INFECTION AND IMMUNITY, Apr. 1991, p. 1402–1408
0019-9567/91/041402-07$02.00/0

Vol. 59, No. 4

Identification of Linear B-Cell Determinants of Pertussis Toxin Associated with the Receptor Recognition Site of the S3 Subunit

M. ALEXANDER SCHMIDT,* BÄRBEL RAUPACH, MIROSLAW SZULCZYNSKI, AND JUTTA MARZILLIER

Zentrum für Molekulare Biologie Universität Heidelberg, D-6900 Heidelberg, Federal Republic of Germany

Received 9 November 1990/Accepted 19 January 1991

Receptor recognition of pertussis toxin is mediated by the B oligomer consisting of subunits S2, S3, 2xS4, and S5. One possible way to interfere with toxin action would be the inhibition of recognition and binding of the cellular receptor(s) by preformed toxin-directed antipeptide antibodies. A prerequisite for this approach is the localization of linear antigenic determinants followed by the identification of inhibitory epitopes. Anti-S2 peptide antibodies have been shown to inhibit binding of the holotoxin to in vitro model receptor systems. For the elucidation of linear antigenic and immunogenic determinants harbored in the S3 subunit, synthetic peptides corresponding to selected linear amino acid sequences of S3 have been prepared and used to raise peptide-specific antibodies in rabbits. All peptides elicited a strong homologous response. Four synthetic peptides reacting with anti-pertussis toxin antibodies (R36-51, R87-95, R134-150, and R147-160) have been identified. Seven synthetic peptides (R1-12, R12-23, R14-29m, R36-51, R95-107, R134-150, and R164-178) induced antibodies recognizing pertussis toxin. Thus, these segments correspond to linear antigenic determinants. Analogous to the S2 subunit, the N terminus of S3 proved to be immunorecessive in the native toxin. The highly homologous S2 subunit was only bound strongly in Western blotting (immunoblotting) by antiserum directed at peptide R164-178, which is identical in the S2 and S3 subunits. A weak recognition of S2 in Western blotting was observed with anti-R95-107 antiserum. The ability of affinity-purified anti-S3 peptide antibodies to interfere with pertussis toxin binding was investigated by hemagglutination of goose erythrocytes as a model receptor system for S3-mediated receptor recognition. Antipeptide antibodies directed at R1-12, R12-23, R14-29m, and R36-51 inhibited hemagglutination of goose erythrocytes. This indicates that the corresponding antigenic regions in the S3 subunit are associated with the formation of the receptor binding domain. Inhibition of B-oligomer-mediated pertussis toxin binding to cellular receptors by preformed antipeptide antibodies of sufficient affinity should not only block the detrimental effects of the S1 subunits, but also interfere with the mitogenic effects attributed to the B oligomer.

Virulent *Bordetella pertussis* organisms exhibit a remarkable array of virulence factors which promote the development of the human respiratory disease whooping cough. Besides cell surface-associated factors such as pili and the filamentous hemagglutinin (32), *B. pertussis* also produces a number of toxins such as pertussis toxin (PT), adenylate cyclase toxin, tracheal cytotoxin, and dermonecrotic toxin (17, 21, 22, 45, 46). The expression of the majority of the known virulence factors has been found to be coordinately regulated (9, 18, 34). Except for paroxysmal coughing and the accumulation of mucus in the respiratory tract, the symptoms of whooping cough can be induced in mice by the action of PT alone (27, 29, 30, 35). Thus, PT not only seems to be the most prominent virulence factor of the organism (19) but also is supposedly responsible for the rare but occasionally severe side effects reported after vaccination with the current whole-cell pertussis vaccines (23, 40). Multiple efforts are therefore under way to develop new and safe pertussis vaccines. These should include PT as one component which has to be inactivated by genetic engineering or efficient chemical detoxification (1, 3, 24, 25, 28, 31, 36).

PT acts through the ADP-ribosylation of the α-subunit of certain G_i proteins, thus inhibiting the regulation of the cellular adenylate cyclase system, which leads to a nonphysiological increase in the level of cyclic AMP (8, 21). The genes coding for PT have been cloned and sequenced (15, 16, 26). The single polypeptide chain of the A protomer (S1 subunit) carries the enzymatic activity, whereas the multisubunit B oligomer (S2, S3, S4 [two], and S5) mediates the docking of the toxin to cellular receptors and is probably also involved in the translocation of the S1 subunit to the cytosol (4, 6, 10, 20, 39, 41). PT and the B oligomer and its two constituting dimers (S2/S4 and S3/S4), but not single isolated (recombinant) subunits (25), have been shown to be protective in the mouse model for *B. pertussis* infections (2, 35). The B oligomer is probably also responsible for the mitogenic effects and the possible "superantigenicity" discussed for PT (42, 44). To investigate the serological properties of subunits constituting the B oligomer and to provide probes for the elucidation and characterization of functional domains, e.g., the carbohydrate binding sites harbored by the B oligomer, synthetic peptides corresponding to selected segments of the S2 subunit have been prepared (38). The antipeptide antibodies have been used as probes to map linear immunogenic and antigenic determinants and to identify domains involved in the formation of the carbohydrate binding pocket (37, 38). Here we report the elucidation of linear antigenic and immunogenic epitopes of the S3 subunit as well as the identification of linear antigenic determinants which are associated with the formation of the second, distinct carbohydrate binding site of PT harbored by the S3 subunit (37).

(Throughout this report, the term antigenic epitope [determinant] refers to domains in the native toxin that are recognized by antibodies engendered by synthetic peptides corresponding to this region. Immunogenic epitope [deter-

* Corresponding author.

minant] is used to describe linear segments in the toxin which are recognized by the immune system and thus give rise to antibodies which recognize the corresponding synthetic peptides.)

MATERIALS AND METHODS

PT. The PT used in this study was either supplied as a gift by the Institut Mérieux, Lyon, France, or purchased from List Biochemicals, Campbell, Calif. The isolated subunits and subunit dimers of PT were a generous gift of C. Capiau, SmithKline, Rixensart, Belgium.

Selection of synthetic peptides. The choice of segments of the S3 amino acid sequence to be prepared as synthetic peptides was biased with regard to hydrophilic β-turns as indicated by secondary-structure predictions according to the algorithms of Chou and Fasman (7), Robson and Suzuki (33), Kyte and Doolittle (13), and Hopp and Woods (11). A natural or an additional cysteine residue was placed at the end of the amino acid sequence distal to the predicted β-turn, thus providing a unique site for coupling of the synthetic peptides to protein carriers in a defined orientation by heterobifunctional cross-linking agents (see below).

Characterization of peptides. Peptides were synthesized with Fmoc technology, using 4-(hydroxymethyl)phenoxymethyl-copol(styrene–1% divinylbenzene) resin as solid support (Novabiochem, Läufelfingen, Switzerland). The coupling of each amino acid was monitored with ninhydrin (12) and, if necessary, repeated until >99% efficiency was achieved. After purification by reverse-phase high-performance liquid chromatography, the identity of each synthetic peptide was verified by amino acid analysis.

Coupling to carrier proteins. Each peptide was coupled to bovine serum albumin (BSA), using succinimidyl-4-(*N*-maleimido-methyl)-cyclohexane-1-carboxylate (SMCC), and to thyroglobulin, using *m*-maleimidobenzoyl *N*-hydroxysuccinimide ester (MBS) as the heterobifunctional cross-linker (37, 38).

PT and peptide antisera. All antisera were prepared in female white chinchilla bastard rabbits. A 25-μg portion of PT or 250 to 300 μg of peptide-carrier conjugate and 50 μg of free peptide were emulsified in 100 μl of RAS adjuvants (PAN Systems, Aidenbach, Germany) and injected subcutaneously at multiple sites. Booster injections were given 4 weeks later. The rabbits were bled from the ear vein 7 to 8 days after the booster injections. For preparation of specific antipeptide antisera, rabbits were only immunized with the respective peptide-MBS-thyroglobulin conjugates.

Affinity purification of antipeptide antibodies. Antibodies directed at synthetic peptides corresponding to linear antigenic determinants of the S3 subunit were isolated by affinity chromatography, using the corresponding peptide-BSA conjugates coupled to EAH-Sepharose (Pharmacia) via 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. Briefly, peptide antiserum (500 μl) diluted 1:1 with 0.05% Brij 35-phosphate-buffered saline (PBS) was recirculated for ≥3 h over a 1.5-ml affinity column. The column was washed extensively with 0.05% Brij 35-PBS followed by PBS, and bound material was eluted with 0.1 M citric acid (pH 2.0) on ice and immediately neutralized with 1 M Tris base. After dialysis against 0.1× PBS, the antibodies were washed and concentrated with PBS, using Centricon (Amicon) concentrators with a cutoff value of about 30 kDa.

Solid-phase antigen-binding assay. Peptide-protein conjugates (1 mg/ml), PT, isolated subunits of PT (1 μg/ml), or free peptides (500 μg/ml) were coated on polystyrene microtiter plates in PBS. Free binding sites were blocked with 3% BSA-PBS at 4°C overnight. The plates were incubated with dilutions of antisera in 0.1% BSA-PBS and washed thoroughly with 0.05% Brij 35-PBS. Bound antibody was detected with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G, followed by *p*-nitrophenyl phosphate as substrate. The enzyme reaction was evaluated in an automated enzyme-linked immunosorbent assay (ELISA) reader (Titertek) at 405 nm. All assays were repeated several times in duplicate.

Western blotting (immunoblotting). For separation of PT subunits by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (14), 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 was performed in 25 mM Tris hydrochloride, pH 7.4, essentially as described by Towbin et al. (43) and Burnette (5). After transfer, the nitrocellulose was blocked with 5% BSA-PBS overnight at 4°C. Subsequently, antiserum was applied in a 1:200 dilution in 0.1% BSA-PBS. After 90 min at ambient temperature, the nitrocellulose was washed four times with 0.03% Brij 35-PBS and incubated with a 1:5,000 dilution of alkaline phosphatase-conjugated second antibody (dianova, Hamburg, Germany) in 0.1% BSA-PBS. Bound antibody was detected with Nitro Blue Tetrazolium–5-bromo-4-chloro-3-indolyl phosphate substrate in AP buffer (100 mM Tris [pH 9.5], 1 mM $MgCl_2$). The reaction was stopped with 20 mM Tris (pH 8.0) containing 5 mM EDTA.

Inhibition of hemagglutination by anti-S3 peptide antibodies. Goose erythrocytes were incubated for 30 min at 37°C with chymotrypsin at a final concentration of 1 mg/ml. The cells were washed repeatedly with PBS containing 1 mM phenylmethylsulfonyl fluoride and resuspended in PBS. These chymotrypsin-sensitized goose erythrocytes (sGRBC; 2×10^7) were incubated with serial dilutions of PT in 0.05% BSA-PBS. In this assay, 2 ng of PT per ml was sufficient for hemagglutination of sGRBC. For binding inhibition, serial dilutions of affinity-purified anti-S3 peptide antibodies were incubated for 2 h in 0.05% BSA-PBS with 15 ng of PT per ml and subsequently added to 2×10^7 sGRBC in 0.05% BSA-PBS.

RESULTS

Choice and synthesis of peptides. Fourteen synthetic peptides (R1-12, R12-23, R23-32, R14-29m, R14-29, R36-51, R53-63, R87-95, R95-107, R104-117, R120-130, R134-150, R147-160, and R164-178) have been prepared by the solid-phase technique for elucidation of linear antigenic and immunogenic determinants in the 199-amino-acid sequence of the S3 subunit of PT. The complete amino acid sequences of the synthetic peptides in comparison with their corresponding segments in the highly homologous S2 subunit are depicted in Table 1. The sequences to be synthesized were chosen according to their probability to encompass hydrophilic β-turns as predicted by different secondary-structure prediction algorithms (7, 11, 13, 33) (Fig. 1). All peptides were synthesized with either a natural or an additional cysteine residue at either end distal to the predicted β-turn, thus facilitating coupling in a unique orientation to a carrier protein.

Peptides as antigens. Each of the 14 peptide-BSA conjugates, as well as the free peptides, was assessed by ELISA with dilutions of polyclonal anti-PT antiserum. Peptides recognized by anti-PT antibodies are defined to encompass

TABLE 1. Sequence comparison and location of the S3 synthetic peptide sequences with the corresponding segments of the S2 subunit

PT subunit	Amino acids	Sequence[a]
S3	1–12	VAPGIVIPPKAL–C
S2		ST-------QEQ
S3	12–23	LFTQQGGAYGRC
S2		QI--H-SP----
S3	23–32	CPNGTRALTV
S2		-A-K------
S3	14–29m	TQQGGAYGRCPNGTRA
S2		--H-SP----A-K---
S3	14–29	C-TQQGGAYGRCPNGTRA
S2		--H-SP----A-K---
S3	36–51	C-RGNAELQTYLRQITPG
S2		--SGD--E---HV-R-
S3	53–63	C-SIYGLYDGTYL
S2		--FA-------
S3	87–95	CITTIYKTG
S2		--M-TRN--
S3	95–107	C-GQPAADHYYSKVT
S2		----T-----N--
S3	104–117	SKVTATRLLASTNS–C
S2		-N-------S----
S3	120–130	CAVFVRDGQSV
S2		------S--P-
S3	134–150	CASPYEGRYRDMYDALR
S2		-T---D-K-WS--SR--
S3	147–160	DALRRLLYMIYMSG–C
S2		SR--KM--L--VA-
S3	164–178	C-RVHVSKEEQYYDYED
S2		---------------

[a] C, Cysteine residue used for specific coupling of the peptide to carrier proteins; -C or C-, a purposely added cysteine residue.

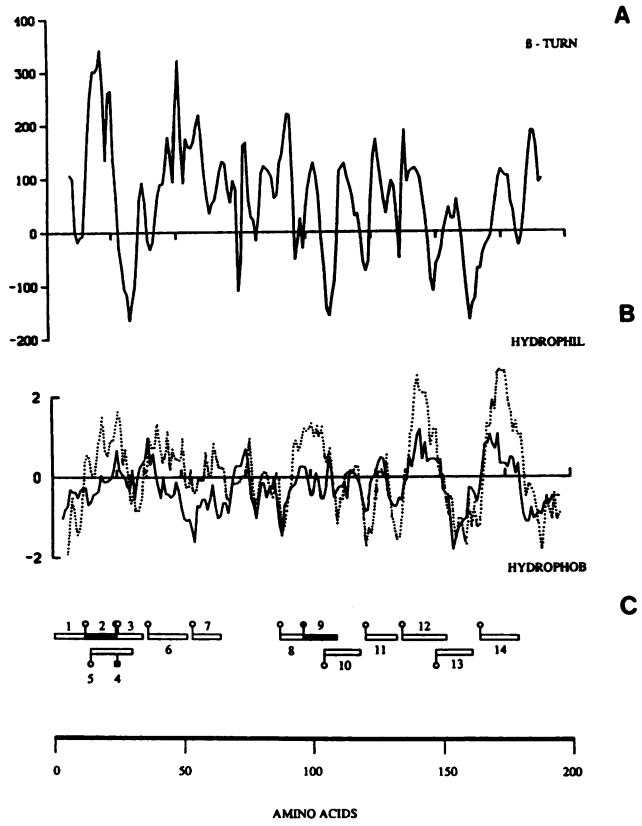

FIG. 1. Predictive analysis of the local average β-turn potentials (A) and hydrophilicity (B) of the S3 amino acid sequence by the methods of Hopp and Woods (11) (——) and Kyte and Doolittle (13) (-----). The hydrophilicity values are derived from the heptapeptide moving average and are plotted at the midpoint of each section. (C) Synthesized amino acid sequences, with the conjugation site via a natural or additional cysteine residue indicated as (○) or (●) in adjacent peptides. (■) denotes the internal cysteine residue used for conjugation. The amino acid numbering is given at the bottom.

immunogenic determinants. Anti-PT antiserum recognized 4 of the 14 peptides (Fig. 2A and B). Sequences R36-51 and R134-150 harbor major linear immunogenic determinants, whereas segments R120-130 and R14-29 encompass rather minor immunogenic epitopes (Table 2). Interestingly, the free peptide R87-95 reacted strongly in ELISA with anti-PT antibodies but was not recognized when the corresponding BSA conjugate was used to coat the solid phase. Peptides corresponding to residues R36-51 and R87-95 show different degrees of recognition whether they are coated onto the microtiter plate as free peptides or as peptide-BSA conjugates (Fig. 2A and B). Synthetic peptides corresponding to the N and C termini of the S3 subunit are not recognized by the anti-PT antiserum, indicating that these regions in the native protein are not available for the immune system and thus are denoted immunorecessive.

Peptides as immunogens. Polyclonal antipeptide antisera were produced in rabbits with peptide-MBS-thyroglobulin conjugates and were assessed for their capacity to recognize the homologous peptide-SMCC-BSA conjugates as well as PT, thus defining antigenic determinants. All peptides elicited a strong antipeptide response (data not shown). Four peptides were found to elicit antibodies cross-reacting with solid-phase bound PT (Fig. 3). Strongly cross-reactive antisera were induced with R1-12, R164-178, R12-23, and R147-164 (Fig. 3), while R14-29m coupled to carrier proteins through an internal cysteine residue induced a medium response. Interestingly, peptide R14-29, which has been conjugated via an additional N-terminal cysteine, did not engender cross-reactive antibodies. A rather weak cross-reactive response was found when peptides R95-107, R134-150, R53-63, R36-51, and R104-117 were used as immunogens and the antipeptide antisera were assayed in ELISA (data not shown). Antibodies engendered against peptides R23-32, R14-29, R87-95, and R120-130 did not recognize the holotoxin either in ELISA or by Western blotting.

Recognition of antigenic determinants by Western blotting. To investigate whether the antipeptide antisera also recognized the S3 subunit under denaturing conditions, PT was boiled in urea–SDS–2-mercaptoethanol by the method of Laemmli (14) and Tamura et al. (41), electrophoresed in 15% SDS-polyacrylamide gels, and transferred to nitrocellulose

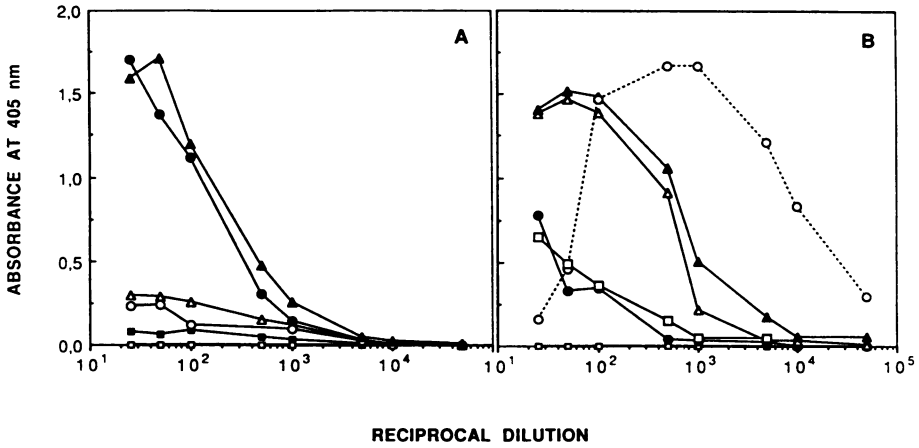

FIG. 2. Reaction of polyclonal anti-PT antiserum with peptide-BSA conjugates (A) and free synthetic peptides (B) by ELISA, as described in Materials and Methods. The A_{405} was corrected for nonspecific binding due to preimmune sera and BSA as indicated (----○----). Symbols: (A) ■, R14-29; ●, R36-51; △, R87-95; ○, R120-130; ▲, R134-150; (B) ●, R36-51; △, R87-95; ▲, R134-150; □, R147-160; ○, PT.

(Fig. 4). Antisera engendered against peptides R1-12, R36-51, R134-150, and R164-178 showed a strong reaction with S3, indicating that these antigenic determinants were not destroyed under harsh denaturing conditions. Antisera raised against peptides R12-23, R14-29m, and R95-107 showed only a weak cross-reactivity with S3 under denaturing conditions.

Recognition of S2 by anti-S3 peptide antibodies. Despite the remarkable degree of sequence homology between S3 and S2 (70%) which is reflected in the sequences chosen for duplication as synthetic peptides (Table 1), only antibodies raised against the synthetic peptide R164-178, which is identical in both subunits, strongly bound S2 in Western blotting (Fig. 4). Antibodies directed at R95-107 showed also a weak reaction with S2. All other antipeptide antisera were specific for the S3 subunit, indicating that even under the relatively strong denaturing conditions the structural heterogeneity of the two homologous subunits is preserved. Isolated subunits of PT (courtesy of C. Capiau) were used to assess the binding of anti-S3 peptide antibodies to S2, S2/S4, or S4 subunits. Only antibodies directed at peptides corresponding to the N terminus (R1-12), to R95-107, and to R164-178 which is identical in S2) recognized isolated S2 and the S2/S4 dimer (Fig. 5). The isolated S4 subunit was not recognized by any anti-S3 peptide antiserum.

Inhibition of hemagglutination by anti-S3 peptide antibodies. As the binding specificity of the S3 subunit of PT is not known, hemagglutination of sGRBC was used as an indicator for S3-mediated binding activity. A concentration of 2 ng of PT per ml was found to be sufficient for agglutination of 10^7 sGRBC. To investigate the ability of anti-S3 peptide antibodies to interfere with PT-mediated hemagglutination of erythrocytes, we preincubated PT (15 ng/ml) with serial

TABLE 2. Linear immunogenic and antigenic determinants of the PT S3 subunit

No.	Amino acids	Sequence	Determinant[a]		
			Immunogenic	Antigenic	
				Western	ELISA
1	1–12	VAPGIVIPPKAL-C[b]	–	++	++
2	12–23	LFTQQGGAYGRC	–	+	+
3	23–32	CPNGTRALTV	–	–	–
4	14–29M	TQQGGAYGRCPNGTRA	–	+	+
5	14–29	C-TQQGGAYGRCPNGTRA	–	–	–
6	36–51	C-RGNAELQTYLRQITPG	+	+	(+)
7	53–63	C-SIYGLYDGTYL	–	–	+
8	87–95	CITTIYKTG	+	–	–
9	95–107	C-GQPAADHYYSKVT	–	+	+
10	104–117	SKVTATRLLASTNS-C	–	–	+
11	120–130	CAVFVRDGQSV	–	–	–
12	134–150	CASPYEGRYRDMYDALR	+	+(+)	+
13	147–160	DALRRLLYMIYMSG-C	+	–	+
14	164–178	C-RVHVSKEEQYYDYED	–	++	++

[a] Immunogenic epitope denotes segments in the native toxin which induce antibodies able to recognize the corresponding synthetic peptides. Antigenic epitopes refers to segments of the protein which as synthetic peptides give rise to antibodies reacting with the holotoxin.

[b] See footnote *a*, Table 1, for explanation of C.

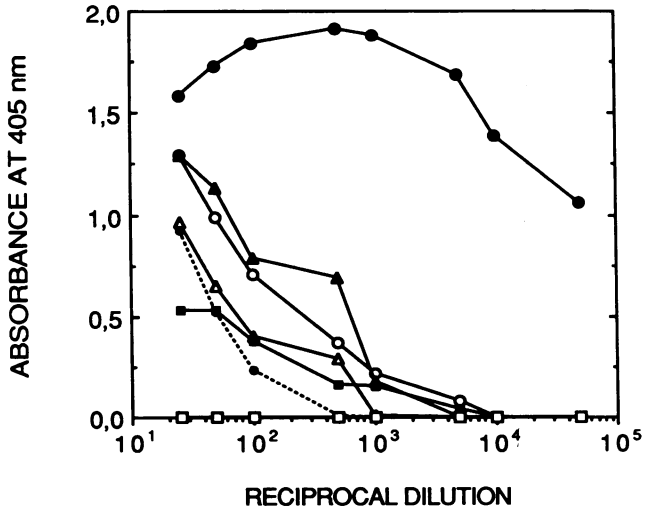

FIG. 3. Recognition of intact PT by antiserum against peptide-MBS-thyroglobulin conjugates and PT by ELISA. Depicted are antipeptide sera exhibiting significant binding to PT. Symbols: ●, binding of antitoxin antiserum to PT; ○, R1-12; △, R12-23; ■, R14-29m; ----●----, R147-160; ▲, R164-178. The A_{405} has been corrected for nonspecific binding as in the legend to Fig. 2.

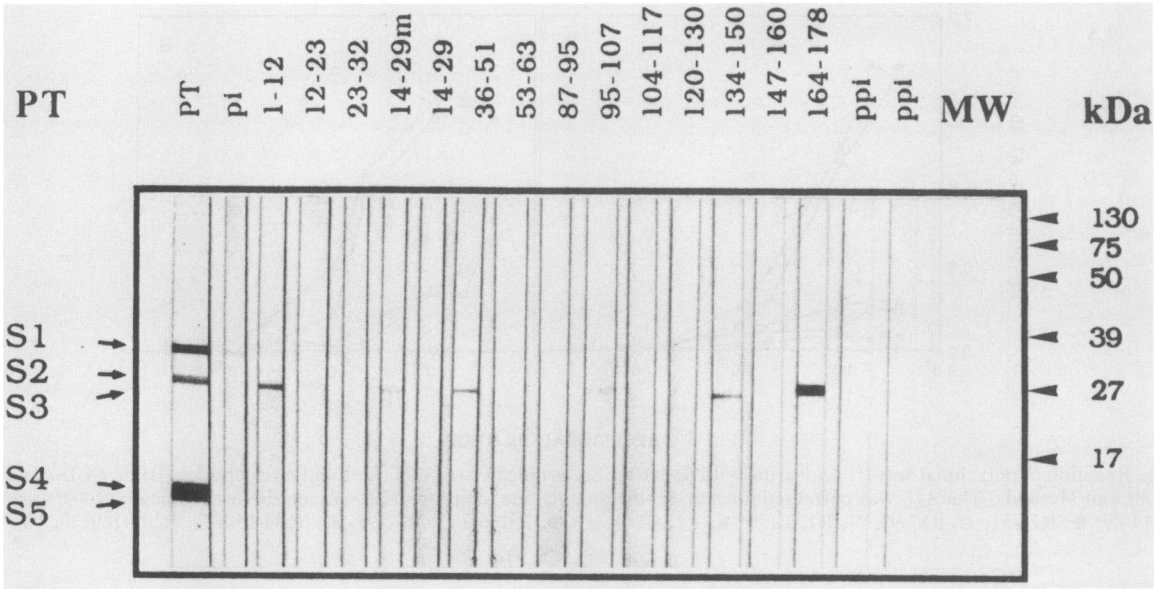

FIG. 4. Western blot of PT with antipeptide antisera diluted 1:200 in 0.1% BSA-PBS. Alkaline phosphatase-conjugated goat anti-rabbit antibodies and Nitro Blue Tetrazolium–5-bromo-4-chloro-3-indolyl phosphate were used to detect bound antibodies.

dilutions of affinity-purified anti-S3 peptide antibodies. Antibodies raised against R1-12, R12-23, R14-29m, and R36-51 were able to inhibit hemagglutination (Table 3). The MICs of antipeptide antibodies were estimated to be 18 μg/ml for anti-R1-12, 14 μg/ml for anti-R12-23, 61 μg/ml for anti-R14-29m, and 33 μg/ml for anti-R36-51. Not all anti-S3 peptide antibodies recognizing PT by ELISA or Western blotting inhibited hemagglutination (Table 3).

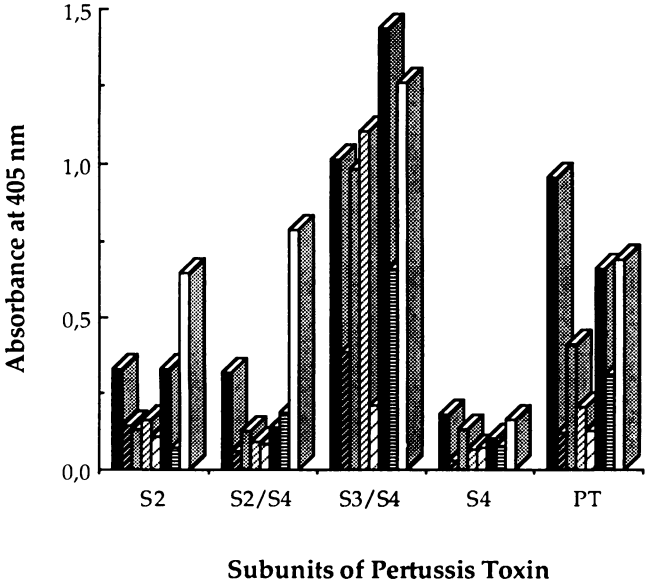

FIG. 5. Recognition of isolated PT subunits and PT dimers by anti-S3 peptide antibodies in ELISA. The microtiter plates were coated with antigen at 1 μg/ml in PBS. Values represent averages from several experiments run in duplicate. Symbols: ■, amino acids 1–12; ▨, 12–23; ▫, 14–29m; ▫, 36–51; ▫, 53–63; ▩, 95–107; ▤, 134–150; □, 164–178.

DISCUSSION

Among the striking number of known virulence factors exhibited by virulent *B. pertussis* organisms, the exotoxin PT is regarded as one of the major factors involved in the development of the pathology of the human respiratory disease whooping cough, acting not only as an exotoxin but also as an agglutinin (21, 22, 39). Contaminating active toxin has been held responsible for the rare, but sometimes severe, complications attributed to pertussis vaccination with the current whole-cell pertussis vaccines. Trials conducted with an acellular component vaccine consisting of filamentous hemagglutinin and PT have shown the component vaccine to be efficient but have failed to settle the issue of possible side effects intrinsic to preparations from biological sources (28). The cloning and sequencing of the genes coding for the expression of PT have opened the possibility of using synthetic peptides for the identification and characterization of immunogenic and antigenic determinants harbored by the single subunits of PT. The identification of segments carrying antigenic epitopes will be helpful for the

TABLE 3. Inhibitory activity of anti-S3 peptide antibodies in hemagglutination

Antibodies	Reactivity[a] in:		MIC (μg/ml)[b]
	ELISA with PT	HA inhibition	
Anti-S3 R1-12	+	+	18
Anti-S3 R12-23	+	+	14
Anti-S3 R14-29m	+	+	61
Anti-S3 R36-51	+	+	33
Anti-S3 R95-107	+	−	
Three others[c]	+	−	
Anti-PT	+	+	ND

[a] +, Reactive; −, nonreactive. HA, Hemagglutination.
[b] MIC for hemagglutination with sGRBC. ND, Not determined.
[c] Three other purified anti-S3 peptide antibodies: anti-R134-150, anti-R147-160, and anti-R164-178.

development of a fully synthetic vaccine against whooping cough, as protective determinants of PT will represent essential components in any new pertussis vaccine.

Though most determinants in proteins are conformational and more often represented by discontiguous rather than contiguous stretches of amino acids, synthetic peptides can be successfully applied as structure-function probes if they elicit antibodies which recognize the native or denatured protein with high enough affinity. To use this approach, it is advantageous when the synthetic peptides adopt or can be induced to adopt conformations which are identical or at least very similar to the conformation of the corresponding segment in the parent protein.

The present study describes the use of 14 synthetic peptides corresponding to linear sequences of the PT S3 subunit predicted to represent hydrophilic β-turns. The S3 subunit has been shown to harbor a second, distinct receptor binding site analogous to the one identified in the S2 subunit (37). Four linear immunogenic and seven linear antigenic determinants have been identified with antipeptide antisera as reagents. The locations of the linear immunogenic and antigenic determinants are summarized in Table 2. Major linear immunogenic determinants have been identified in peptides R36-51, R87-95, and R134-150, while a minor immunogenic epitope was detected in peptide R147-160. It is interesting to note that the recognition of two of the synthetic peptides by antitoxin antiserum changes remarkably depending on the coating of the ELISA plates with either the peptide-BSA conjugates or free peptides. Peptide R87-95 is only weakly recognized when coated as a BSA conjugate but shows strong binding of antitoxin antiserum when applied as a free synthetic peptide. The opposite is true for peptide R36-51, which is strongly recognized as a BSA conjugate but reacts rather weakly as a free peptide. These differences in reactivity of the two antigen preparations might be attributed to different sites available for recognition which are not occupied due to hydrophobic interactions of the peptides with the polystyrene support. Thus, for evaluation of ELISA results, both types of assays should be taken into account.

Only peptides R36-51 and R134-150 correspond to linear segments in the S3 subunit which harbor both immunogenic and antigenic determinants. Particularly, peptides corresponding to the segments at the N terminus and near the C terminus of the S3 subunit are not recognized by polyclonal antitoxin antiserum. Thus, these segments are denoted to be immunorecessive in the native PT, while as synthetic peptides they elicit antisera which show strong binding to PT in Western blotting and ELISA. Antigenic determinants have been detected in peptides R1-12, R12-23, R14-29m, R36-51, R95-107, R134-150, and R164-178. This is yet another example that, with synthetic peptides, cross-reactive antibody populations can be induced which are not available by immunization with the native protein.

As has been found with synthetic peptides corresponding to segments of the S2 subunit, only one of the seven antipeptide antisera reactive with the S3 subunit in Western blotting also binds strongly to the S2 subunit (R164-178). The amino acid sequence of this segment is identical in the S2 and S3 subunits (Table 1). Anti-S3 R95-107 antiserum reacts weakly with the S2 subunit, which is reminiscent of the antibodies raised against the corresponding segment in the S2 subunit. The alterations between the S3 and S2 amino acid sequences in all other synthetic peptides might interfere with recognition by heterologous antipeptide antibodies. Another possibility might be that the two PT subunits retain structural heterogeneity even under the strong denaturing conditions experienced during SDS-PAGE and Western blotting.

It has been shown that the interaction of PT with a model receptor system such as fetuin or haptoglobin is dependent on the binding specificity of the S2 subunit. Thus, as there is yet no receptor specificity known which is exclusively mediated by the S3 subunit, we used hemagglutination of sGRBC to assess the inhibitory capacity of affinity-purified anti-S3 peptide antibodies. Bivalency and thus also hemagglutination could be inhibited by four of the antipeptide antibodies used in this study (Table 3) which therefore recognize inhibitory epitopes. With the exception of antibodies directed at R95-107, these antibodies bind to segments of the S3 subunit which are analogous to the inhibitory regions identified in the S2 subunit. Though one has to be aware of the possible effects of steric hindrance, we conclude in analogy to the situation in the S2 subunit that in the S3 subunit the N terminus as well as the region around R36-51 is involved in the formation of the carbohydrate binding pocket of the S3 subunit in the native toxin. Whether the region around R95-107, as in the S2 subunit, also takes part in the formation of the binding site in the S3 subunit awaits further investigation.

However, the efficacy of the antipeptide antibodies engendered by the synthetic peptides developed in this study seems not to be sufficient to inhibit efficiently the binding of PT to its cellular receptors in an in vivo situation, e.g., the PT-mediated CHO cell clustering effect. Thus, the quality of antibodies raised by synthetic peptides corresponding to inhibitory determinants as identified in this study for S3 as well as in the preceding study for S2 (37) has to be improved. Experiments addressing these questions are under way in our laboratory. Second-generation synthetic peptides mimicking inhibitory determinants of PT might constitute successful candidates for a completely synthetic pertussis vaccine.

ACKNOWLEDGMENTS

We are indebted to Elke Bernarth and Rüdiger Pipkorn (Zentrum für Molekulare Biologie Universität Heidelberg) for peptide synthesis and to Ute Seitz for technical assistance. The generous gift of isolated PT subunits by Carine Capiau (SmithKline, Rixensart, Belgium) is gratefully acknowledged.

This work was supported by grant BCT 0381-5 of the Bundesministerium für Forschung und Technologie, Germany, and by a grant from the Institut Mérieux, Lyon, France.

REFERENCES

1. **American Society for Microbiology.** 1988. Status of the acellular vaccine: Swedish trail update leaves pertussis vaccine issue unsettled. ASM News **54**:164–166.
2. **Arciniega, J. L., D. L. Burns, E. Garcia-Ortigoza, and C. R. Manclark.** 1987. Immune response to the B oligomer of pertussis toxin. Infect. Immun. **55**:1132–1136.
3. **Black, W. J., J. J. Munoz, M. G. Peacock, P. A. Schad, J. L. Cowell, J. J. Burchall, M. Lim, A. S. Kent, and S. Falkow.** 1988. ADP-ribosyltransferase activity of pertussis toxin and immunomodulation by *Bordetella pertussis*. Science **240**:656–659.
4. **Brennan, M. J., J. L. David, J. G. Kenimer, and C. R. Manclark.** 1988. Lectin-like binding of pertussis toxin to a 165-kilodalton Chinese hamster ovary cell glycoprotein. J. Biol. Chem. **263**:4895–4899.
5. **Burnette, W. N.** 1981. Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112**:195–203.
6. **Capiau, C., J. Petre, J. Van Damme, M. Puype, and J. Vande-**

kerckhove. 1986. Protein-chemical analysis of pertussis toxin reveals homology between the subunits S2 and S3, between S1 and the A chains of enterotoxins of *Vibrio cholerae* and *Escherichia coli* and identifies S2 as the haptoglobin binding subunit. FEBS Lett. **204**:336–340.

7. **Chou, P. Y., and G. D. Fasman.** 1978. Empirical predictions of protein conformation. Annu. Rev. Biochem. **47**:251–276.
8. **Gilman, A. G.** 1984. G proteins and dual control of adenylate cyclase. Cell **36**:577–579.
9. **Gross, R., B. Aricò, and R. Rappuoli.** 1989. Genetics of pertussis toxin. Mol. Microbiol. **3**:119–124.
10. **Hewlett, E. L., and J. L. Cowell.** 1989. Evaluation of the mouse model for study of encephalopathy in pertussis vaccine recipients. Infect. Immun. **57**:661–663.
11. **Hopp, T. P., and K. R. Woods.** 1981. Prediction of protein antigenic determinants from amino acid sequences. Proc. Natl. Acad. Sci. USA **78**:3824–3828.
12. **Kaiser, E., R. L. Colescott, C. D. Bossinger, and P. I. Cook.** 1970. Color test for detection of free terminal amino groups in the solid phase synthesis of peptides. Anal. Biochem. **34**:595–598.
13. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157**:105–132.
14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T 4. Nature (London) **227**:680–685.
15. **Locht, C., P. A. Bostad, T. E. Coligan, L. Mayer, J. J. Munoz, S. G. Smith, and J. M. Keith.** 1986. Molecular cloning of pertussis toxin genes. Nucleic Acids Res. **14**:3251–3261.
16. **Locht, C., and J. M. Keith.** 1986. Pertussis toxin gene: nucleotide sequence and genetic organization. Science **232**:1258–1263.
17. **Manclark, C. R., and J. R. Cowell.** 1985. Pertussis. Bacterial Vaccines **3**:69–106.
18. **Melton, A. R., and A. Weiss.** 1989. Environmental regulation of expression of virulence determinants in *Bordetella pertussis*. J. Bacteriol. **171**:6206–6212.
19. **Monack, D., J. J. Munoz, M. G. Peacock, W. J. Black, and S. Falkow.** 1989. Expression of pertussis toxin correlates with pathogenesis in *Bordetella* species. J. Infect. Dis. **159**:205–210.
20. **Montecucco, C., M. Tomasi, G. Schiavo, and R. Rappuoli.** 1986. Hydrophobic photolabelling of pertussis toxin subunits interacting with lipids. FEBS Lett. **194**:301–304.
21. **Munoz, J. J.** 1985. Biological activities of pertussigen (pertussis toxin), p. 1–19. *In* R. D. Sekura, J. Moess, and M. Vaughan (ed.), Pertussis toxin. Academic Press, Inc., New York.
22. **Munoz, J. J., and M. G. Peacock.** 1989. Role of pertussigen (pertussis toxin) on the mouse protective activity of vaccines made from *Bordetella* species. Microbiol. Immunol. **33**:341–355.
23. **Munoz, J. J., M. G. Peacock, and W. J. Hadlow.** 1987. Anaphylaxis or so-called encephalopathy in mice sensitized to an antigen with the aid of pertussigen (pertussis toxin). Infect. Immun. **55**:1004–1008.
24. **Nencioni, L., M. Pizza, M. Bugnoli, T. De Magistris, A. Di Tommaso, F. Giovannoni, R. Manetti, I. Marsili, G. Matteucci, D. Nucci, R. Olivieri, P. Pileri, R. Presentini, L. Villa, J. G. Kreeftenberg, S. Silvestri, A. Tagliabue, and R. Rappuoli.** 1990. Characterization of genetically inactivated pertussis toxin mutants: candidates for a new vaccine against whooping cough. Infect. Immun. **58**:1308–1315.
25. **Nicosia, A., A. Bartoloni, M. Perugini, and R. Rappuoli.** 1987. Expression and immunological properties of the five subunits of pertussis toxin. Infect. Immun. **55**:963–967.
26. **Nicosia, A., M. Perugini, C. Franzini, M. C. Casagli, M. G. Borri, G. Antoni, M. Almoni, P. Neri, G. Ratti, and R. Rappuoli.** 1986. Cloning and sequencing of the pertussis toxin genes: operon structure and gene duplication. Proc. Natl. Acad. Sci. USA **83**:4631–4635.
27. **Oda, M., J. L. Cowell, D. G. Burstyn, and C. R. Manclark.** 1984. Protective activities of the filamentous hemagglutinin and the lymphocytosis-promoting factor of *Bordetella pertussis* in mice. J. Infect. Dis. **150**:823–833.
28. **Ölin, P.** 1986. Clinical trials of acellular pertussis vaccine in Sweden—an attempt to solve the problem of pertussis vaccination. Proceedings of the Selavo International Conference on Bacterial Vaccines and Local Immunity. Ann. Sclavo **1-2**:191–197.
29. **Pittman, M.** 1984. The concept of pertussis as a toxin mediated disease. Pediatr. Infect. Dis. **3**:467–486.
30. **Pittman, M., B. L. Furman, and A. C. Wardlaw.** 1980. *Bordetella pertussis* respiratory tract infections in the mouse: pathophysiological responses. J. Infect. Dis. **142**:56–66.
31. **Pizza, M., A. Covacci, A. Bartoloni, M. Perugini, L. Nencioni, M. T. De Magistris, L. Villa, D. Nucci, R. Manetti, M. Bugnoli, F. Giovannoni, R. Olivieri, J. T. Barbieri, H. Sato, and R. Rappuoli.** 1989. Mutants of pertussis toxin suitable for vaccine development. Science **246**:497–500.
32. **Relman, D. A., M. Domenghini, E. Tuomanen, R. Rappuoli, and S. Falkow.** 1989. Filamentous hemagglutinin of *Bordetella pertussis*: nucleotide sequence and crucial role in adherence. Proc. Natl. Acad. Sci. USA **86**:2637–2641.
33. **Robson, B., and E. Suzuki.** 1976. Conformational properties of amino acid residues in globular proteins. J. Mol. Biol. **107**:327–356.
34. **Roy, C. R., J. F. Miller, and S. Falkow.** 1990. Autogenous regulation of the *Bordetella pertussis bvgABC* operon. Proc. Natl. Acad. Sci. USA **87**:3763–3767.
35. **Sato, H., and Y. Sato.** 1984. *Bordetella pertussis* infection in mice: correlation of specific antibodies against two antigens, pertussis toxin, and filamentous hemagglutinin with mouse protectivity in an intracerebral or aerosol challenge system. Infect. Immun. **46**:415–421.
36. **Sato, H., and Y. Sato.** 1986. Japanese acellular pertussis vaccine. Proceedings of the Sclavo International Conference on Bacterial Vaccines and Local Immunity. Ann. Sclavo **1-2**:191–197.
37. **Schmidt, M. A., and W. Schmidt.** 1989. Inhibition of pertussis toxin binding to model receptors by antipeptide antibodies directed at an antigenic domain of the S2 subunit. Infect. Immun. **57**:3828–3833.
38. **Schmidt, W., and M. A. Schmidt.** 1989. Mapping of linear B-cell epitopes of the S2 subunit of pertussis toxin. Infect. Immun. **57**:438–445.
39. **Sekura, R. D., Y.-L. Zhang, and M.-J. Quentin-Millet.** 1985. Pertussis toxin: structural elements involved in the interaction with cells, p. 45–64. *In* R. D. Sekura, J. Moess, and M. Vaughan (ed.), Pertussis toxin. Academic Press, Inc., New York.
40. **Steinman, L., A. Weiss, N. Adelman, M. Lim, R. Zuniga, J. Oehlert, E. Hewlett, and S. Falkow.** 1985. Pertussis toxin is required for pertussis vaccine encephalopathy. Proc. Natl. Acad. Sci. USA **82**:8733–8736.
41. **Tamura, M., K. Nogimori, S. Murai, M. Yajima, K. Ito, T. Katada, M. Ui, and S. Ishii.** 1982. Subunit structure of islet-activating protein, pertussis toxin, in conformity with the A-B model. Biochemistry **21**:5516–5522.
42. **Tamura, M., K. Nogimori, M. Yajima, K. Ase, and M. Ui.** 1983. A role of the B-oligomer moiety of islet-activating protein, pertussis toxin, in development of the biological effects on intact cells. J. Biol. Chem. **258**:6756–6761.
43. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
44. **Ui, M., K. Nogimori, and M. Tamura.** 1985. Islet-activating protein, pertussis toxin: subunit structure and mechanism for its multiple biological actions, p. 19–43. *In* R. D. Sekura, J. Moess, and M. Vaughan (ed.), Pertussis toxin. Academic Press, Inc., New York.
45. **Weiss, A., E. L. Hewlett, G. A. Myers, and S. Falkow.** 1984. Pertussis toxin and extracytoplasmic adenylate cyclase as virulence factors of *Bordetella pertussis*. J. Infect. Dis. **150**:219–222.
46. **Weiss, A. A., and E. L. Hewlett.** 1986. Virulence factors of *Bordetella pertussis*. Annu. Rev. Microbiol. **40**:661–686.