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NOTES

Characterization of Murine Monoclonal Antibodies That Recognize Defined Epitopes of Pertussis Toxin and Neutralize Its Toxic Effect on Chinese Hamster Ovary Cells

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Three murine monoclonal antibodies (MAb), E19, E205, and E251, raised against pertussis toxin reacted in Western blots (immunoblots) with the S1, S4, and S2-S3 subunits, respectively, and neutralized the Chinese hamster ovary cell-clustering activity of pertussis toxin. MAb E251 recognized a linear synthetic peptide corresponding to amino acids 107 to 120 of the S2 subunit, suggesting a role for this region in receptor binding.

Pertussis toxin (PT) is secreted from virulent *Bordetella pertussis* and is responsible for a number of biological effects, including histamine sensitization, islet activation, and lymphocyte promotion, which may contribute significantly to the severity of whooping cough (18, 19, 23). Pertussis holotoxin contains subunits S1, S2, S3, S4, and S5 in a ratio of 1:1:1:2:1 and consists of an A protomer (S1 subunit), which contains the enzymatic ADP ribosyltransferase and NAD glycohydrolase activities of the toxin, and a B oligomer (S2 to S5 subunits),which is involved in receptor binding (9). The PT receptor-binding pockets are contained within the S2 and S3 subunits (24, 25). Although PT is one of the major protective antigens against whooping cough (18, 19), concern regarding the observed side effects of immunization has resulted in strategies for the chemical and genetic detoxification of PT (2, 12, 15, 17). To identify regions of the toxin molecule that contribute to the biological activity of PT, we have raised monoclonal antibodies (MAb) that recognize linear epitopes of the S1, S2-S3, and S4 subunits of PT as determined by Western blot (immunoblot) analysis and that neutralize the toxic effect of PT on Chinese hamster ovary (CHO) cells.

BALB/c mice were immunized with glutaraldehyde-detoxified PT (16) and MAb raised essentially as previously described (30). The myeloma used in the fusion experiment was X63Ag8 (10). Hybridoma supernatant fluids were screened for PT subunit recognition by Western blot analysis carried out essentially as described by Burnette (3) and by inhibition of CHO cell clustering (7, 8) as described by Sato et al. (22). PT was electrophoresed through a polyacrylamide gel by the method of Laemmli (13) by using a 3.85% acrylamide stacking gel and a 15% acrylamide separating gel. MAb E19 bound to the S1 subunit (Fig. 1A, lane 2), MAb E251 reacted strongly with the S2 subunit and weakly with the S3 subunit (Fig. 1A, lane 3), and MAb E205 reacted with the S4/S5 doublet (Fig. 1A, lane 4). To determine which of the subunits was recognized by MAb E205, the reaction was repeated with purified S4 and S5; this revealed that E205 recognizes the S4 subunit of PT (Fig. 1B). E19, E205, and E251 were subclassed by use of an MAb subclassing kit (Dianova) as immunoglobulin G1 heavy-chain and kappa light-chain antibodies (results not shown). The three anti-PT antibodies were purified from hybridoma supernatant fluids by using a Pharmacia protein A-Sepharose CL-4B column (4). The antibody preparations consisted of a 26-kDa light-chain band and a 55-kDa heavy-chain band after electrophoresis (data not shown). All three MAb neutralized PT clustering of CHO cells, whereas antibody 13C4 (28), directed against the B subunit of Shiga-like toxin, did not (Table 1).

The ability of MAb E19 to inhibit the ADP ribosylation of bovine transducin was compared with that of rabbit polyclonal anti-S1 antiserum. The MAb (1.1 mg/ml) or antiserum was serially diluted in phosphate-buffered saline, and 20 μl of each antibody dilution was incubated with 1 μl of PT (0.1 μg/ml) for 30 min at room temperature. After toxin activation by the addition of 5 μl of 250 mM dithiothreitol for 1 h at room temperature, 4 μl of bovine eye transducin (G protein substrate for PT-mediated ADP ribosylation; 1-mg/ml concentration), 2.5 μl of 1 M Tris-Cl (pH 8.0), 10 μl of 10 mM ATP, 7 μl of H_2O, and 0.1 μl of [adenylate-^{32}P]NAD (50 mCi/ml; NEN) were added and the mixture was incubated at room temperature for 2 h. The mixtures were passed through cellulose nitrate filters (Schleicher & Schuell) which were subsequently washed with 50 mM Tris-Cl–5 mM $MgCl_2$ (pH 8.0). ADP-ribosylated transducin was detected after scintillation counting of filters. Clearly, the preincubation of PT with MAb E19 inhibits the ADP ribosylation activity of the toxin (Fig. 2).

The epitope recognized by MAb E251 was identified by screening for binding to a bank of synthetic peptides corresponding to regions of the S2 subunit protein sequence predicted to contain surface-exposed hydrophilic β turns (25, 26). E251 reacted strongly with a peptide corresponding to amino acids 107 to 120 (TATRLLSSTNSRLC; amino acid one-letter code) of the mature protein in enzyme-linked immunosorbent assays (ELISAs) when either the free pep-

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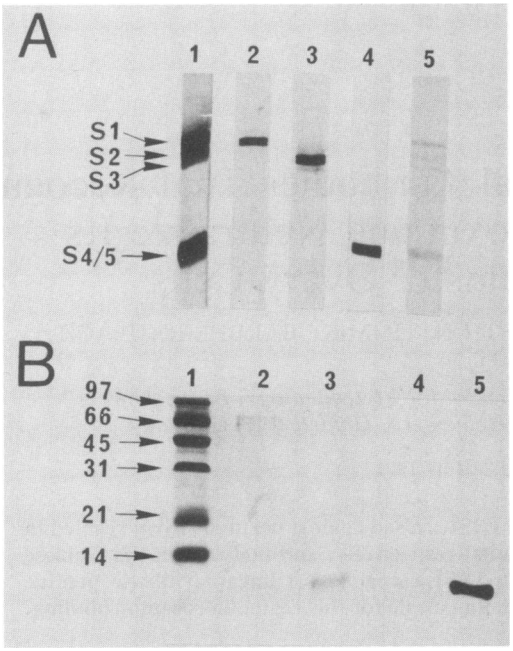

FIG. 1. Western blot analysis of PT binding by MAb. (A) PT was subjected to electrophoresis and blotted with polyclonal mouse antiserum (lane 1) and MAb E19 (lane 2), E251 (lane 3), and E205 (lane 4). Coomassie blue-stained toxin is shown in lane 5. PT subunits are indicated by arrows. (B) Purified subunits S4 and S5 were subjected to electrophoresis and blotted with MAb E205. Lane 1 contains Coomassie blue-stained Bio-Rad molecular weight markers (sizes indicated by arrows), lanes 2 and 4 contain the S5 subunit, and lanes 3 and 5 contain the S4 subunit. Lanes 2 and 3 are stained with Coomassie blue, whereas lanes 4 and 5 are Western blots with E205.

tide or its bovine serum albumin (BSA) conjugate was used to coat the ELISA plate (Fig. 3). Previous work employing antibodies raised against peptides as sequence-specific probes for the localization of the receptor-binding pocket of PT suggested involvement of amino acid residues 1 to 7, 35 to 59, and 91 to 106 of the S2 subunit (25, 26) and corresponding regions in the S3 subunit (24).

Numerous PT-specific MAb have been obtained (1, 5, 6, 11, 14, 20–22, 27), but few have been characterized with respect to their ability to interfere with PT binding activity (5, 14). Frank and Parker (6) described an S2-reactive MAb which did not neutralize PT in an in vivo histamine-sensitizing assay. Sato et al. (20) described another S2-specific MAb which was inhibitory in the CHO cell-clustering assay, and S3-reactive MAb which neutralized PT in the CHO cell-clustering assay and/or in a histamine-sensitizing assay have been reported by Anwar et al. (1) and Schou et al. (27). Lang et al. (14) have described MAb that recognize conformational epitopes of S2-S4 and S3-S4 dimers which inhibited PT-mediated CHO cell clustering and binding of PT to CHO cells. However, none of the epitopes recognized by neutral-

TABLE 1. Neutralization of the PT-mediated clustering of CHO cells by MAb E19, E205, and E251

Final MAb concn (μg/ml)	CHO cell-clustering activity of PT[a] preincubated with the following MAb for 5 min at room temp[b]:			
	E19	E205	E251	13C4
10.0	++	++	++	−
1.0	++	++	++	−
0.1	−	++	+	−
0.01	−	+	−	−

[a] Final concentration, 6 ng/ml.

[b] PT-MAb mixtures were added to 1 ml of CHO cells diluted to 10^5 cells/ml in 24-well tissue culture plates. Inhibition of PT-mediated CHO cell clustering was scored after growth at 37°C for 2 days. Symbols: ++, inhibition of clustering activity; +, partial inhibition of clustering activity; −, no inhibition of CHO cell-clustering activity.

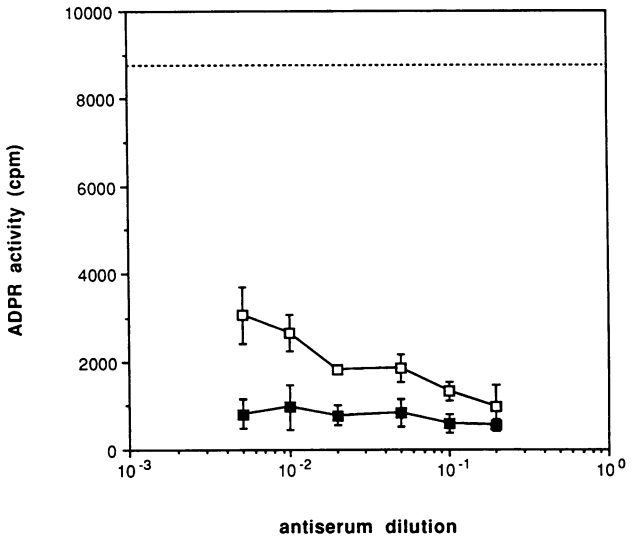

FIG. 2. Inhibition of ADP ribosylation (ADPR) activity of PT by purified MAb E19 (filled squares) or anti-S1 rabbit polyclonal antiserum (open squares). The level of PT ribosylation of bovine eye transducin (G protein substrate for PT-mediated ADP ribosylation) without the addition of antibodies is indicated by a dashed line. Standard deviations of triplicate samples are indicated by vertical bars.

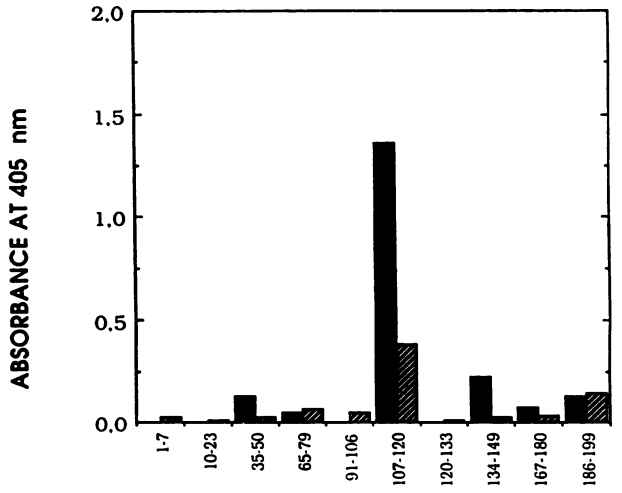

FIG. 3. Determination of the epitope recognized by MAb E251. An ELISA of BSA-conjugated peptides (hatched bars) and free peptides (filled bars) with E251 as the primary antibody (undiluted hybridoma supernatant) is shown. The peptides correspond to regions of the S2 subunit protein sequence and have been described elsewhere (25, 26). Undiluted hybridoma supernatant from E55, which bound the S2 and S3 subunits of PT in Western blots but did not neutralize CHO cell-clustering activity, did not bind any of the peptides tested (results not shown).

izing MAb reported thus far has been mapped. Here we report localization of the binding site of the inhibitory MAb E251 to amino acids 107 to 120 of the S2 subunit, which is adjacent to the segment of S2 previously implicated in receptor recognition (25). Interestingly, in an ELISA, E251 recognizes the corresponding segment of the S3 subunit containing amino acids 104 to 117 only very weakly as a free peptide and not at all when the peptide is conjugated to BSA (results not shown). The core region of the two peptides differs only by a conservative alanine (S3) to serine (S2) exchange at position 113.

The identification of regions that are involved in the biological activity of PT not only provides insights into the structure-function relationship of the toxin molecule but also specifies epitopes in which an immune response may play an important role in protection. This, combined with recent advances in the "humanization" of murine MAb (29), should lead to the production of anti-PT antibodies applicable to humans that may prove effective in providing passive protection (21) against pertussis in the nonimmunized newborn.

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