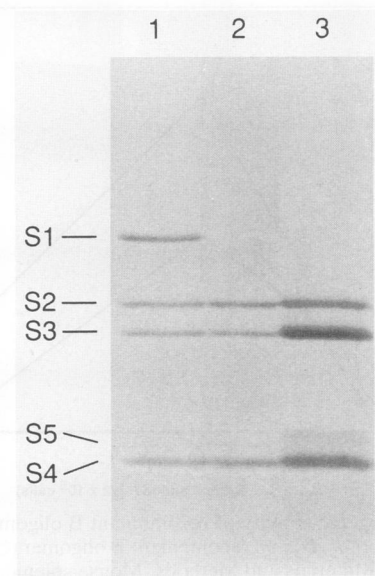

FIG. 1. SDS-PAGE of purified recombinant B oligomer. Lanes: 1, native PT (10 μg, as supplied by List Biologicals); 2, native B oligomer (15 μg, as supplied by List Biologicals); 3, recombinant B oligomer (15 μg, by Bio-Rad protein assay) prepared as described in Materials and Methods.

By virtue of its ability to bind complex glycoproteins (4, 37), recombinant B oligomer was purified to near homogeneity by affinity chromatography on fetuin-Sepharose (30). The composition of this material was evaluated by SDS-PAGE (Fig. 1); all of the B oligomer subunits were found to be present in the purified recombinant preparation. In the gel shown in Fig. 1, S3 appears to be slightly overrepresented in the recombinant multimer relative to that in native B oligomer, suggesting that free S3 subunit copurifies with the recombinant multimer. Extensive comparison by densitometric gel scanning of a number of preparations indicated, however, that differences detected between the relative subunit compositions of native and recombinant B oligomer were inconsistent and within standard error. None of the individual recombinant subunit proteins were capable of binding to the affinity resin, indicating that free subunits did not copurify with the B oligomer. Moreover, no significant multimer formation was obtained in the absence of any one of the recombinant subunits (data not shown). It has long been recognized, and it is confirmed by the gel shown in Fig. 1, that the S5 subunit migrates anomalously in SDS-PAGE and stains poorly with Coomassie blue.

The molar yield of B oligomer by our experimental protocol was estimated to be 26% in the example described in Materials and Methods. The weight ratio of recombinant subunit proteins employed here was empiric; although similar yields have been obtained for a number of preparations,

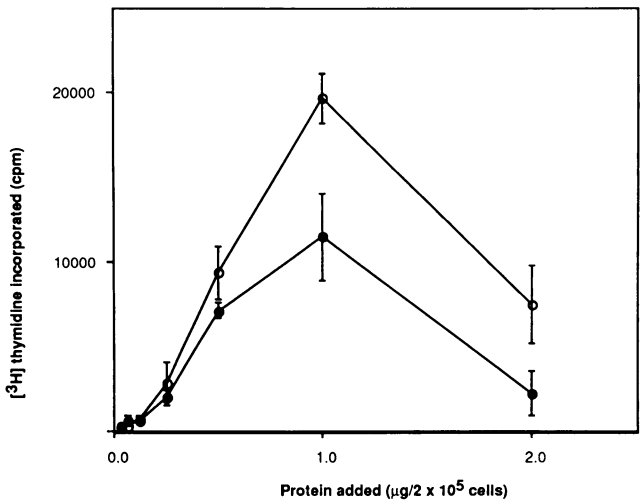

FIG. 2. Mitogenic activity of recombinant B oligomer. Dilutions of native B oligomer (●) and recombinant B oligomer (○) were made as described in Materials and Methods. Mouse splenic lymphocytes were then added to the B oligomer preparations to yield B oligomer concentrations indicated on the abscissa. The mean counts per minute (± 1 standard deviation) of [^{3}H]thymidine incorporated in two assays was plotted. Cells not subjected to B oligomer treatment incorporated 230 ± 111 cpm (n = 6) of [^{3}H]thymidine.

we have not exhaustively examined the effects of various ratios on recovery. The only significant losses of free recombinant subunits occurred following removal of the GuHCl solubilizer and during affinity chromatography. Less than 8% of the total subunit protein precipitated during the exchange of 2 M urea for 6 M GuHCl and 50 μM Cu_2SO_4; this precipitate was recycled through the solubilization procedure, and a small but measurable amount of purified B oligomer was obtained by affinity chromatography. Approximately 90% (by weight) of the total subunit-related proteins applied to fetuin-Sepharose failed to adhere after two applications, perhaps representing subunits that had been inadequately or inappropriately folded; we are currently investigating whether these proteins may likewise be recycled to participate in multimer formation. It should be noted that the amount of the S2 subunit protein added to the association mixture was limiting; it was thus not unexpected to find free, unassociated S3, S4, and S5 in the void and wash volumes from the affinity chromatography column. Although the current yield indicates that our process may be further improved, such recovery is consistent with efficient manufacturing standards. Furthermore, the purity of the recombinant B oligomer was >90% as assessed by SDS-PAGE; the endotoxin contents of two separate purified preparations (measured by *Limulus* amoebocyte assay) were found to be 0.06 and 0.09 endotoxin U/μg of protein.

Western blotting with polyclonal antitoxin serum and individual monoclonal antibodies (data not shown) confirmed that each of the B subunit polypeptides was present in the fetuin column-adhering fraction. The presence of B multimers was substantiated by examining the agglutinating and mitogenic activities of the recombinant protein preparation, as previously described (13). The recombinant preparation agglutinated goose erythrocytes at a minimum concentration of 0.24 μg/ml, compared with 0.12 μg/ml for native B oligomer. As shown in Fig. 2, the recombinant protein preparation exhibited marked mitogenic activity, similar to that of the native B oligomer.

TABLE 1. Antibody response to the recombinant B oligomer[a]

Antigen	ELISA titer of antibody to:		Neutralizing titer
	B oligomer	PT	
Native B oligomer			
Immunization 1	4,838	2,478	226
Immunization 2	223,664	41,085	1,810
Recombinant B oligomer			
Immunization 1	12,068	1,933	113
Immunization 2	144,813	79,834	2,506

[a] ELISAs were performed by using microtiter plates coated with either PT or native B oligomer. Values for ELISA antibody titers were calculated as the inverse of the antilog of the x intercept extrapolated from the linear portion of each curve relating $\log_{10}$ serum dilution versus A_{405} and represent the results obtained with pools of sera from five mice. Sera from mice injected with PBS–0.02% gelatin plus $Al(OH)_3$ did not exhibit detectable titers to either the B oligomer or PT. Neutralization of PT-induced clustering of CHO cells was measured, and the titers are expressed as the reciprocal of the maximal dilution of serum (pooled from five mice) which neutralized the CHO cell-clustering activity of PT. The geometric means of two values are shown. Sera from mice injected with PBS–0.02% gelatin plus $Al(OH)_3$ did not exhibit detectable antibody titers.

Recombinant B oligomer was then assessed for the ability to stimulate immune responses in experimental animals. Immunization of mice with the recombinant preparation induced antibody titers comparable to those elicited by native B oligomer (Table 1). Furthermore, the titers of toxin-neutralizing antibodies induced by recombinant B oligomer were similar to those observed when native B oligomer was used as the immunogen. Vaccination with recombinant B oligomer, moreover, protected mice against the leukocytosis-promoting activity of pertussis toxin (Fig. 3); the dose of recombinant B oligomer required for this protection corresponded to that of the native preparation.

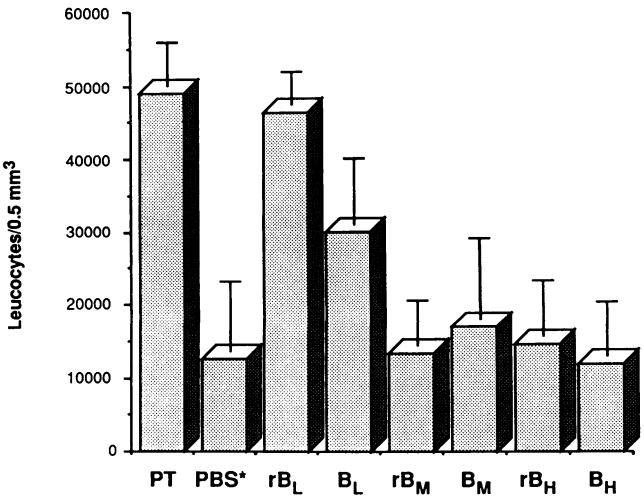

FIG. 3. Protection of mice against the leukocytosis-promoting activity of PT by immunization with recombinant B oligomer. Mice were injected with preparations of either recombinant B oligomer (rB) or native B oligomer (B) at a dose of 8 (rB_H and B_H), 1 (rB_M and B_M), or 0.1 (rB_L and B_L) μg as described in Materials and Methods. Each mouse was challenged i.p. with PT, except those in the PBS* group. Mean leukocyte counts (± 1 standard deviation) are shown.

DISCUSSION

Inactivated PT has been shown to protect humans against pertussis disease (1) and will therefore be an important component of newer generations of pertussis vaccines. Current acellular pertussis vaccines contain PT that has been inactivated by chemical treatment (28). Discovery that a specific amino acid substitution in the S1 subunit of PT could result in an inactive toxin that retains its immunogenic potential (3, 5, 11) implied that a safe and effective toxoid vaccine component could be manufactured by recombinant means. Recently, a strain of *B. pertussis* has been molecularly modified to produce such a genetic toxoid (25); this protein has been purified and included in vaccines presently undergoing clinical evaluation (26). Production of a toxoid entirely from *E. coli*-synthesized subunits would have the added advantage that the potential for contamination with other *B. pertussis* toxins (36) would be eliminated. Unfortunately, toxin is not assembled and secreted from recombinant *E. coli* (12), nor does in vitro assembly of holotoxin yet yield sufficient product (10).

The observations that B oligomer alone is apparently sufficient to stimulate protective immune responses in animals (31), that such immunity is equivalent to that conferred by a genetically attenuated holotoxin (3), and that the purified B moiety lacks the potent activities attributed to PT (2) suggested to us that this multimeric protein might be a suitable vaccine component. However, methods currently available for obtaining B oligomer are impractical for commercial-scale production. The yield, expense, and potential for contamination by active holotoxin mitigate biochemical purification of B oligomer from native PT as a means of manufacture. Furthermore, *B. pertussis* strains in which S1 subunit synthesis has been disabled are incapable of efficient extracellular transport of B oligomer (24).

In this report, we describe the in vitro assembly of the B oligomer of PT from individual subunits produced in *E. coli*. This recombinant B oligomer exhibited the agglutinating and mitogenic activities of native B oligomer. Earlier studies had demonstrated that multimeric forms of the subunits constituting the B oligomer exhibit certain of these activities: S2-S4 and S3-S4 dimers and the B oligomer itself can each agglutinate erythrocytes, whereas only the intact B oligomer is mitogenic (20, 34). Therefore, our preparation of recombinant B subunits must contain intact B oligomer. Support for this conclusion is complemented by the results obtained from immunization of mice with this preparation. It had been previously shown that individual recombinant subunits of PT do not induce neutralizing antibodies to the toxin (12, 19); in addition, S2-S4 and S3-S4 dimers are known to be less effective than B oligomer in protecting mice against the leukocytosis-promoting effects of PT (14). We found that the recombinant product elicited immune responses in mice that were virtually indistinguishable from those of native B oligomer. The immunogenic properties of the recombinant B subunit multimer thus reinforce the notion that this molecule has a structure comparable to that of native B oligomer. However, fundamental to the concept of the B oligomer as a suitable vaccine component is the fact that this recombinant protein, in the absence of any contaminating S1 subunit, was capable of providing toxin-neutralizing immune responses in experimental animals that protected them against the lymphocytosis-promoting activities of PT.

The results presented herein demonstrate that it is possible to produce a nontoxic and highly immunogenic subspecies of the PT molecule without resorting to destructive chemical treatment. Removal of the pathogenic organism from the manufacturing process eliminates the potential for contamination by other *B. pertussis* toxic components which may contribute to adverse reactions to pertussis vaccines. Moreover, we have illustrated the feasibility of creating complex heteromeric proteins from recombinant DNA-derived subunits, suggesting that other natural multifunctional macromolecules may likewise be constructed in vitro from insoluble recombinant products.

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