SHORT COMMUNICATION

Antigenic Heterogeneity in Subunit S1 of Pertussis Toxin

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Culture supernates containing pertussis toxin (PT) from four strains of *Bordetella pertussis* were examined for both immunological reactivity and biological activity. PT from all four strains sensitized mice to histamine and toxin was detectable in supernates of all strains when examined by Western blotting with polyclonal antiserum to PT. In supernates of three of the four strains, PT was detectable by an enzyme-linked immunosorbent assay (ELISA) using mouse monoclonal antibody to subunit S1 of PT as the third antibody layer. However, supernates from one strain, 18323, failed to react in ELISA. Electroblots probed with the monoclonal antibody labelled subunit S1 of PT from all strains except that of strain 18323. PT of strain 18323, whilst retaining histamine-sensitizing activity, differed antigenically from that of other strains.

INTRODUCTION

Pertussis toxin (PT) is one of the protein toxins produced by phase 1, toxigenic strains of *Bordetella pertussis* (Wardlaw & Parton, 1983). This toxin is reported to consist of two functional moieties: the A protomer (S1) and a B oligomer with four dissimilar subunits S2, S3, S4 and S5 (Tamura *et al.*, 1982). Evidence from peptide mapping suggests that subunit S2 is structurally related to S1 (Perera *et al.*, 1985). As with several other bacterial toxins which conform to the A–B model (Gill, 1978), the S1 subunit of PT catalyses the ADP-ribosylation of membrane proteins (Tamura *et al.*, 1982).

Watanabe (1984) reported that PT from five strains of *B. pertussis* was antigenically homogeneous when examined against polyclonal antibodies to native toxin in Ouchterlony immunodiffusion gels. Our objective was to use monoclonal and polyclonal antibodies to explore the antigenic relatedness of PTs, particularly the S1 subunits, from four strains of *B. pertussis*, in view of the potential use of monoclonal antibodies in immunological techniques for diagnosis of pertussis.

METHODS

Bacterial strains and growth conditions. *B. pertussis* phase 1 strains 18334, 77/18319, 18323 and Tohama were grown in shaken cyclodextrin medium as described previously (Perera *et al.*, 1985).

Concentration of PT in culture supernates. Bacteria from 10 ml culture medium were removed by centrifugation (10000 g for 30 min) and the supernate was dialysed against distilled water for 24 h at 4 °C. The retentate was lyophilized and reconstituted in 250 µl 0.01 M-sodium phosphate buffer, pH 7.4, containing 0.15 M-NaCl (PBS).

Monoclonal and polyclonal antibodies to PT. Mouse monoclonal antibody to subunit S1 of PT was kindly provided by A. Robinson, CAMR, PHLS, Porton Down, UK. Purified PT (kindly provided by M. Ui, Department of Physiological Chemistry, Hokkaido University, Sapporo, Japan) from strain Tohama was used to raise polyclonal antibodies in rabbits as follows. PT (130 µg) was dissolved in 1.3 ml PBS and emulsified with an equal volume of

Abbreviations: PT, pertussis toxin; TMB, 3,3',5,5'-tetramethylbenzidine.

Freund's complete adjuvant (Difco) by ultrasonication for 1 min at 4 °C. Two female New Zealand White rabbits were bled for pre-immune sera and immunized with the above emulsion. Each animal received ten subcutaneous injections (0.1 ml emulsion per site) distributed over the back and limbs. Three weeks later, the animals received a second injection in Freund's incomplete adjuvant. The animals were bled 6 weeks after the second immunization and the antisera were stored at -20 °C. A globulin-rich fraction was prepared from the antiserum by $(\text{NH}_4)_2\text{SO}_4$ precipitation (Livingston, 1974).

Enzyme-linked immunosorbent assay (ELISA) for PT. The NUNC-transferable solid phase (TSP) screening system utilizing pins (see below) as the binding phase was adapted for an ELISA method for PT. The pins were incubated in microwells (of a non-binding type) containing the reagents. Briefly, the globulin fraction of rabbit anti-PT serum was diluted 1 in 500 in 0.1 M-sodium carbonate/bicarbonate buffer, pH 9.6, and added to wells of a flat-bottom microwell plate (NUNC, bacteriological grade). The volume of reagent per well throughout the assay was 150 µl unless otherwise stated. Polystyrene pins (NUNC-TSP) were coated with antibody by immersion in globulin solution for 18 h at 4 °C and both microwell plate and pins were washed three times with PBS containing 0.05% (v/v) Tween 20 (PBST). The pins were then incubated with serial twofold dilutions of culture supernates (unconcentrated) for 2 h at 37 °C, washed three times in PBST and incubated with a 1 in 150 dilution of mouse monoclonal antibody to PT for 3 h at 37 °C. After a further washing step, the pins were incubated with a 1 in 3000 dilution of sheep anti-mouse γ -globulin conjugate of horseradish peroxidase (Scottish Antibody Production Unit, Carlisle, Lanarkshire, UK) for 1 h at 37 °C, washed, and peroxidase activity was assayed using 3,3',5,5'-tetramethylbenzidine (TMB) (Miles) as the substrate, which was prepared as follows. TMB (10 mg) was dissolved initially in 1 ml dimethyl sulphoxide and added dropwise to 100 ml 0.1 M-sodium acetate/citric acid buffer, pH 6.0. Hydrogen peroxide was then added to a final concentration of 1.3 mM to the above solution of TMB just prior to use. After incubation for 15 min at 25 °C, the microwell plates containing the pins were gently shaken to disperse the colour and the pins were removed. After addition of 50 µl 2 M- H_2SO_4 to each well, the absorbance was read at 450 nm. The concentration of PT in the culture supernates was estimated using purified PT as the standard. Control wells contained PBST. Samples were assayed in duplicate.

SDS-PAGE and immunoblot analysis of toxin subunits. Concentrated culture supernates (70 to 100 µg protein) containing PT were solubilized in SDS and 2-mercaptoethanol and subjected to SDS-PAGE (Perera *et al.*, 1985; Laemmli, 1970). The peptides separated, some of which were toxin subunits, were electrophoretically transferred to nitrocellulose paper (Towbin *et al.*, 1979) in transfer buffer containing 0.7% (w/v) SDS. The nitrocellulose paper was subsequently treated with 3% (w/v) bovine serum albumin (Sigma) in 0.01 M-Tris/HCl buffer, pH 7.4, containing 0.15 M-NaCl (TBS) for 1 h at 45 °C. The strips were washed briefly in TBS containing 0.05% (v/v) Tween 20 (TBST) and incubated with either a 1 in 100 dilution of rabbit anti-PT serum diluted in TBST or undiluted mouse hybridoma supernate containing 0.05% (v/v) Tween 20 for 16 h at 4 °C. After rigorous washing with TBST (four to five changes) for 16 h at 25 °C, the immunoblots were incubated with a 1 in 3000 dilution of a horseradish peroxidase conjugate of either donkey anti-rabbit or sheep anti-mouse γ -globulin (both from Scottish Antibody Production Unit) for 2 h at 25 °C. The immunoblots were washed with TBST and the colour was developed for 10 to 15 min with 3-amino-9-ethylcarbazole (Sigma) as the chromogen (Irons *et al.*, 1983).

Histamine-sensitizing assay. Culture supernates (unconcentrated) were heated to 56 °C for 15 min and serial twofold dilutions were made using PBS as diluent. Groups of ten 7 to 8 week old female mice (strain HAM-1/CR, weight 28 to 30 g) were injected intraperitoneally (0.5 ml per mouse) with each dilution. Five days later, each animal was injected intraperitoneally with 0.5 ml histamine dihydrochloride (6 mg ml⁻¹) (Sigma) and deaths were recorded after 2 h. Histamine-sensitizing dose per ml giving 50% death (HSD_{50} ml⁻¹) was estimated graphically from a plot of probit of percentage mortality against log₁₀ dilution of sample.

RESULTS AND DISCUSSION

Culture supernates (containing PT) from all four strains of *B. pertussis* sensitized mice to histamine (HSD_{50} ml⁻¹ ranged between 80 and 280). Electroblots probed with polyclonal antibodies showed all subunits of PT (subunits S2 and S3 formed diffuse bands) from all four strains examined (Fig. 1a). Subunits S4 and S5 were not separable in this gel system, and gave a single diffuse band. This peptide was previously designated as S4,5 (Perera *et al.*, 1985). In addition, subunit S1a, reported previously to be a product of degradation of S1 (Perera *et al.*, 1985), was detectable in the culture supernates. The amount of PT in culture supernates of strains 18334, Tohama and 77/18319, detectable by ELISA using mouse monoclonal antibody to subunit S1 as the third antibody layer, was between 2.6 and 4.7 µg ml⁻¹. However, PT could not be detected in culture supernates of strain 18323 when examined by ELISA. Likewise, electroblots probed with the monoclonal antibody (Fig. 1b) showed homology with subunit S1 of strains Tohama, 77/18319 and 18334 (lanes 1, 3 and 4 respectively) but not 18323 (lane 2). This

Fig. 1. Immunoblot analysis of culture supernates from four strains of *B. pertussis*. Lanes: 1, Tohama; 2, 18323; 3, 77/18319; 4, 18334. (a) Reactivity with rabbit polyclonal antibodies to PT; (b) reactivity with mouse monoclonal antibody to PT. Positions of subunits S1, S1a, S2, S3 and S4,5 of PT are shown.

suggested that the subunit S1 of strain 18323 differed antigenically from that of other strains even though the M_r of this subunit was apparently identical to that of other strains (Fig. 1a) and culture supernates of strain 18323, like those of the other strains, sensitized mice to histamine. Such a subtle difference in the antigenic structure was apparent only after treatment with monoclonal antibody since neither immunoblotting (Fig. 1a) nor immunodiffusion gels (Watanabe, 1984) with polyclonal antibodies revealed any difference in the reactivity of PT from strain 18323. The epitope reactive with the monoclonal antibody is either modified or absent in subunit S1 of strain 18323. It is not known whether the other subunits of PT from strain 18323, if examined with monoclonal antibodies, would show antigenic micro-heterogeneity.

Strain 18323 is unusual in that it is highly virulent in the mouse intracerebral test (Kendrick *et al.*, 1949; Standfast, 1958a) and is widely used as the challenge strain for potency-testing of pertussis vaccines (Preston, 1966, 1967; Standfast, 1958b). Two other strains (GL 353 and its derivative 353/Z) have been reported as virulent in the mouse intracerebral challenge test (Preston, 1966). It is not known whether the reactivity of subunit S1 from these strains differs in a similar manner to 18323.

Antigenic differences in PT have not been previously documented. The evidence presented here substantiates for the first time the existence of antigenic micro-heterogeneity in PT. It is not known whether PT from other strains of *B. pertussis*, including clinical isolates, would show minor antigenic differences. If heterogeneity is widespread, then clearly this would limit the use of monoclonal antibodies in immunological techniques and also hamper the selection of strains for purification of PT for use in acellular vaccines.

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