INFECTION AND IMMUNITY, Nov. 1992, p. 4640–4647
0019-9567/92/114640-08$02.00/0

Vol. 60, No. 11

Identification of T- and B-Cell Epitopes of the S2 and S3 Subunits of Pertussis Toxin by Use of Synthetic Peptides

PELE CHONG,* GLORIA ZOBRIST, CHARLES SIA, SHEENA LOOSMORE, AND MICHEL KLEIN

Connaught Centre for Biotechnology Research, Connaught Laboratories Ltd., 1755 Steeles Avenue West, Willowdale, Ontario, Canada M2R 3T4

Received 10 June 1992/Accepted 24 August 1992

To design an optimized synthetic vaccine against whooping cough, we have studied the biological and immunological properties of three peptides of the S2 subunit and nine overlapping synthetic peptides covering the entire sequence of the S3 subunit of pertussis toxin (PT). Synthetic peptides corresponding to sequences 18 to 41, 78 to 108, 134 to 154, and 149 to 176 of S3 were found to be consistently capable of stimulating the proliferation of PT-specific T-cell lines primed with pertussis toxoid in both BALB/c and A/J strains of mice. All synthetic peptides were recognized by rabbit antisera raised against PT or pertussis toxoid. Both S2 and S3 peptide-keyhole limpet hemocyanin (KLH) conjugates in the presence of complete Freund's adjuvant induced peptide-specific antibody responses in rabbits, and the antisera raised against S2(1-23), S3(18-41), S3(37-64), and S3(149-176) peptide-KLH conjugates cross-reacted with both subunits in the immunoblots. All antisera except those against S2(123-154) and S3(103-127) reacted with native PT in an enzyme-linked immunosorbent assay (ELISA) with PT directly coated onto microtiter wells. In contrast, antisera raised against S2(123-154), S3(1-23), S3(18-41), S3(37-64), S3(60-87), and S3(103-127) peptide-KLH conjugates recognized native PT in a fetuin-PT capture ELISA. S2(78-98), S3(1-23), and S3(149-176) peptide-KLH conjugates elicited good PT-neutralizing antibody responses as judged by the antitoxin CHO cell assay. Identification of these B-cell neutralization epitopes and T-cell immunodominant determinants represents a first step towards the rational design of a synthetic vaccine against whooping cough.

Antibodies raised against pertussis toxin (PT) have been shown to protect mice against both intracerebral and respiratory challenges with virulent *Bordetella pertussis* (20). Thus, PT is a prime candidate for inclusion in an acellular pertussis vaccine. Pertussis toxin is an A-B type toxin. The A or S1 subunit is ADP-ribosyltransferase and mediates most of PT's biological and pharmacological functions (3, 8, 33, 36). The B-oligomer (S2 to S5 subunits) is responsible for the binding of the holotoxin to target cell receptors (3, 33). The DNA sequences of the PT gene have recently been established (18, 21), and the amino acid sequences of S2 and S3 subunits are found to be 70% similar.

PT can be detoxified by chemical treatment with aldehydes, but formalin-inactivated PT has been shown to revert to its toxic form (15). Therefore, the development of a synthetic peptide vaccine represents an attractive alternative. Sato's group reported that mouse monoclonal antibodies raised against S1, S2, and S3 subunits inhibited several PT-mediated activities, including promotion of lymphocytosis, insulin secretion, and clustering of Chinese hamster ovary (CHO) cells (16, 26, 27). These data suggest that these subunits are important immunogens and may contain neutralization epitopes. We and others (1, 6, 23) have recently identified the major PT-neutralization epitopes on S1. To design an optimized synthetic vaccine against whooping cough, we studied the functional and immunological properties of nine overlapping synthetic peptides covering the entire S3 sequence. These peptides were selected from the protein regions containing hydrophilic β-turns as judged by the secondary structure prediction analysis, and therefore are likely to be exposed and antigenic. Also, three peptides were chosen from the regions most different between S2 and S3 subunits (Table 1).

(The preliminary studies have been presented at the Sixth International Symposium on Pertussis, National Institutes of Health, Bethesda, Md., 26 to 28 September 1990, abstr. 11.)

MATERIALS AND METHODS

Peptide synthesis and peptide-carrier conjugation. Peptides from PT S2 and S3 subunits were synthesized by using an ABI 430A peptide synthesizer and optimized t-Boc chemistry as described by the manufacturer and then cleaved from the resin by hydrofluoric acid. The peptides were purified by reversed-phase high-performance liquid chromatography (HPLC) on a Vydac C4 semipreparative column (1 by 30 cm) with a 15 to 55% acetonitrile gradient in 0.1% trifluoryl acetic acid developed over 40 min at a flow rate of 2 ml/min. All synthetic peptides (Table 1) used in biochemical and immunological studies were >95% pure as judged by analytical HPLC. Amino acid composition analyses performed on a Waters Pico-Tag system were in good agreement with the theoretical compositions. Individual peptides were conjugated to keyhole limpet hemocyanin (KLH) by using sulfosuccinimyl(4-iodoacetyl)-amino-benzoate (Pierce) as a cross-linker at a 10:1 molar ratio of peptide over carrier as previously described (6, 17). An additional cysteine was added to the C-terminal end of peptides S3(37-64) and S3 (169-176) for conjugation purposes. Three peptides, BE3 (LPTPRGPDRPEGIEEEGGGERDRDRS), HIV1-P24 (GP KEPFRDYVDRFYKTLRAEQASQEV), and RSV-P1 (CSI SNIETVIEFQQKNNRLLEITRE), were synthesized as negative controls.

Preparation of pertussis toxin, B-oligomer, and pertussis toxoid. Pertussis toxin and B-oligomer were prepared by fetuin-Sepharose affinity chromatography by the method of

* Corresponding author.

TABLE 1. Amino acid sequences of overlapping peptides of pertussis toxin S2 and S3 subunits

Peptide	Sequence[a]
S2(9-23)	PQEQITQHGSPYGRC
S3(9-23)	PKALFTQQGGAYGRC
S2(1-23)	STPGIVIPPQEQITQHGSPYGRC
S3(1-23)	VAPGIVIPPKALFTQQGGAYGRC
S3(18-41)	GAYGRCPNGTRALTVAELRGNAEL
S3(37-64)	GNAELQTYLRQITPGWSIYGLYDGTYLG(C)
S3(60-87)	GTYLGQAYGGIIKDAPPGAGFIYRETFC
S3(87-108)	CITTIYKTGQPAADHYYSKVTA
S2(78-98)	GAFDLKTTFCIMTTRNTGQPA
S3(78-108)	AGFIYRETFCITTIYKTGQPAADHYYSKVTA
S3(103-127)	YSKVTATRLLASTNSRLCAVFVRDG
S3(134-154)	CASPYEGRYRDMYDALRRLLY
S2(123-154)	FVRSGQPVIGACTSPYDGKYWSMYSRLRKMLY
S3(123-154)	FVRDGQSVIGACASPYEGRYRDMYDALRRLLY
S3(149-176)	LRRLLYMIYMSGLAVRVHVSKEEQYYDY(C)
S3(173-199)	YYDYEDATFQTYALTGISLCNPAASIC

[a] The amino acid residues differing between the S2 and S3 subunits are in boldface.

Chong and Klein (5). Pertussis toxoid was prepared by treating native PT (300 μg/ml) with glutaraldehyde (0.2% [vol/vol]) for 2 h at room temperature. The reaction was quenched by 1 M lysine-HCl at pH 7.0. Pertussis toxoid then was dialyzed against phosphate-saline buffer to remove excess glutaraldehyde-lysine complexes and stored at 4°C.

Immunization protocols. Monospecific rabbit anti-native PT (RB-56 and RB-68) and anti-PT toxoid (RB-258, RB-263, and RB-266) antisera were prepared as previously described (5). To produce peptide-specific antisera, two New Zealand White rabbits (Maple Lane Farm, Ontario, Canada) were immunized intramuscularly with 20 to 100 μg of individual peptide or 50 to 500 μg of peptide-KLH conjugates emulsified in Freund's complete adjuvant. Rabbits were boosted with the same immunogens administered in incomplete Freund's adjuvant 14 and 28 days after the first injection. BALB/c mice were immunized with 20 μg of individual free peptides in Freund's complete adjuvant and given booster injections at 10 and 28 days with the same amount of peptide emulsified in incomplete Freund's adjuvant. Rabbit and mouse sera were collected 2 weeks after the final booster injection, heat inactivated at 56°C, and then stored at −20°C. Preimmune sera were collected from each animal 1 day before the immunization and were used to establish baseline reactivities.

Human serum samples. Eight serum samples were collected from children who had received four immunizations with a prototype Connaught acellular vaccine in which >80% of the proteins were pertussis toxoid (13). The sera were kindly provided by the department of Medical Affairs, Connaught Laboratories Limited (Ontario, Canada). All sera recognized PT in a PT-specific enzyme-linked immunosorbent assay (ELISA) and had CHO cell antitoxin neutralization titers ranging from <1/2 to 1/2,048. Purified human polyclonal immunoglobulin Gs (IgGs) were purchased from Bio/Can Scientific Inc. (Ontario, Canada).

CHO cell antitoxin neutralization assay. Inhibition of PT-induced CHO cell clustering by synthetic peptides and antisera raised against PT, free peptide, or peptide-KLH conjugates was assayed by the method of Gillenius et al. (12). Each assay was performed in triplicate and was repeated two to three times.

Proliferation assay for synthetic T-cell epitopes. T-cell epitope mapping was performed by priming BALB/c and A/J mice with 5 μg of pertussis toxoid in $AlPO_4$. Three weeks later, the spleens were removed and the splenocytes were cultured in RPMI 1640 (Flow Lab) supplemented with 10% heat-inactivated fetal calf serum (GIBCO), 2 mM L-glutamine (Flow Lab), 100 U of penicillin (Flow Lab) per ml, 100 μg of streptomycin (Flow Lab) per ml, 10 U of recombinant interleukin-2 per ml and 50 μM 2-mercaptoethanol (Sigma) for 5 to 7 days. Proliferative responses of the primed splenocytes to the panel of S2 and S3 peptides were determined in a standard in vitro assay (30). Briefly, 10^6 splenocytes were cocultured in a 96-well microtiter plate with 5×10^5 irradiated (1,700 rads) fresh syngeneic spleen cells used as a source of antigen-presenting cells in the presence of increasing molar concentrations (0.03 to 3 μM) of peptide dissolved in the culture medium without interleukin-2. Cultures were kept for 40 h in a humidified 5% CO_2–air incubator maintained at 37°C. During the final 16 h of culture, 0.5 μCi of [^{3}H]thymidine (5 Ci/mmol; NEN) was added to each of the microculture wells. The cells were then harvested onto glass-fiber filters, and the incorporation of [^{3}H]thymidine into cellular DNA was measured by a scintillation β-counter (Beckman). Results are expressed as the mean of triplicate cultures performed for each peptide concentration. The standard deviation was always <15%. Proliferative responses were considered positive when [^{3}H]thymidine incorporation was threefold above that obtained with either irrelevant peptides or the culture medium.

ELISA. Rabbit anti-peptide antisera were screened for reactivity with PT in two different ELISA methods: PT-coated ELISA and fetuin-PT-coated ELISA. In one ELISA, microtiter wells were directly coated with 400 ng of PT, while the fetuin-PT ELISA wells were coated with 400 ng of fetuin, and subsequently PT was captured as previously described (10, 15). Serial dilutions of rabbit or mouse antisera were added to the wells, and PT-bound antibodies were detected by protein A conjugated to horseradish peroxidase. Goat IgG $F(ab')_2$ anti-mouse IgG conjugated to the same enzyme was used to quantitate mouse antibodies. Peptide-specific ELISAs were essentially performed as previously described by Chong et al. (6). Microtiter plates were coated with 1 μg of free peptides per well instead of 5 μg per well. Purified rabbit polyclonal IgGs were purchased from Bio/Can Scientific Inc. The minimum titer is defined as the dilution at which the antiserum gives at least twofold above the absorbance obtained with the negative control.

Immunoblot analysis. The immunospecificity of antisera raised against peptides and peptide-KLH conjugates was determined by immunoblot analysis as previously described (6, 34).

RESULTS

Peptide selection. The objectives of the study were twofold: (i) to map the immunodominant epitopes of PT S2 and S3 subunits to guide the rational design of a synthetic peptide-based pertussis vaccine, and (ii) to determine their topographical relationship with B-oligomer functional domains to engineer toxin mutants by site-specific mutagenesis. The sequences of the nine peptides covering the entire S3 sequence were chosen for their high index of hydrophilic β-turns as judged by secondary structure prediction analysis by the methods of Chou and Fasman (7) and Hopp and Woods (14). These regions are likely to be exposed to the solvent and form antigenic determinants. Three regions of S2 (residues 1 to 23, 78 to 108, and 123 to 154) were also

TABLE 2. Immunological properties of rabbit antisera raised against peptide-KLH conjugates

Immunogen	Reactive titer[a]			Anti-PT CHO assay[b]	Subunit recognized in Western blots[c]
	Peptide	PT	Fetuin-PT		
S3(9-23)-KLH	+++	+++	+++	1/128	S3
S3(1-23)-KLH	+++	++++	+++	1/32	S3
S3(18-41)-KLH	+++	++++	++	1/8	S2,S3
S3(37-64)-KLH	+++	+++	+++	1/4	S2,S3
S3(60-87)-KLH	+++	+++	+++	1/4	S3
S3(87-108)-KLH	+++	++++	−	1/4	S2,S3
S3(78-108)-KLH	+++	++++	−	1/4	S3
S3(103-127)-KLH	+++	−	++	1/4	S3
S3(123-154)-KLH	+++	++++	−	1/4	S3
S3(149-176)-KLH	+++	++++	−	1/8	S2,S3
S3(173-199)-KLH	+++	+++	−	<1/2	S3
S2(9-23)-KLH	+++	+++	−	<1/2	S2
S2(1-23)-KLH	+++	+++	∓	<1/2	S2,S3
S2(78-98)-KLH	+++	+++	−	1/128	S2
S2(123-154)-KLH	+++	∓	++	<1/2	S2

[a] +, ++, +++, and ++++, average reactive titers of two individual rabbit antisera at 1/1,000, 1/5,000, 1/10,000, and >1/20,000, respectively; − and ∓, nonreactive and weakly reactive, respectively.

[b] The value reported here was the lowest PT-neutralization titer obtained from two individual rabbit anti-peptide antisera in the antitoxin CHO cell assay. The average PT-neutralization titer of rabbit anti-PT sera is 1/8,192.

[c] Subunits were recognized by the anti-peptide antisera in the Western blot with PT.

selected because (i) they correspond to the variable amino acid sequences distinguishing the S2 from the S3 subunit (Table 1), and (ii) they may be involved in different PT-receptor binding sites, as suggested by other workers (4, 22, 29, 29a). Peptides greater than 20 residues in length were synthesized, since it has been suggested that native linear epitopes are better mimicked by long peptides rather than by short peptides (35).

Reactivity of S2 and S3 peptides with anti-native PT and anti-toxoid antisera. Numerous studies have demonstrated that good ELISA results can be obtained by coating microtiter wells directly with synthetic peptides (2, 6, 10, 11). However, it is critical to ascertain that peptides are noncovalently bound to microtiter wells. Thus, to validate the peptide-specific ELISA, individual peptides were coated onto the polystyrene microtiter wells and probed with peptide-specific rabbit antisera raised against peptide-KLH conjugates. All polystrene bound peptides used in this study were recognized by their respective antisera diluted at least 10,000-fold (Table 2). These results clearly established that all synthetic peptides were adsorbed to the microtiter wells and their antigenic determinants were accessible to antibody recognition. To rule out the possibility of natural anti-PT antibodies or nonspecific interaction between synthetic peptides and rabbit immunoglobulins, peptides were screened with a pool of normal rabbit IgGs and rabbit preimmune sera. No reactivity was observed either with preimmune sera at a dilution above 1 in 200 or with IgGs at a concentration below 24 μg/ml. Only peptides S3(78-108) and S3(123-154) were found to react with rabbit IgGs at 12 μg/ml and preimmune sera at a 1/400 dilution (data not shown).

The antigenicity of the synthetic peptides was assessed by peptide-specific ELISA with rabbit monospecific antisera raised against either purified pertussis toxin or pertussis toxoid. These antisera were previously shown to react with native PT in a PT-specific ELISA with reactive titers ranging from 1/1,000,000 to 1/6,400,000 and on immunoblots (5). Although rabbit antisera had variable reactivity with S2 and S3 peptides as shown in Fig. 1, the results suggested that synthetic peptides S2(1-23), S2(78-98), S2(123-154), S3(78-108), S3(149-176), and S3(173-199) were the linear B-cell epitopes, since they reacted with both anti-PT and anti-toxoid antisera. All anti-PT antisera reacted poorly with S3(18-41) peptide. This peptide may thus represent one of the immunorecessive epitopes of PT. Interestingly, synthetic peptides corresponding to the S3 sequence encompassing residues 37 to 87 of S3 were recognized by one of two anti-native PT antisera but reacted poorly with all three anti-toxoid antisera (Fig. 1). These results suggest that the epitopes localized within this region of S3 may have been altered by glutaraldehyde treatment.

One essential objective was to identify the linear B-cell epitopes recognized by human antisera. Figure 2 shows the typical reactivities of antisera from a child immunized with the whole-cell pertussis vaccine and three children vaccinated with an acellular pertussis vaccine against the panel of synthetic peptides. Antisera from all vaccinees reacted strongly with peptides S2(78-98), S3(78-108) and S3(103-127), indicating that these peptides represent major human linear B-cell epitopes. Since the S3(173-199) peptide reacted weakly with all children's sera, it could be a minor B-cell determinant. It is worthwhile to point out that the S2 and S3 synthetic peptides were not recognized by commercially available human IgGs. In addition, rabbit and human anti-PT antisera did not react with the three irrelevant peptides included as negative controls in this study.

Immunogenicity of S2 and S3 synthetic peptide-KLH conjugates. To determine whether the synthetic B-cell determinants were able to elicit antibodies against native PT structure, rabbits were immunized with individual S2 and S3 peptide-KLH conjugates in the presence of complete Freund's adjuvant. All conjugates were highly immunogenic and induced strong and specific antibody responses against the immunizing peptides in peptide-specific ELISAs (Table 2). Peptide-antibody interactions were specifically blocked by the immunizing peptide in its unconjugate form. However, rabbit antisera raised against the S3(78-108) and S3(123-154) peptide-KLH conjugates showed strong cross-reactivity with S2 peptides S2(78-98) and S2(123-154), respectively. In contrast, rabbit antisera raised against peptide S2(78-98) and S2(123-154) conjugates were found to be monospecific and showed very little if any cross-reactivity

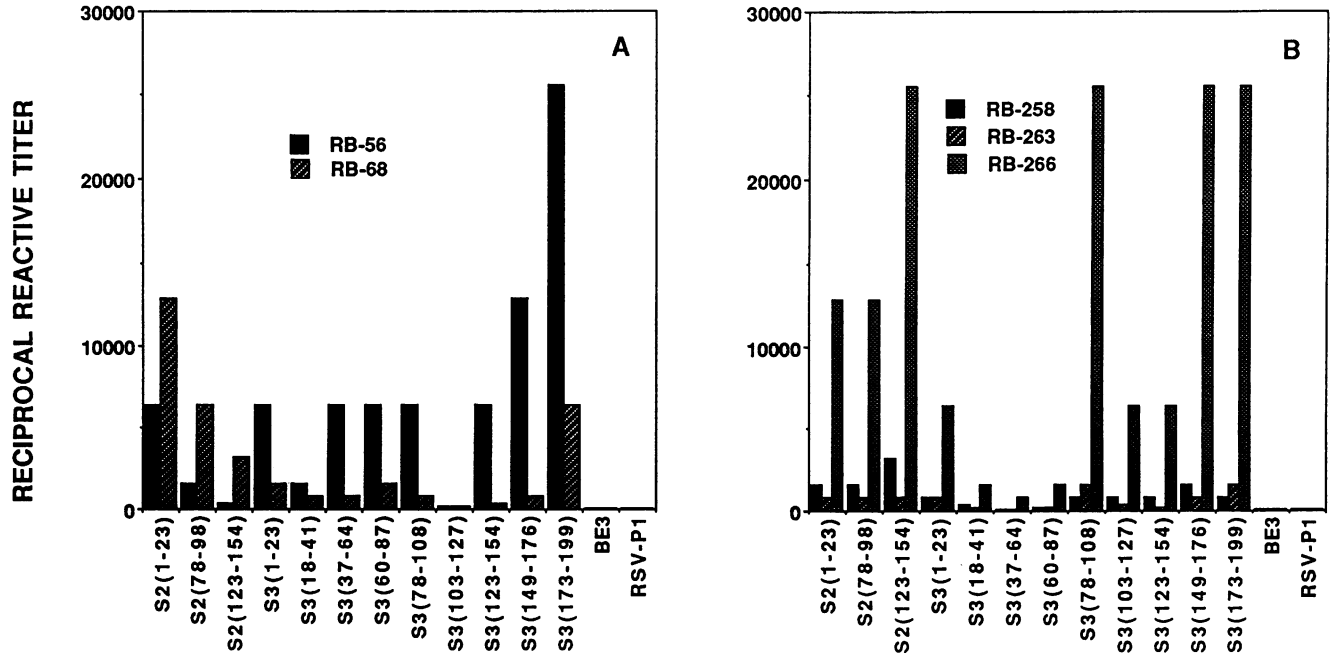

FIG. 1. (A) ELISA reactivity of rabbit anti-PT antisera (RB-56 and RB-58); (B) antisera raised against pertussis toxoid (RB-258, -263, and -266) with S2 and S3 synthetic peptides.

with the corresponding S3 peptides. Although the sequences of peptides S2(78-98) and S3(78-108) are 50% similar and those of S2(123-154) and S3(123-154) are 60% similar (Table 1), the low cross-reactivity of anti-S2 peptide antibodies suggests that the S2 variable residues form the S2-specific epitope whereas the S3 epitope involves conserved residues. Similarly, the cross-reactivity of antisera against S2(1-23) and S3(1-23), which are 60% homologous, was observed only at a 1/100 dilution, indicating that these antisera essentially recognized the variable residues.

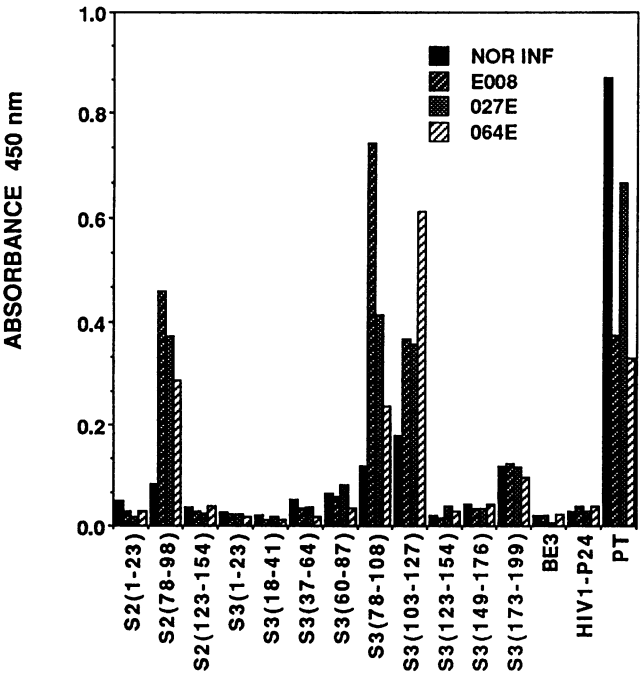

FIG. 2. ELISA reactivity of human sera (E008, 024E, and 064E) from babies vaccinated with Connaught acellular pertussis vaccine with S2 and S3 synthetic peptides. NOR INF is serum from a baby immunized with the normal whole-cell pertussis vaccine. In this assay, human sera were used at a 1/800 dilution.

The specificity of anti-peptide antisera was further assessed by immunoblots. Most peptide-specific antisera specifically recognized either the S2 or the S3 subunit on immunoblot analysis even at a 1/200 dilution (Table 2). Only antisera raised against S2(1-23), S3(18-41), S3(37-64), and S3(149-176) conjugates reacted with both S2 and S3 subunits in the blots (Table 2). Interestingly, both anti-S3(78-108) and anti-S3(123-154) antisera which had previously been shown to cross-react with S2 peptides S2(78-98) and S2(123-154) in peptide-specific ELISAs did not recognize the S2 subunit on immunoblots.

Mapping of surface-exposed epitopes. To map the surface-exposed epitopes of PT, the peptide-specific antisera were first assayed for their reactivities with native PT in a direct PT-coated ELISA. All anti-peptide antisera except those raised against S2(123-154) and S3(103-127) showed strong reactivity with native PT in the PT-coated ELISA (Table 2). These results indicate that regions around residues 1 to 23 and 78 to 98 of S2 and residues 1 to 23, 18 to 41, 37 to 64, 60 to 87, 78 to 108, 123 to 154, 149 to 176, and 173 to 199 of S3 are exposed on the surface of native PT and readily accessible to antibody recognition.

To further establish the topographical relationship of S2 and S3 peptides with the B-oligomer functional domain, we tested the reactivities of anti-peptide antisera with fetuin-bound PT. Antisera against peptides S2(123-154), S3(1-23), S3(18-41), S3(37-64), S3(60-87), and S3(103-127) reacted strongly with fetuin-bound PT in the PT-fetuin capture ELISA (Table 2). Although S2(1-23)-specific antibodies still recognized PT in this assay, their binding was reduced as judged by ELISA titers. The other antisera did not react with

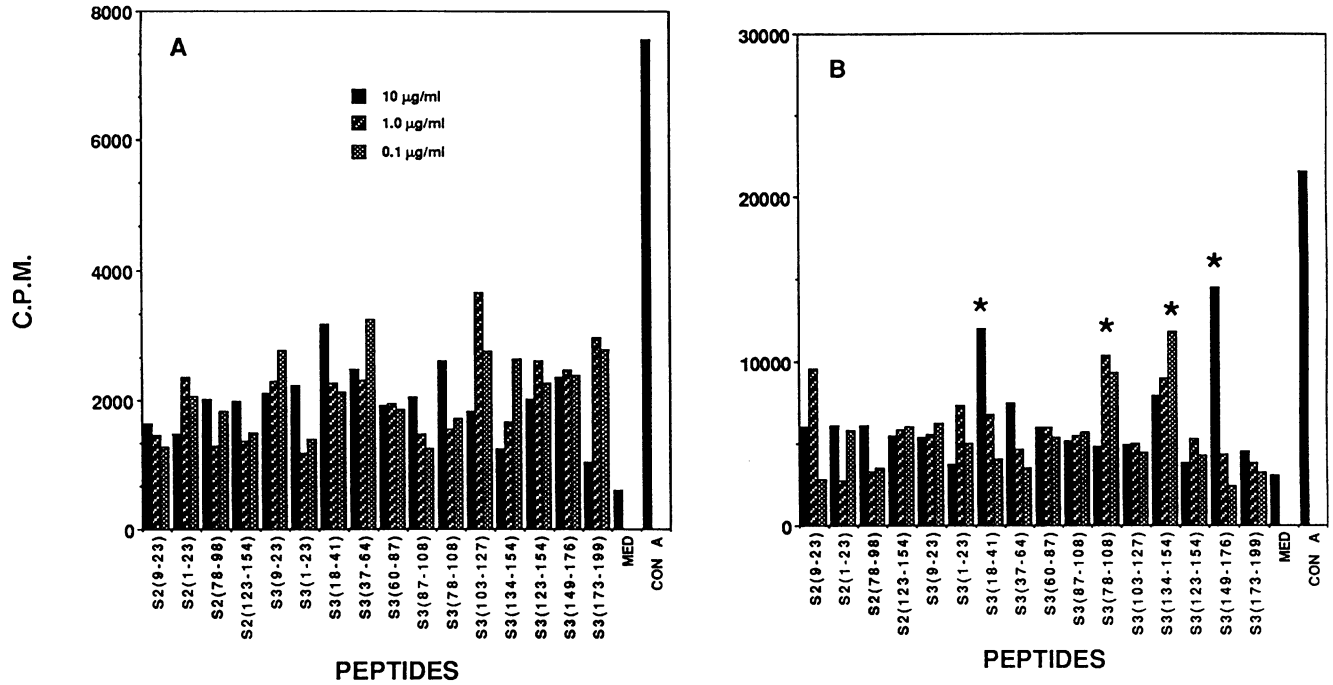

FIG. 3. Proliferative response of PT-specific murine T-cells to S2 and S3 synthetic peptides. T-cell proliferation assay was performed with primed splenocytes from A/J strain mice (A) and with BALB/c mice (B). Results are expressed as mean counts per min (C.P.M.) of triplicate cultures. All standard deviations were less than 15%. Immunodominant T-cell epitopes are highlighted with an asterisk.

PT in this capture assay. These data suggest that the structural conformation around residues 78 to 98 of S2 and 78 to 108 and 123 to 199 of S3 are very sensitive to fetuin-PT binding, so that anti-peptide antibodies could not recognize PT as they do in the direct PT-coated ELISA. It is also worthwhile to point out that upon fetuin binding the region around residues 123 to 154 of S2 and 103 to 127 of S3 in PT had changed from buried to surface exposed and accessible for antibody binding.

Neutralization epitope mapping. The functional properties of the peptide-specific antisera were determined by the CHO cell antitoxin neutralization assay (12). Rabbit antisera raised against S2(78-98), S3(1-23), S3(18-41), and S3(149-176) were found to be capable of neutralizing PT toxicity in the CHO cell clustering assay at a >1/8 dilution (Table 2), whereas anti-S3(37-64), -S3(60-87), -S3(78-108), -S3(103-127), and -S3(123-154) antisera inhibited CHO cell clustering only at a lower dilution (1/4). Preimmune sera and the remaining peptide-specific antisera had no neutralizing activity. To test whether synthetic peptides could bind to the PT receptor on CHO cells and directly block PT toxicity, individual free peptides (0.1 to 200 μg/ml) were tested in the CHO cell clustering assay. Although peptide S3(149-176) was found to be capable of inhibiting PT-induced CHO cell clustering at 12.5 μg/ml, it was at about a 2,500-fold molar excess of peptide over PT. These results further support that the region around residues 149 to 176 of S3 is in close proximity to the PT receptor sites.

T-cell epitope mapping. The presence of T-cell epitopes was assessed by the ability of free peptides to stimulate the proliferation of mouse (BALB/c and A/J strains) T lymphocytes primed with pertussis toxoid in a standard in vitro T-cell proliferation assay (30). The results summarized in Fig. 3A showed that all synthetic peptides tested at molar concentrations ranging from 0.03 to 3 μM were capable of inducing a good proliferative response in T lymphocytes from the A/J strain. At this time we could not explain this unusual finding. In contrast, only synthetic peptides S3(18-41), S3(78-108), S3(134-154), and S3(149-176) were found to stimulate the proliferation of primed T cells from BALB/c mice (Fig. 3B). These data indicate that four murine T-cell epitopes are located within residues 18 to 41, 78 to 108, 134 to 154, and 148 to 176 of the S3 subunit.

DISCUSSION

Numerous studies have suggested that synthetic peptides can function as inexpensive candidate vaccines against infectious diseases (31). Recently, we and others (1, 6, 8, 19, 23, 36) have identified the critical regions and the neutralization epitopes on S1. In this study, we have examined the reactivity of 12 synthetic peptides from the S2 and S3 subunits of PT with rabbit anti-PT antisera and sera from children vaccinated with acellular pertussis vaccine. We have identified the immunodominant T-cell epitopes by using spleen cells from mice primed with pertussis toxoid. Also, individual synthetic peptide-KLH conjugates were assessed for their immunogenicities in rabbits. Rabbit anti-peptide antisera were tested for their immunological properties (i) in the CHO cell antitoxin neutralization assay, (ii) by PT- and peptide-specific ELISA, and (iii) by immunoblot analysis.

Free peptide has an advantage over peptide-carrier conjugate as an antigen in ELISA in that it has no anticoupling reagent antibody interference and is time-saving because there is no need to conjugate the peptide to another carrier with different chemical cross-linkers. In addition, we often observed lower reactive titers of the anti-peptide antisera against peptide in the peptide-carrier-coated ELISA (data not shown), and this reduction of titer could be due to the precipitation of peptide-carrier conjugates using glutaralde-

hyde as a chemical cross-linker. As in our previous study (6), we found that all 12 synthetic peptides coated the microtiter wells under standard conditions (pH 9.6, 1 μg of peptide per well) and their antigenic determinants were accessible to antibody binding. Using this peptide-specific ELISA, we have successfully determined that residues 78 to 98 of S2 and 78 to 108 of S3 are the immunodominant B-cell epitopes of the S2 and S3 subunits of PT for both humans and rabbits. However, the high reactivity of human sera with these peptides does not correlate with the CHO cell antitoxin neutralization titers, which range from <1/2 to 1/2,048. Further studies showed that the infant serum reactivity with peptides S3(87-108) and S2(78-98) could efficiently be inhibited by peptide S3(78-108) in a competition assay (data not shown). These results suggest that human sera recognized the common amino acids within residues 78 to 87 of both subunits, and these residues seem to be not critical to PT biological activity. Altogether this implies that the neutralization epitopes recognized by human sera are conformational or located in other subunits such as S1. Interestingly, the amino acid sequences around residues 37 to 87 of S3 were recognized by rabbit anti-PT antisera, but the peptides from the same region reacted poorly with both rabbit antitoxoid antisera and sera from infants vaccinated with acellular pertussis toxoid. This region (residues 37 to 87) of S3 could be modified by glutaraldehyde detoxification, which would cause the loss of B-cell epitopes.

All synthetic peptides, chosen according to the high index of hydrophilic β-turns in secondary structure prediction analysis, were highly immunogenic as indicated by the induction of antisera reactive with their respective peptides in the peptide-specific ELISA and the corresponding subunits of PT in immunoblots. Although the S2 and S3 subunits are 70% homologous in their amino acid sequences, we observed that only antisera raised against S2(1-23) and the four S3 peptide conjugates [S3(18-41), S3(37-64), S3(87-108), and S3(149-176)] cross-reacted with both subunits in the immunoblots. The cross-reactivity of antisera raised against peptides S3(18-41) and S3(149-176) can be explained by the sequence similarity (>90%) between the two subunits (Fig. 1). However, the present results are somewhat different from the epitope mapping studies on S2 reported by Schmidt and Schmidt (29a), who observed that only antisera against residues 91 to 106 of S2 recognized both subunits in the immunoblots. One possible explanation could be that the antibodies generated from S2(78-98) only recognized the variable residues within 78 to 93 of S2 since they showed very little cross-reactivity against the S3(78-108) peptide. The antisera raised against 91 to 106 of S2 in the Schmidt study reacted with the C-terminal half of the peptide (residues 98 to 106) in a manner similar to the reactivity of antisera raised against the S3(87-108)-KLH conjugate mentioned above. However, both studies (ours and the Schmidts') showed, even under the reduced and denatured conditions (sodium dodecyl sulfate [SDS]-polyacrylamide gel electrophoresis [PAGE] and electroblotting), the structural conformation differences between the two subunits are retained so that the corresponding regions are not presented or exposed to the same extent. These structural differences are also predicted by the secondary structure analysis. The structural heterogeneity is consistent with the concept and observation of the existence of two distinct PT-receptor binding sites in the S2-S4 dimer and the S3-S4 dimer (4, 16, 22, 29).

We and others have successfully reduced the toxicity of PT by site-directed mutagenesis of the S1 subunit (8, 19, 36), but additional studies need to be done to verify whether the remaining T-cell mitogen effect of the B-oligomer might have deleterious consequences in vivo. Therefore, it is of interest to know the receptor-binding sites, and PT-fetuin binding was chosen as a model for investigation. The reactivities of PT with the peptide-specific antisera were determined in the presence and absence of fetuin. Antisera against the N-terminal region residues 1 to 78 of S3 recognized PT in both PT-coated and PT-fetuin capture ELISAs. These results suggest that the structural conformation of the N-terminal region (residues 1 to 78) of S3 is not significantly affected upon PT-fetuin binding, and this region is still accessible for antibody binding. However, the regions around residues 1 to 23 and 78 to 98 of S2 and the C-terminal half of S3 are shown to be very sensitive to PT-fetuin binding, and antibody recognition sites are either buried or destroyed because of fetuin-induced conformational changes. Similarly, either the sites around residues 123 to 154 of S2 and 103 to 127 of S3 which become surface exposed and accessible for antibody binding upon fetuin binding may be buried in the native PT or the structural conformation in these regions is not recognized by the anti-peptide antibodies. These results are in agreement with a recent study by Schmidt and Schmidt (29) that showed antibodies raised against residues 1 to 7, 35 to 50, and 91 to 105 of S2 could inhibit PT-fetuin interaction, and they suggested that these regions could be the PT-fetuin binding sites. In preliminary experiments, we observed direct binding between biotinylated fetuin or biotinylated ceruloplasmin and synthetic peptides S2(78-98), S3(78-108), and S3(149-176). Furthermore, PT mutants generated from site-directed mutagenesis around these sites reduce the toxicity to CHO cells and the affinity to fetuin binding (18a).

Schmidt et al. (28, 29, 29a) found that rabbit antisera raised against residues 1 to 7, 35 to 50, and 91 to 106 of S2 and residues 1 to 12, 12 to 23, 14 to 29, and 36 to 51 of S3 were capable of inhibiting PT-mediated hemagglutination of goose erythrocytes, but these antisera failed to show neutralization activity in the antitoxin CHO cell assay. In this study, we have located four linear B-cell neutralization epitopes, residues 78 to 98 of S2 and residues 9 to 23, 18 to 41 and 149 to 176 of S3. One possible explanation for this discrepancy would be that the antibodies generated from S2(78-98) recognize the variable residues within 78 to 98 of S2 and these residues are involved in CHO cell PT-receptor binding. The antisera raised against 91 to 106 of S2 in the Schmidt study react with the C-terminal half of the peptide (residues 98 to 106), which is in close enough proximity to the receptor binding site that it shows marginal neutralization activity. It is worthwhile to point out that the production of the different population of antibodies against these two peptides could be due to both the orientation and the method of conjugation, as shown in other studies (6, 10, 24). The natural cysteine (residue 87) was used in the present study, and an additional cysteine was added at the N-terminal end of peptide R91-106 used in the Schmidt study. Although the antisera raised against the N-terminal end of S3 in the Schmidt studies had no antitoxin activities in the CHO cell assays, their inhibition of PT-mediated hemagglutination pointed out that this region, especially residues 9 to 23, may be involved in the specific target cell binding; any antibodies against this site can directly block the PT-receptor binding. This is also consistent with the idea of a second distinct receptor binding site on the B-oligomer of PT.

Synthetic peptides, beside having well-defined chemical structures and being nontoxic, have another significant advantage over native toxin as an immunogen in that they quite

often induce functional antibody populations different from those obtained with the native toxin. This advantage is clearly demonstrated in the present study of the induction of PT-neutralizing antibodies against S2(78-98), S3(9-23), and S3(149-176) peptide-KLH conjugates. Preliminary evidence from experiments with synthetic peptides corresponding to the S4 and S5 subunits suggests that antitoxin neutralizing antibodies could be generated by immunizing rabbits with peptides or peptide-KLH conjugates.

Synthetic peptides corresponding to the S1 subunit of PT are capable of eliciting major histocompatibility complex class II restricted T-cell immune responses in both humans and mice (23). In the present study, we observed that all 12 synthetic peptides induced strong proliferative responses in the A/J strain T cells, but S3(18-41), S3(78-108), S3(134-154), and S3(149-176) were the only peptides capable of stimulating the proliferation of the PT-specific T cell of BALB/c mice. From these results we conclude that there are four immunodominant murine T-cell epitopes located within residues 18 to 41, 78 to 108, 134 to 154, and 148 to 176 of the S3 subunit. Although neither peptide S3(18-41) nor peptide S3(148-176) is identified as a T-cell epitope by the amphipathicity algorithm of Delisi and Berzofsky (9), they do contain the T-cell receptor binding motifs as described by Rothbard and Taylor (25) and the properties of a T-helper cell determinant as predicted by Stille et al. (32). On the other hand, peptides S3(78-108) and S3(134-154) were both predicted as T-cell epitopes by all three algorithms. This is further supported by our preliminary studies that BALB/c mice and rabbits immunized with these four individual unconjugated peptides in the presence of complete Freund's adjuvant elicit strong antibody response against S3 as judged by immunoblots, indicating that these regions of S3 contain potent functional T-helper cell epitopes.

Certainly, identification of these potent T-cell epitopes and surface-exposed B-cell neutralizing epitopes represents a first step towards the rational design of a synthetic whooping cough vaccine and provides the target amino acid residues for site-directed mutagenesis to generate detoxified PT mutants.

ACKNOWLEDGMENTS

We thank E. Wu, P. Fan, W. Li, M. Flood, W. Williams, and T. van den Elshout for their technical assistance, the Q.C. department of Connaught Laboratories Ltd. for performing the CHO cell antitoxin assay, and H. Boux and S. Wilson for having critically reviewed the manuscript.

REFERENCES

1. **Askelof, P., K. Rodmalm, G. Wrangsell, U. Larsson, S. B. Svenson, J. L. Cowell, A. Unden, and T. Bartfai.** 1990. Protective immunogenicity of two synthetic peptides selected from the amino acid sequence of *Bordetella pertussis* toxin subunit S1. Proc. Natl. Acad. Sci. USA **87**:1347–1351.
2. **Briand, J. P., S. Muller, and M. H. V. Van Regenmortel.** 1985. Synthetic peptides as antigens: pitfalls of conjugation methods. J. Immunol. Methods **78**:59–69.
3. **Burns, D. L., J. G. Kenimer, and C. R. Manclark.** 1987. Role of the A subunit of pertussis toxin in alteration of Chinese hamster ovary cell morphology. Infect. Immun. **55**:24–28.
4. **Capiau, C., J. Petre, J. Van Damme, M. Puype, and J. Vandekerckhove.** 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 identified S2 as the haptoglobin-binding subunit. FEBS Lett. **204**:336–340.
5. **Chong, P., and M. Klein.** 1989. Single-step purification of pertussis toxin and its subunits by heat-treated fetuin-Sepharose affinity chromatography. Biochem. Cell Biol. **67**:387–391.
6. **Chong, P., M. Sydor, E. Wu, G. Zobrist, H. Boux, and M. Klein.** 1991. Structural and functional analysis of the S1 subunit of pertussis toxin using synthetic peptides. Mol. Immunol. **28**:239–245.
7. **Chou, P. Y., and G. D. Fasman.** 1978. Empirical prediction of protein conformation. Annu. Rev. Biochem. **47**:251–276.
8. **Cieplak, W., W. N. Burnette, V. L. Mar, K. T. Raljot, C. F. Morris, K. K. Chen, H. Sato, and J. M. Keith.** 1988. Identification of a region in the S1 subunit of pertussis toxin that is required for enzymatic activity and that contributes to the formation of a neutralizing antigenic determinant. Proc. Natl. Acad. Sci. USA **85**:4667–4671.
9. **Delisi, C., and J. A. Berzofsky.** 1985. T-cell antigenic sites tend to be amphipathic structure. Proc. Natl. Acad. Sci. USA **82**:7048–7052.
10. **Dyrberg, T., and M. B. A. Oldstone.** 1986. Peptides as antigens: importance of orientation. J. Exp. Med. **164**:1344–1349.
11. **Geerligs, H. J., W. J. Weijer, W. Bloemhoff, G. W. Welling, and S. Welling-Wester.** 1988. The influence of pH and ionic strength on the coating of peptides of herpes simplex virus type 1 in an enzyme-linked immunosorbent assay. J. Immunol. Methods **106**:239–244.
12. **Gillenius, P., E. Jaattmaa, P. Askelof, M. Grandstrom, and M. Tiru.** 1985. The standardization of an assay for pertussis toxin and antitoxin in microplate culture of Chinese hamster ovary cells. J. Biol. Stand. **13**:61–67.
13. **Gordon, L., D. Stainer, S. Johnson, and E. Pearson.** 1986. Presented at the Workshop on Acellular Pertussis Vaccines, 22 to 24 September 1986, Washington, D.C.
14. **Hopp, T. P., and K. R. Woods.** 1981. Prediction of protein antigenic determinants from amino acid sequence. Proc. Natl. Acad. Sci. USA **78**:3824–3828.
15. **Iwasa, S., S. Ishida, S. Asakawa, and K. Akama.** 1985. A curious histamine-sensitizing activity shown by the newly developed Japanese acellular pertussis vaccine. Dev. Biol. Stand. **61**:453–460.
16. **Lang, A. B., M. T. Ganss, and S. J. Cryz, Jr.** 1989. Monoclonal antibodies that define neutralizing epitopes of pertussis toxin: conformational dependence and epitope mapping. Infect. Immun. **57**:2660–2665.
17. **Liu, F.-T., M. Zinnecker, T. Hamaoke, and D. H. Katz.** 1979. New procedure for preparation and isolation of conjugates of proteins and a synthetic copolymer of D-amino acids and immunochemical characterization of such conjugates. Biochemistry **18**:690–697.
18. **Locht, C., and J. M. Keith.** 1986. Pertussis toxin gene: nucleotide sequence and genetic organization. Science **232**:1258–1264.

18a. **Loosmore, S. M., et al.** Unpublished data.

19. **Loosmore, S. M., G. R. Zealey, H. A. Boux, S. A. Cockle, K. Radika, R. E. F. Fahim, G. J. Zobrist, R. K. Yacoob, P. C.-S. Chong, F.-Y. Yao, and M. H. Klein.** 1990. Engineering of genetically detoxified pertussis toxin analogs for development of a recombinant whooping cough vaccine. Infect. Immun. **58**:3653–3662.
20. **Manclark, C. R., and J. R. Cowell.** 1985. Pertussis. Bacterial Vaccine **3**:69–106.
21. **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.
22. **Nogimori, K., M. Tamura, T. Nakamura, M. Yajima, K. Ito, and M. Ui.** 1985. Dual mechanisms involved in the development of diverse biological activities of islet-activating protein, as revealed by chemical modification of the toxin molecule. Dev. Biol. Stand. **61**:51–61.
23. **Oksenberg, J. R., C. Ko, A. K. Judd, M. Lim, A. Kent, G. K. Schoolnik, and L. Steinman.** 1989. Multiple T and B cell epitopes in the S1 subunit ("A"-monomer) of the pertussis toxin molecule. J. Immunol. **143**:4227–4231.
24. **Rhodes, H., R. Houghten, J. P. Taulane, D. Carson, and J. Vaughan.** 1985. The immune response to Epstein-Barr nuclear

antigen: conformational and structural features of antibody binding to synthetic peptides. Mol. Immunol. **21**:1047.

25. **Rothbard, J. B., and W. R. Tayor.** 1988. A sequence pattern common to T cell epitopes. EMBO J. **7**:93–100.
26. **Sato, H., A. Ito, J. Chiba, and Y. Sato.** 1984. Monoclonal antibody against pertussis toxin: effect on toxin activity and pertussis infections. Infect. Immun. **46**:422–428.
27. **Sato, H., Y. Sato, A. Ito, and I. Ohishi.** 1987. Effect of monoclonal antibody to pertussis toxin on toxin activity. Infect. Immun. **55**:909–915.
28. **Schmidt, M. A., B. Rauoach, M. Szulczynski, and J. Marzillier.** 1991. Identification of linear B-cell determinants of pertussis toxin associated with the receptor recognition site of the S3 subunit. Infect. Immun. **59**:1402–1408.
29. **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.

29a. **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.

30. **Sia, D. Y., and J. L. Chou.** 1987. In vivo activated lymphoid cells (IVALC) affect the cloning efficiency of human T lymphocytes reactive to a soluble antigen, purified protein derivative. Scand. J. Immunol. **26**:683–690.
31. **Steward, M. W., and C. R. Howard.** 1987. Synthetic peptides: a next generation of vaccine? Immunol. Today **8**:57–58.
32. **Stille, C. J., L. J. Thomas, V. E. Reyes, and R. E. Humphreys.** 1987. Hydrophobic strip-of-helix algorithm for selection of T cell-presented peptides. Mol. Immunol. **24**:1021–1027.
33. **Tamura, M., K. Nogimori, 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**:5512–5516.
34. **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.
35. **Van Regenmortel, M. H. V., S. Muller, V. F. Quesniaux, D. Altchuh, and J. P. Briand.** 1988. Operational aspects of epitope identification: structural features of proteins recognized by antibodies, p. 113–122. *In* H. Kohler and P. T. LaVerde (ed.), Vaccines: new concepts and developments. Longman, London.
36. **Zealey, G., S. Loosmore, S. Cockle, H. Boux, K. Radika, R. Yacoob, P. Chong, F. L. Yao, and M. Klein.** 1989. Construction of B. pertussis strains that secrete inactive pertussis toxin analogs, p. 259–263. *In* R. M. Chanock and R. A. Lerner (ed.), Vaccine 89. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.