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Mapping of Linear B-Cell Epitopes of the S2 Subunit of Pertussis Toxin

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The linear immunogenic and antigenic structure of the S2 subunit of pertussis toxin was investigated with synthetic peptides corresponding to regions of the protein sequence predicted to contain surface-exposed hydrophilic β turns. Five peptides as peptide-bovine serum albumin conjugates were recognized by anti-pertussis toxin antiserum and were thus designated "immunogenic epitopes." Two prominent immunogenic epitopes were specified by peptides corresponding to sequences spanning R107-120 and R186-199, whereas peptides corresponding to residues R35-50 and R91-106 were only bound in low titer. Three peptides as thyroglobulin conjugates elicited antisera in rabbits that bound intact pertussis toxin by enzyme-linked immunosorbent assay and immunoblot. These peptides were designated "antigenic epitopes." The most prominent antigenic determinant was localized to the N-terminal end of the S2 sequence encompassing residue R1-7. Peptides R35-50 and R91-106 represented two minor antigenic epitopes. Antisera to two additional peptides corresponding to residues R134-149 and R186-199 recognized the S2 subunit only by Western blotting (immunoblotting). Only antiserum raised against peptide R91-106 also recognized the S3 subunit by Western blotting, indicating a marked antigenic and probably also structural difference between the two highly homologous subunits.

Virulent organisms of *Bordetella pertussis*, the causative agent of whooping cough, exhibit a number of virulence factors promoting the development of the disease. Besides cell surface-associated proteins such as pili and filamentous hemagglutinins which are involved in the adherence of bacteria to target cells, *B. pertussis* also exhibit secretory components such as adenylate cyclase and several toxins, e.g., pertussis toxin (PT), dermonecrotic toxin, and tracheal cytotoxin (14, 17, 25, 38). Except for the paroxysmal coughing and the accumulation of mucus in the respiratory tract, most of the symptoms of whooping cough can be induced in mice (20, 24, 27) by the action of PT alone (22). Thus, pertussis is widely regarded as a toxin-mediated disease (23). PT seems not only to be the main virulence factor of the organism but also to be responsible for the severe side reactions seen occasionally upon pertussis vaccination with the vaccines currently in use (16, 18, 31–33). These complications have been attributed to the presence of active PT in conjunction with active adenylate cyclase in some vaccine preparations. Initial efforts to develop a new, safe PT have therefore focused on different and possibly more secure ways to detoxify PT to be included in a multicomponent acellular vaccine (30).

PT acts through ADP-ribosylation of the $G_{i\alpha}$ subunit, thus inhibiting the cellular adenylate cyclase regulatory system (1, 6, 7, 17). The single polypeptide chain of the A protomer (S1 subunit) carries the enzymatic activity (34, 37). The B (binding) oligomer is a pentamer consisting of the S2, S3, two S4, and S5 subunits (29, 34, 35). The PT-encoding genes were recently cloned, and the amino acid sequences of all five subunits were deduced from the corresponding DNA sequences (12, 13, 19).

To investigate the serological properties of PT and to provide probes for the elucidation and characterization of functional domains, synthetic peptides corresponding to selected segments of the different subunits were prepared. We report the identification of linear antigenic and immunogenic epitopes of the PT S2 subunit which has been implicated in receptor recognition of target cells (4, 15).

(Throughout this report, the term "antigenic epitope" refers to domains in the native toxin that are recognized by antibodies engendered by synthetic peptides corresponding in sequence to the amino acid sequence of these particular domains. "Immunogenic epitope" is used to describe segments in the native toxin that are recognized by the immune system and give rise to antibodies able to bind the corresponding synthetic peptides.)

MATERIALS AND METHODS

PT. The PT used in this study was either purchased from List Biochemicals, Campbell, Calif., or obtained as a gift from the Institut Merieux, Lyon, France.

Selection of synthetic peptides. The choice of peptides corresponding to segments of the S2 subunit sequence for synthesis was biased with regard to a combination of secondary-structure predictions according to the algorithms of Chou and Fasman (5) and Robson and Suzuki (26). Predictions of hydrophilicity according to algorithms of Hopp and Woods (8) and Kyte and Doolittle (10) were also taken into account. Regions were selected that were predicted to incorporate hydrophilic and possibly acrophilic β turns. A natural or additional cysteine residue was placed at either end distal to the predicted reverse turn, thus providing a specific site to couple the peptide in a defined orientation to the carrier protein(s) via heterobifunctional cross-linkers (see below).

Synthesis and characterization of peptides. Peptides were synthesized by solid-phase techniques, using either t-Boc- or Fmoc technology depending on the respective sequences.

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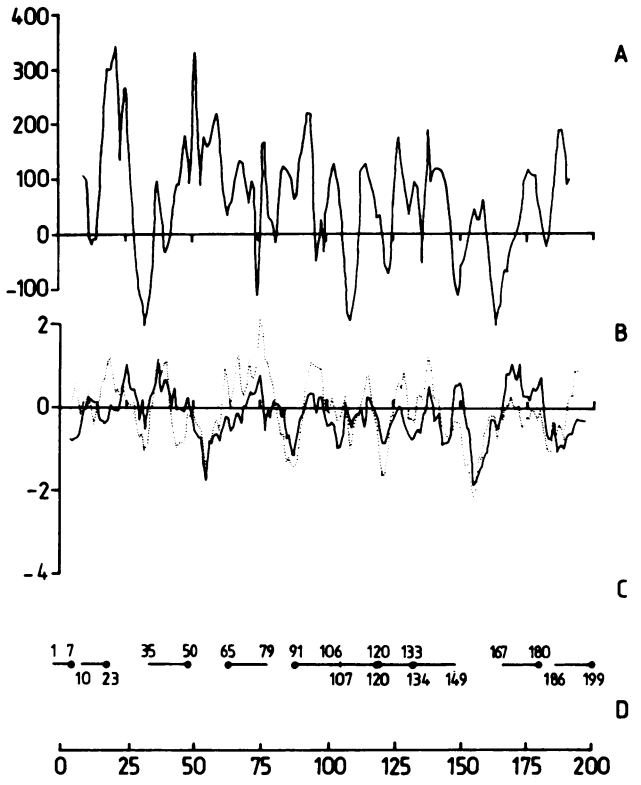

FIG. 1. Predictive analysis of the local average β-turn potentials (A) and the hydrophilicity (B, ——) and acrophilicity (B, ---) of the PT S2 subunit amino acid sequence, based on algorithms by Robson and Suzuki (26), Hopp and Woods (8), and Hopp, respectively. The hydrophilicity and acrophilicity values are derived from heptapeptide moving average and are plotted at the midpoint of each section. Hydrophilic and acrophilic regions appear as positive peaks above the zero line. (C) Synthesized amino acid sequences, with the conjugation site via a cysteine residue depicted as (—●). (D) Amino acid numbering of the sequence.

t-Boc amino acids were coupled to phenylacetamidomethyl resin and Fmoc amino acids were coupled to 4-(hydroxymethyl)phenoxymethyl-copoly(styrene-1% divinylbenzene) resin (Novabiochem, Läufelfingen, Switzerland). The coupling of each amino acid was monitored with ninhydrin (9), and, when necessary, the coupling was repeated until >99% efficiency was achieved. The completed peptide chain was cleaved from the resin with either anhydrous hydrogen fluoride in the presence of dimethylsulfide and anisole as scavengers (t-Boc method) or a mixture of trifluoracetic acid and trifluormethane sulfonic acid, using ethanedithiol and methyl-phenyl sulfide as scavengers (Fmoc-method). The crude peptide was precipitated with ether, redissolved in 5% acetic acid, incubated with Dowex 1×8 anion-exchange resin, and lyophylized after filtration. The peptides were assessed for homogeneity by analytical reverse-phase high-performance liquid chromatography on a Nucleosil C_{18} column (250 by 4 mm; 10 to 70% acetonitrile gradient for 30 min). When necessary, the peptides were further purified by chromatography on Sephadex LH-20 (5% acetic acid) and subsequently isolated by preparative high-performance liquid chromatography (Nucleosil C_{18} column [250 by 20 mm]; 10 to 90% acetonitrile gradient for 40 min). The purity of the final product was determined by amino acid analysis.

Conjugation of peptides to carrier proteins. Each peptide was conjugated to both bovine serum albumin (BSA), using succinimidyl-4-(*N*-maleimido-methyl)-cyclohexane-1-carboxylate, and thyroglobulin, using *m*-maleinimidobenzoyl *N*-hydroxysuccinimide ester (MBS) as heterobifunctional cross-linkers. Briefly, 10 mg of the carrier protein was dissolved in 3 ml of phosphate-buffered-saline (PBS; pH 7.4) and mixed with 1 ml of fresh *N*,*N*-dimethylformamide containing 5 mg of the respective cross-linker. After 2 h with stirring at room temperature, the conjugate was separated from unreacted cross-linker by gel filtration on Sephadex G-25 in 0.1 M phosphate buffer (pH 6.0). To ensure a free sulfhydryl group on the peptide, reduction with sodium borohydride was carried out for 15 min on ice. Excess borohydride was subsequently destroyed with acid. The neutralized and reduced peptide was combined with the carrier–cross-linker conjugate and reacted with stirring at room temperature overnight. The resulting peptide-carrier conjugate was separated from unreacted free peptide by gel filtration on Sephadex G-25 in 0.1 M ammonium hydrogen carbonate buffer (pH 7.4) and adjusted to a protein concentration of 1 mg/ml.

Specific PT and peptide antisera. All hyperimmune sera were prepared in female chinchilla bastard rabbits. A 300-μg portion of peptide-carrier conjugate (protocol A) or 25 μg of PT was emulsified with complete Freund adjuvant and injected subcutaneously at multiple sites. Booster injections with the same dose were prepared with incomplete Freund adjuvant and administered 4 weeks later. For some peptides a mixture of 300 μg of peptide conjugate and 100 μg of free peptide was used for immunization (protocol B) with Freund adjuvants. At 7 to 8 days after the booster injection, the rabbits were bled from the ear vein. For preparation of specific antipeptide antisera, rabbits were immunized solely with the appropriate peptide-MBS-thyroglobulin conjugates.

TABLE 1. Location and sequence comparison of synthetic peptides in PT S2 and S3 subunits

PT subunit	Amino acids	Sequence
S2	1–7	STPGIVI–C
S3		VA-----
S2	10–23	QEQI TQHGSPYGRC
S3		KALF--Q-GA----
S2	35–50	LRGSGDLQEYLRHVTR–C
S3		---NAE--T---QI-P
S2	65–79	C–GEYGGVI KDGTPGGA
S3		QA---I---AP--AG
S2	91–106	C–TRNTGQPATDHYYSNV
S3		I YK-----A-----K-
S2	107–120	TATRLLS STNSRLC
S3		------A-------
S2	120–133	CAVFVRSGQPVI GA
S3		------D--S----
S2	134–149	CTS PYDGKYWSMYSRL
S3		-A---E-R-RD--DA-
S2	167–180	VSKEEQYYDYEDAT–C
S3		----------
S2	186–199	LTG I S I CNPGSSLC
S3		-----L---AA-I-

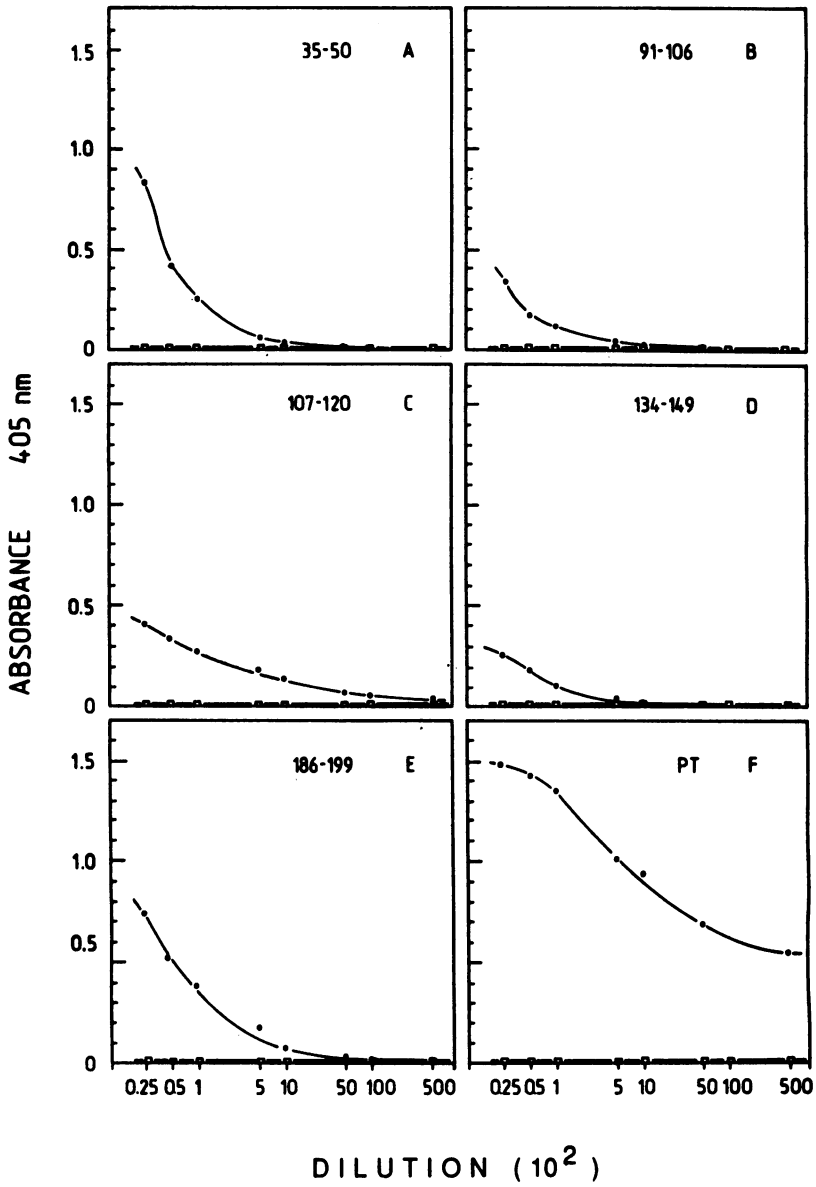

FIG. 2. Reaction of polyclonal antiserum against PT with peptide-BSA conjugates by ELISA, as described in Materials and Methods. The anti-PT antiserum was assayed for its ability to bind peptides R35-50 (A), R91-106 (B), R107-102 (C), R134-149 (D), and R186-199 (E) relative to the binding to PT (F). The A_{405} was corrected for unspecific binding due to preimmune sera and BSA as indicated (□). Peptides R1-7, R10-23, R65-79, R120-133, and R167-180 were not bound by anti-PT antiserum.

The resulting antisera were evaluated in a solid-phase binding assay, using the corresponding peptide–succinimidyl-4-(*N*-maleimido-methyl)-cyclohexane-1-carboxylate–BSA conjugates as antigens.

Solid-phase antigen-binding assay. Peptide-protein conjugates (1 mg/ml) or PT (1 μg/ml) was coated on polystyrene microdilution plates in carbonate buffer or PBS. Free binding sites were blocked with 5% BSA–PBS at 4°C overnight. The plates were treated with dilutions of antisera in 0.1% BSA–PBS and washed several times with 0.05% Brij 35–PBS, and bound antibody was detected enzymatically with alkaline phosphatase-conjugated second antibody, followed by *p*-nitrophenyl phosphate as substrate. The enzyme reaction was evaluated by determining the optical density at 405 nm, using a Titertek enzyme-linked immunosorbent assay (ELISA) reader. All assays were repeated several times in duplicate.

Western blotting (immunoblotting). For separation of PT subunits by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (11), the toxin was denatured by heating for 15 min at 100°C in sample buffer (4 M urea, 2% SDS, 0.6 M 2-mercaptoethanol, 4 mM EDTA, 10% glycerol). The electrophoretic transfer of proteins was performed in 25 mM Tris buffer (pH 7.2) essentially as described by Towbin et al. (36) and Burnette (3). After transfer, the nitrocellulose was blocked with 5% BSA–PBS overnight at 4°C. Subsequently, the antiserum was applied in a 1:200 dilution in 0.1% BSA–PBS. After 90 min at room temperature, the paper was washed four times, 15 min each, with 0.1% Brij 35–PBS and incubated with a 1:2,000 dilution of alkaline phosphatase-

conjugated second antibody (Bio-Yeda, Israel) in 0.1% BSA–PBS. Bound antibody was visualized after repeated washings by incubating the nitrocellulose with Nitro Blue Tetrazolium–5-bromo-4-chloro-3-indolyl phosphate substrate in AP buffer (100 mM Tris, pH 9.5, 1 mM Mg). After color development, the reaction was stopped with 20 mM Tris (pH 8.0) containing 5 mM EDTA.

RESULTS

Choice and synthesis of peptides. For identification of linear antigenic and immunogenic determinants in the 199-amino-acid sequence of the PT S2 subunit, 10 peptides were prepared by solid-phase synthesis, corresponding to residues R1-7, R10-23, R35-50, R65-79, R91-106, R107-120, R120-133, R134-149, R167-180, and R186-199. The peptides were chosen from areas of the protein sequence with a high probability to contain a β turn according to secondary-structure predictions (Fig. 1). As the S2 and S3 subunits share a 70% amino acid homology, the amino acid sequences of the S2 peptides chosen for synthesis are also highly homologous to the corresponding segments of the S3 subunit. The complete amino acid sequences of the 10 synthetic peptides as well as their counterparts in the S3 subunit sequence are depicted in Table 1. All peptides were synthesized with either a natural or an additional cysteine residue at either end distal to the predicted reverse turn, thus making it possible to couple the peptides in a unique orientation to the respective carrier proteins.

Peptides as antigens. Each of the 10 peptide-BSA conjugates was assessed by ELISA with dilutions of polyclonal anti-PT antiserum. We define synthetic peptides recognized by anti-PT antibodies as immunogenic epitopes. Anti-PT antiserum recognized 5 of the 10 peptides (Fig. 2). Segments R35-50 and R186-199 are shown to represent major linear immunogenic epitopes, while residues R91-106, R107-120, and R134-149 correspond to rather minor immunogenic determinants of the PT S2 subunit.

Peptides as immunogens. Polyclonal antipeptide antisera were engendered by immunizing rabbits with peptide-MBS-thyroglobulin conjugates and were assayed for capacity to bind the homologous BSA conjugates and native PT, thus defining the corresponding peptide as an antigenic epitope. All peptides elicited a strong specific antipeptide response as judged by ELISA when the corresponding BSA conjugates were used to sensitize the solid phase (Fig. 3). Three peptides were found to elicit antisera cross-reacting with intact PT bound to the solid phase (Fig. 4). Strongly cross-reacting antisera were induced by R1-7 and R35-50 peptide conjugates, whereas antisera to peptide R91-106 exhibited only weak cross-reactivity. Furthermore, for the amount of cross-reactive antibodies engendered by the peptide corresponding to the N-terminal segment of the S2 subunit (R1-7), the immunization protocol seems to play a crucial role. Cross-reactivity was largely increased when a mixture of free peptide (100 μg) and peptide-MBS conjugate (300 μg) was used for immunization compared with when conjugate alone was used (Fig. 5). Recognition of the homologous peptide-BSA conjugate was not affected by this change in protocol.

To compare the relative cross-reactivities of the three peptides (R1-7, R35-50, and R91-106) corresponding to antigenic determinants in the S2 subunit, a solid-phase binding assay was conducted, using ^{125}I-labeled protein A to detect bound antibody. More than half of the total counts bound by intact PT were due to antibodies directed towards the

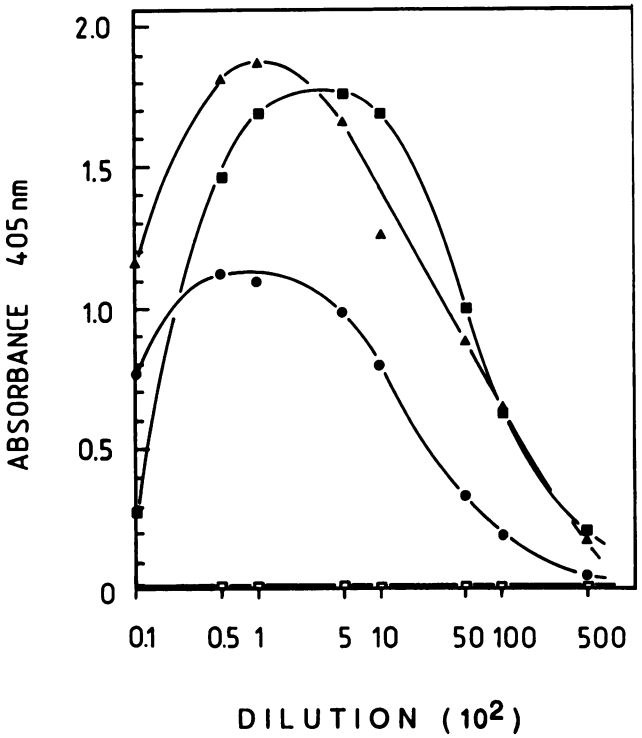

FIG. 3. Reactions of antisera against peptide-MBS-thyroglobulin conjugates with homologous peptide-BSA conjugates. The ELISA data for R1-7 (●), R35-50 (■), and R91-106 (▲) are shown as examples. The A_{405} was corrected for nonspecific binding to BSA and for the reactivity of the preimmune sera (□).

N-terminal peptide R1-7 (data not shown), thus denoting this region to harbor a major linear antigenic determinant in the S2 subunit of PT. However, peptide R1-7 was not recognized by polyclonal anti-PT antiserum, indicating that this domain is immunorecessive in the native toxin.

Recognition of antigenic determinants by Western blotting. To determine whether the antisera generated against peptides corresponding to segments of the PT S2 subunit bind S2 also under denaturing conditions, PT was boiled in urea–SDS–2-mercaptoethanol by the method of Laemmli (10) and Tamura et al. (34), electrophoresed in 15% SDS-polyacrylamide gels, and transferred to nitrocellulose (Fig. 6). Antisera engendered by peptide conjugates R1-7, R35-50, and R91-106, corresponding to linear antigenic epitopes, recognized S2 under denaturing conditions, indicating that these determinants are not destroyed by denaturation. Also, only antisera raised against synthetic peptides R134-149 and R186-199 bound to the S2 subunit in Western blotting, indicating that these regions are not accessible in the intact toxin but are only available under denaturing conditions. To see whether binding of PT to nitrocellulose alone can alter the conformation of the protein, PT was spotted onto nitrocellulose paper and antipeptide antisera were assayed for recognition of bound toxin. Only peptides corresponding to antigenic epitopes also induced antibodies recognizing bound PT on nitrocellulose (Fig. 7).

Shared antigenicity between S2 and S3. The S2 and S3 subunits are about 70% homologous with the amino acid exchanges dispersed along the primary structure. The corresponding synthetic peptide sequences also reflect this homology (Table 1). Thus, it was interesting to see whether antisera raised against S2 segments would cross-react with

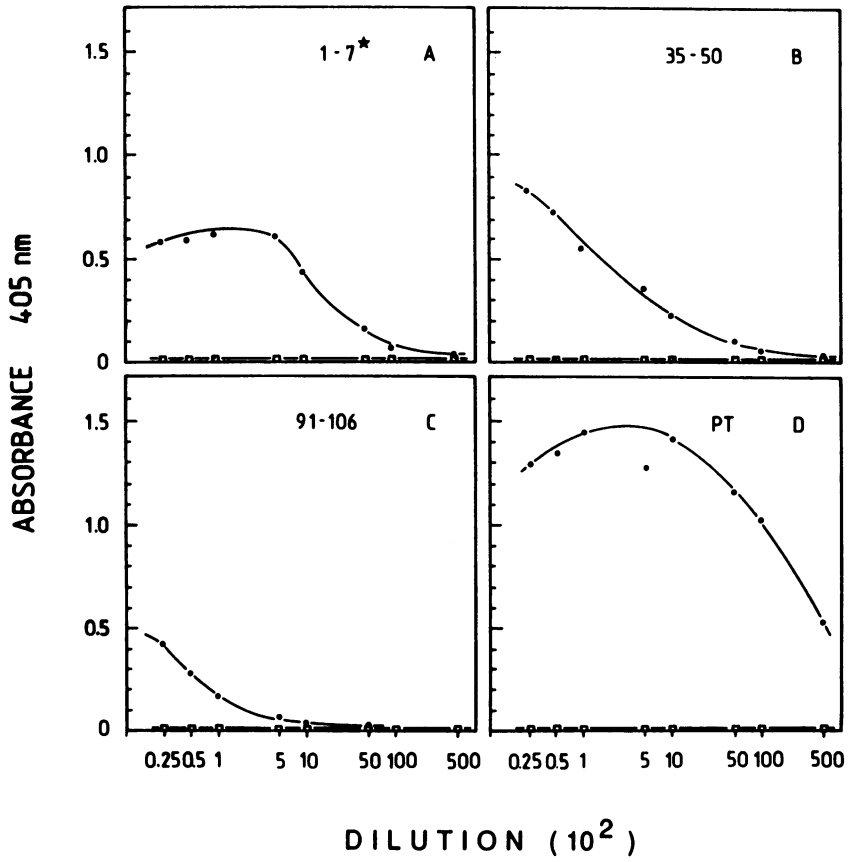

FIG. 4. Recognition of intact PT by antiserum against peptide-MBS-thyroglobulin conjugates. Antisera against peptides R1-7 (A), R35-50 (B), and R91-106 (C) showed significant binding. (D) Binding of antitoxin serum to PT. Antiserum against peptide R1-7* was obtained by immunization protocol B (see Materials and Methods). The A_{405} has been corrected as in the legend to Fig. 3.

the S3 subunit under denaturing conditions. However, only antiserum raised against peptide R91-106 showed a weak cross-reaction with the S3 subunit in Western blotting (Fig. 6), indicating that there might be residual structural heterogeneity of the two subunits, even under the relatively harsh denaturing conditions used.

DISCUSSION

B. pertussis organisms exhibit a number of virulence factors involved in the pathogenesis of whooping cough. The secretory exotoxin, PT, is regarded as one of the major virulence factors not only involved in the pathology of the disease, but also thought to be mainly responsible for the side effects sometimes seen upon pertussis vaccination. Recent trials with an acellular composite candidate vaccine developed by Japanese workers (28) consisting of filamentous hemagglutinin and formaldehyde-detoxified PT performed in Sweden (21) have shown the acellular vaccine to be effective (2). These trials have, however, failed to settle the issue of possible side effects intrinsic to vaccines prepared from biological sources. The genes coding for PT have recently been cloned and sequenced, and the amino acid sequences of the different subunits have been deduced from the corresponding DNA sequences (13, 19). Thus, knowing the amino acid sequences, it is now possible to synthesize peptides which might carry antigenic determinants of the native toxin and might be helpful in the development of a toxin-based fully synthetic vaccine against whooping cough.

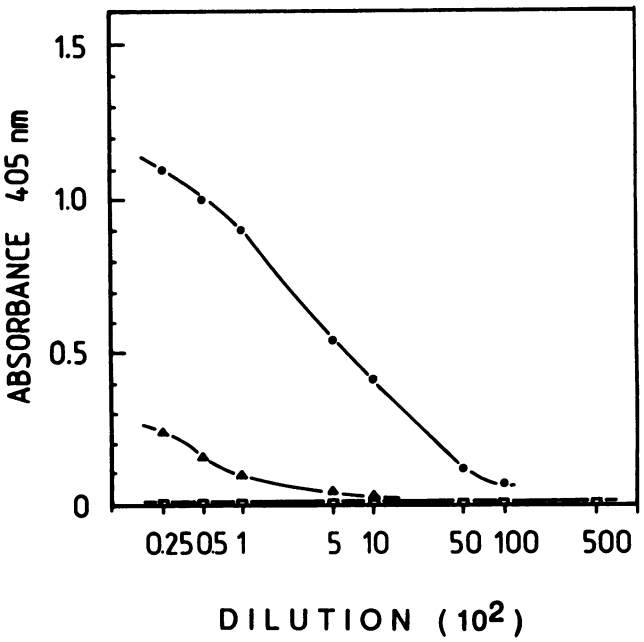

FIG. 5. Increased recognition of intact PT by antiserum obtained with R1-7–MBS–thyroglobulin conjugate according to immunization protocols A (▲) and B (●). Values were corrected for unspecific binding as described in the legend to Fig. 3.

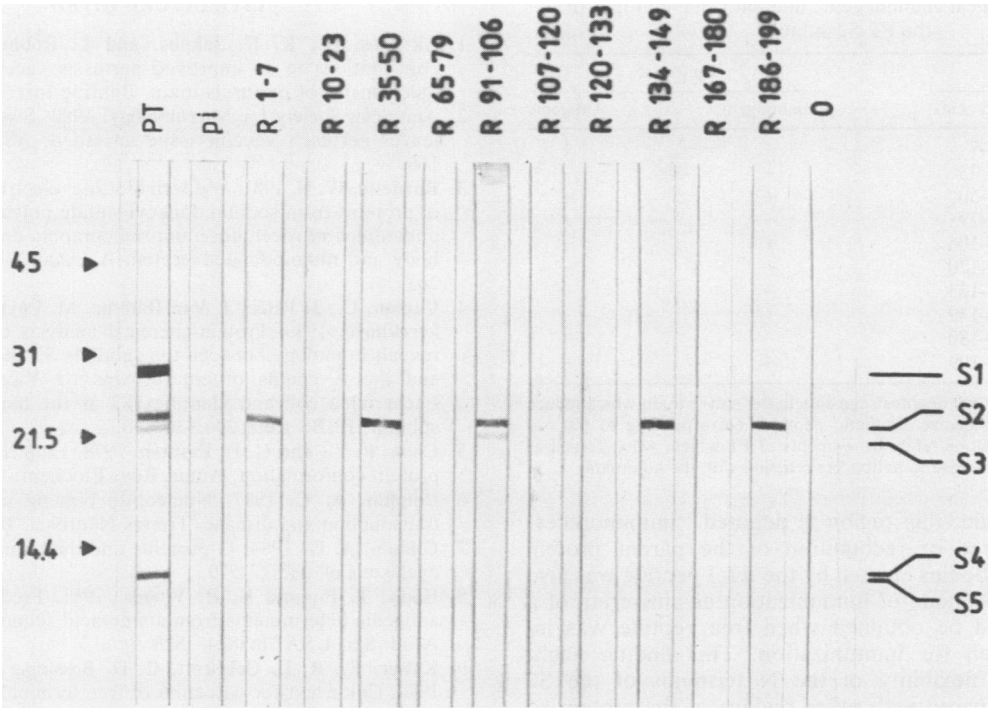

FIG. 6. Western blot of PT with antipeptide antisera diluted 1:200 in 0.1% BSA–PBS. Alkaline phosphatase-conjugated goat anti-rabbit antibodies and Nitro Blue Tetrazolium–5-bromo-4-chloro-3-indolyl phosphate as substrate was used to detect bound antibodies.

Synthetic peptides for the identification of antigenic and immunogenic epitopes are in a first approach only applicable for the detection of epitopes encoded by linear segments of the sequence. In most proteins, however, linear determinants comprise only a relatively small fragment of the overall antigenicity of the entire molecule. Most of the antigenicity of a given protein seems to reside in segments which are not contiguous but are juxtaposed in space due to the three-dimensional folding of the amino acid chain. Nevertheless, antibodies engendered by synthetic peptides can be successfully used for the elucidation of antigenic determinants and as sequence-specific probes for structure-function analysis provided the antibodies elicited by these peptides recognize the parent protein with high enough affinity. Only if a synthetic peptide adopts or can be induced to adopt a conformation identical or very similar to the conformation of the corresponding segment of the parent protein will it engender those antibodies to the parent protein or be likely be recognized by antiprotein antibodies. This also implies that the linear antigenic and immunogenic determinants detected by this approach might not represent the complete repertoire of linear antigenic and immunogenic determinants in a given protein.

In the present studies, we used synthetic peptides corresponding to 10 linear segments predicted to contain hydrophilic β turns of the PT S2 subunit which has been implicated with the recognition of PT receptors on target cells (4, 15). Five linear immunogenic and three linear antigenic determinants were identified with these reagents. The locations of linear antigenic and immunogenic determinants are summarized in Table 2. Peptides R35-50 and the C-terminal peptide R186-199 contain major linear immunogenic epitopes, whereas peptides R91-106, R107-120, and R134-149 contain rather minor linear immunogenic determinants. Only peptides R35-50 and R91-106 correspond to segments of the S2 sequence consisting of both linear antigenic and immunogenic determinants. Quantitative analysis of the recognition of PT by antibodies induced by the three synthetic peptides corresponding to PT S2 antigenic epitopes showed that more than half of the collective binding of antipeptide antibodies to PT is due to antibodies elicited by the N-terminal peptide R1-7. As antibodies to native PT do not recognize the

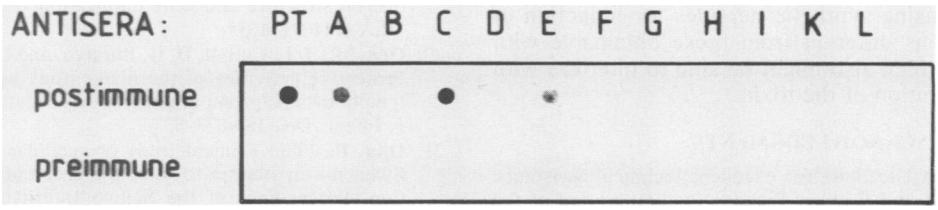

FIG. 7. Immunoblot of PT with antipeptide antisera. A 500-ng portion of PT was spotted on nitrocellulose paper, air dried, and incubated with the respective antisera: A, anti-R1-7; B, anti-R10-23; C, anti-R35-50; D, anti-R65-79; E, anti-R91-106; F, anti-R107-120; G, anti-R120-133; H, anti-R134-149; I, anti-R167-180; K, anti-R186-199; L, preimmune anti-PT. The peptide antisera were diluted 1:200 in 0.1% BSA–PBS. Bound antibody was detected as described in the legend to Fig. 6.

TABLE 2. Linear immunogenic and antigenic epitopes of the PT S2 subunit

Peptides		Epitopes[a]	
No.	Amino acids	Immunogenic	Antigenic
1	1–7	–	+
2	10–23	–	–
3	35–50	+	+
4	65–79	–	–
5	91–106	+	+
6	107–120	+	–
7	120–133	–	–
8	134–149	+	–
9	167–180	–	–
10	186–199	+	–

[a] Immunogenic epitope denotes segments in the native toxin which induce antibodies able to recognize synthetic peptides corresponding to this sequence. Antigenic epitope refers to segments of PT which, when copied as synthetic peptides, give rise to antibodies reacting with the holotoxin.

N-terminal peptide, this region is denoted "immunorecessive." The degree of recognition of the parent protein exhibited by antibodies elicited by the R1-7 peptide was also dependent on the mode of immunization as antiserum of a higher titer could be obtained when free peptide was included as antigen for immunization. This finding might reflect a higher flexibility of the N terminus of the S2 polypeptide compared with other regions of the protein so that the addition of free peptide as immunogen might be helpful in approaching more dynamic conformations. The C-terminal region of the S2 subunit harbors a major linear immunogenic determinant which is, however, not recognized by antibodies raised against the corresponding peptide. This is yet another example of the separation of linear antigenic and immunogenic determinants as detected by synthetic peptides.

Surprisingly, only antisera raised against synthetic peptide R91-106 corresponding to a linear antigenic epitope recognized the S3 subunit in Western blotting. A possible explanation might be that, even under the relatively harsh denaturing conditions used during SDS-polyacrylamide gel electrophoresis and Western blotting, the structural heterogeneity of the S2 and S3 subunits is conserved so that corresponding linear segments are not exposed to the same extent. This structural difference of subunits that are also probably functionally highly homologous raises the possibility of the existence of two distinct, different receptor sites in the PT. This possibility is supported by an earlier finding (4) describing affinity chromatography of dissociated PT with a haptoglobin affinity column in which only the S2-S4 dimer was retained. Preliminary evidence from experiments in our laboratory that addressed this question also suggests a second, distinct binding site in the B oligomer of PT.

Another aspect of the characterization of linear antigenic determinants of the S2 subunit under investigation concerns the possibility of using synthetic peptides for induction of antibody populations different from those obtainable with the native toxin, which also might be able to interfere with the receptor recognition of the toxin.

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