Site-specific Alterations in the B Oligomer that Affect Receptor-binding Activities and Mitogenicity of Pertussis Toxin

By Yves Lobet, Christiane Feron, Guy Dequesne, Etienne Simoen,* Pierre Hauser,* and Camille Locht‡

*From the Research Department and *Preclinical Development Department, SmithKline Beecham Biologicals, B-1330 Rixensart, Belgium; and ‡Laboratoire de Microbiologie Génétique et Moléculaire, Institut National de la Santé et de la Recherche Médicale CJF 9109, Institut Pasteur, F-59019 Lille, France*

Summary

Pertussis toxin plays a major role in the pathogenesis of whooping cough and is considered an important constituent of vaccines against this disease. It is composed of five different subunits associated in a molar ratio 1S1:1S2:1S3:2S4:1S5. The S1 subunit is responsible for the ADP-ribosyltransferase activity of the toxin. The B moiety, composed of S2 through S5, recognizes and binds to the target cell receptors and has some ADP-ribosyltransferase–independent activities such as mitogenicity. Site-directed mutagenesis of subunits S2 and S3 allowed us to identify amino acid residues involved in receptor binding. Of all the modifications generated, the deletion of Asn 105 in S2 and of Lys 105 in S3 resulted in the more drastic reduction of binding to haptoglobin and CHO cells, respectively. A holotoxin carrying both deletions presented a mitogenicity reduced to an undetectable level. The combination of these B oligomer mutations with two substitutions in the S1 subunit led to the production of a toxin analog with reduced ADP-ribosyltransferase–dependent and –independent activities including mitogenicity. As shown by immunoprecipitation with various monoclonal antibodies, the mutant holotoxin was correctly assembled and antigenically similar to the native toxin. This toxin analog induced toxin-neutralizing antibodies at the same level as the holotoxin carrying only mutations in the S1 subunit, and may therefore be considered a useful candidate for the development of a new generation vaccine against whooping cough.

Bordetella pertussis, the etiologic agent of whooping cough, produces a number of toxins responsible for the local and systemic manifestations of this important childhood disease (1). Among these toxins, pertussis toxin (PTX)[1] plays a major role in the pathogenesis, in that it expresses a wide variety of biological activities, such as histamine sensitization, activation of insulin secretion, promotion of lymphocytosis, and others (2). In addition, immunization with PTX has been shown to protect mice against subsequent lethal challenge with virulent *B. pertussis* (3). It is therefore considered an important constituent in vaccines against pertussis, and is the major component of the new acellular vaccines currently being tested or used in several countries (4, 5).

Paradoxically, this toxin may itself be responsible for the harmful side effects associated with the current vaccines (6). These side effects range in severity from minor local reactions and simple flushing, to permanent neurological damage and, in some rare cases, death. Before PTX can effectively and safely be used in pertussis vaccines, complete and irreversible detoxification is therefore required.

The toxin is composed of five different subunits, named S1 to S5, based on their decreasing molecular weights. The subunits associate in a molar ratio of 1S1:1S2:1S3:2S4:1S5 to form the holotoxin. Functionally, PTX can be divided into the A protomer, or S1 subunit, capable of expressing enzymatic ADP-ribosyltransferase activity, and the B moiety, responsible for the target cell receptor binding activities (7). The B oligomer is in turn composed of two dimers, D1 and D2, connected by subunit S5. Dimer D1 comprises subunits S2 and S4, and dimer D2 subunits S3 and S4 (7).

As for many bacterial protein toxins with an A-B structure, toxicity of PTX relies on three molecular steps. First, the toxin binds via its B moiety to the target cell receptors. Then, the enzymatically active A moiety is translocated through the target cell membrane. Finally, the A protomer expresses its ADP-ribosyltransferase activity using NAD as the ADP-ribose donor. Elimination of any of these steps should drastically reduce the biological activities of PTX. Altera-

[1] *Abbreviations used in this paper:* CRM, cross-reactive mutant; NAD, nicotinamide adenine dinucleotide; PTX, pertussis toxin; WT, wild type.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/93/01/0079/09 $2.00
Volume 177 January 1993 79–87

tions by site-specific mutagenesis of several critical residues in the active site of the S1 subunit (8–11) have indeed yielded PTX–cross-reactive mutant proteins (PTX-CRM) drastically affected in many of the biological activities (12–14). However, some biological activities are independent of the ADP-ribosyltransferase activity (15). Of concern is PTX-associated mitogenicity, one of the activities that are not reduced by inactivation of the S1 subunit (16). Since it is unclear which precise molecular activities especially cause the rare but severe side effects of current pertussis vaccines, it is desirable to abolish all measurable biological activities of the PTX-CRM molecules used in the future vaccines.

Whereas abolition of the ADP-ribosyltransferase activity, the last of the three molecular steps in cytotoxicity, only affects some toxin activities, reduction of all biological activities can be expected if the initial, B oligomer-mediated binding step of the toxin to the target cell receptors is abolished.

Although the receptors of PTX have not been identified yet, several studies have indicated that they are glycoproteins of variable sizes depending on the target cell (17–19). Witvliet et al. (18) showed that a 165-kD protein on the surface of CHO cells was preferentially recognized by dimer 2 (S3-S4), but not by dimer 1 (S2-S4). On the other hand, sialoglycoproteins haptoglobin and fetuin have been used as model systems for the interaction of PTX with at least one class of receptors. It has been shown, using an enzyme-linked immunosorbent assay, that dimer 1 but not dimer 2 is able to bind to haptoglobin (20, 21). Even the S2 subunit alone was able to bind to haptoglobin, albeit less efficiently than the S2-S4 dimer (21). Taken together, these results suggest that the receptor-binding sites of PTX are likely to involve subunits S2 and S3. The high degree of sequence similarity of these two subunits (22, 23) further suggests that the binding sites share some common features. On the other hand, since there appears to be some specificity in the interaction of the individual dimers to their respective receptors (18, 21), receptor binding activities most likely also involve some of the nonconserved residues.

In this communication, we describe the identification of amino acid residues involved in B oligomer activities, their site-directed modification, and the construction of an immunogenic vaccine candidate PTX-CRM devoid of cytotoxic and mitogenic activities.

Materials and Methods

Strains, Plasmids, and Culture Medium. *B. pertussis* BPRA, from which the *ptx* operon has been deleted, and plasmid pRIT13070, containing the entire *ptx* operon inserted into the EcoRI site of pUC7, were described by Antoine and Locht (24). *Escherichia coli* strain SM10 (25) was provided by S. Stibitz (Food and Drug Administration, Bethesda, MD). Broad-host-range plasmid pLAFRII (26) was provided by J. J. Mekalanos (Harvard University Medical School, Cambridge, MA). Phage φRIT20001 and plasmid pPTX42 were described in Locht et al. (11 and 27, respectively). Phage M13mp9 and plasmid pUC9 were purchased from Pharmacia LUB Biotechnology (Uppsala, Sweden). *E. coli* strains were grown in Luria-Bertani broth (28) supplemented with the appropriate antibiotics. *B. pertussis* strains were grown in flasks or in fermentors as described previously (24, 29).

DNA Manipulations. DNA manipulations were performed under standard conditions, as described by Maniatis et al. (28). DNA modifying enzymes were purchased from Boehringer Mannheim (Mannheim, Germany), Bethesda Research Laboratories (Bethesda, MD), Pharmacia LKB (Uppsala, Sweden), or New England Biolabs (Beverly, MA) and were used according to the suppliers' recommendations. DNA sequencing was performed with Sequenase (US Biochemical Corporation, Cleveland, OH) as recommended by the supplier.

Mutagenesis of the ptx Operon. All mutations in the *ptx* operon, except deletion of asparagine 105 of subunit S2, were performed with the Oligonucleotide-directed in vitro Mutagenesis System (Amersham International, Bucks, UK). To mutagenize the S1 subunit, we used phage φRIT20001 containing the coding region of the S1 subunit, and the oligonucleotides CCTCCGGCGGCAGGGAGTCATA and TGCCAGATAGCCGCTCTGGTAG to replace arginine 13 by a leucine residue (R13L) and glutamic acid 129 by a glycine residue (E129G), respectively. The mutations were subsequently introduced in plasmid pRIT13070, either by exchanging the AccI fragment (to yield pPTS1R13L) or the SalI/Sau3AI fragment (to yield pPTS1E129G) with their respective mutated sequences.

To mutagenize the S2 cistron, the 750-bp XbaI/AvaI fragment of pPTX42 was inserted into M13mp19 digested with XbaI and XmaI to yield phage φRIT20200. The deletion of the Tyr 102 codon was performed with the oligonucleotide CGTTGCTGTAGTGATCCGTT. The resulting phage, designated φRIT20201, was then used to specifically delete Tyr 103 in addition to Tyr 102 with the help of the synthetic oligonucleotide TGACGTTGCTGTGATCCGTT. The resulting phage was named φRIT20202. The 750-bp XbaI/SmaI fragment of pRIT13070 was then exchanged by the mutated XbaI/SmaI fragment from phages φRIT20201 and φRIT20202 to yield pPTΔS2Y102 and pPTΔS2Y102-103, respectively. To delete the Asn 105 codon from S2, the PCR technology was employed using the flanking oligonucleotides CGTTCTAGACCTGGCCCAGCCCCG and TGTCCCGGGGCGGTGGTTCGAGTG, that contain, respectively, a XbaI and a SmaI site, and the mutagenic and partially complementary (over 14 bp) synthetic oligonucleotides TGGCGGTGACGCTGTAGTAG and CAGCGTCACCGCCACTCGCCTGCTCTCCAG. After in vitro amplification, the DNA fragment was digested with XmaI and XbaI and ligated into the same sites in pRIT13070. The recombinant mutant plasmid was named pPTΔS2N105.

To perform mutagenesis of the S3 cistron, the 2510 bp XmaI/BamHI of pRIT13070 was first subcloned into pUC9, thereby yielding pPT3. Then, a 960-bp PstI fragment from pPTX42 carrying the coding region for the S3 subunit was subcloned into M13mp9 to yield phage φRIT20300. To delete Lys 10, Tyr 92, Lys 93, Tyr 102, Tyr 103, and Lys 105 of S3, the synthetic oligonucleotides TGAACAGTGCCGGCGGGATG, GCCCGGTCTTTATGGTCGTG, GTTGCCCGGTGTATATGGTC, CCTTGCTGTAGTGATCCGCA, TGACCTTGCTGTGATCCGCA, and TGGCCGTGACGCTGTAGTAG were used, respectively. After mutagenesis as described above, the 690-bp BglII/MluI fragment of pPT3 was exchanged with the corresponding mutated fragments from the M13mp9-derived phages. The mutated 1750-bp BglII/BamHI segment from the resulting recombinant plasmids were then exchanged with the BglII/BamHI region of pRIT13070. The final recombinant plasmids containing the desired mutations in the S3 cistron of the PTX operon were named pPTΔS3K10, pPTΔS3Y92, pPTΔS3K93, pPTΔS3Y102, pPTΔS3Y102–103, and pPTΔS3K105, respectively.

All the mutagenized inserts were sequenced to insure that the correct mutations had occurred and that no unwanted mutations were introduced into the exchanged fragments.

To create pPT-RE, the two mutations in the S1 cistron were combined by exchanging the SalI/XbaI fragment of pPTS1R13L with the mutant SalI/XbaI region of pPTS1E129G. The deletions of residue 105 in S2 and in S3 were combined to yield plasmid pPT-NK by replacing the 1591 bp MluI fragment of pPTΔS2N105 with the mutagenized fragment from pPTΔS3K105. Finally, pPT-RENK was constructed by exchanging the 887-bp KpnI/XbaI fragment of pPT-NK for the same fragment from pPT-RE.

Expression of the Mutated ptx Genes in B. pertussis. The 4.7-kb EcoRI fragments of the pRIT13070-derived plasmids containing the described mutations in the S2 or S3 cistrons were inserted into the EcoRI site of the broad host range plasmid pLAFRII. The recombinant plasmids were introduced into *B. pertussis* strain BPRA by conjugation as described before (24) using *E. coli* SM10 as a donor strain. Recombinant *B. pertussis* were grown in modified Stainer Scholte medium (30) containing cyclodextrin (24) and tetracycline. After growth, the cells were separated from the culture medium by centrifugation and the supernatant was used directly in ELISA and CHO cell clustering assays.

Alternatively, plasmids pPT-RE, pPT-NK, or pPT-RENK were introduced into *B. pertussis* strain BPRA by electroporation (31). The transformed cells were plated on Bordet-Gengou medium containing ampicillin (24). As these pUC-derived plasmids are unable to replicate in *B. pertussis*, the resulting colonies were expected to contain a copy of the plasmids integrated into the chromosome via a single recombination event. The recombinant strains were named respectively BPRA/RE, BPRA/NK, and BPRA/RENK. After growth of these strains in a 10-liter fermentor, PTX or the PTX-CRMs were adsorbed on hydroxylapatite (HA-ultrogel) and, upon elution, further purified by hydrophobic interaction chromatography (Butyl TSK), dye-ligand chromatography (Trisacryl blue), and gel filtration (Sephacryl S-200 HR) (29).

Immunoprecipitation. PTX (500 ng) were incubated for 2 h at room temperature with 5 μg of various mAbs in 250 μl PBS containing 0.1% Tween 20. 50 μl of suspension of Immunobeads rabbit anti-mouse Ig (H + L) (Bio-Rad Laboratories, Richmond, CA) were added and the mixture was incubated overnight at 4°C. The beads were then collected by centrifugation, resuspended in loading buffer, and submitted to SDS-PAGE (32). Upon separation, the proteins were transferred onto nitrocellulose membranes, and the PTX subunits were detected with mAbs (21) or by a mixture of biotinylated rabbit and goat anti-PTX polyclonal antibodies. These polyclonal antibodies were obtained from animals hyperimmunized with formaldehyde-detoxified PTX.

Analytical Procedures. ELISA using polyclonal antibodies or mAbs and haptoglobin-binding assays were carried out as described before (24). CHO cell cytotoxicity of PTX, PTX-CRMs, or the various *B. pertussis* culture supernatants was estimated as described by Hewlett et al. (33). To measure the inhibition of CHO cell activity of PTX by mAbs, serial dilutions of ascitic fluids containing the antibodies were added to 1.5 μg of PTX. The mixture was incubated for 3 h at 37°C before addition to the CHO cells. A mAb is considered neutralizing when the corresponding ascitic fluid is able to inhibit the CHO cell-clustering activity of PTX at a dilution equal or superior to 160.

Nicotinamide adenine dinucleotide (NAD)-glycohydrolase assays were performed as described by Locht et al. (34). The inhibition of the NAD-glycohydrolase activity by mAbs was evaluated by preincubating the 0.2 μg of the S1 subunit to be introduced in the test with 1 μg of purified antibody for 30 min at room temperature. In the inhibition assay, we used a recombinant S1 subunit carrying the mutation Cys41→Ser (35). Using this molecule, the NAD-glycohydrolase assay is performed in the absence of reducing agent. A mAb is considered neutralizing when 1 μg of purified antibody is able to reduce the NAD glycohydrolase activity of 0.2 μg of S1 subunit by at least a fourfold factor.

IL-2 Secretion. Freshly prepared spleen cells from BALB/c mice were suspended at a final density of 10^6 cells/ml in complete medium (RPMI 1640 medium supplemented with 1% normal mice serum, 1% L-glutamin (043-05030H), 1% penicillin-streptomycin (043-01350D), and 1% β-ME (043-05140H) all from Gibco Laboratories (Grand Island, NY). 100 μl of this suspension was distributed in each well of a 96-well plate. To these wells, 100 μl of PTX solutions at various concentrations were added, and the plates were incubated for 24 h at 37°C. 100 μl of supernatant were then harvested, transferred to new microtiter plates, and frozen for at least 24 h to kill surviving cells. The concentration of IL-2 present in these supernatants was evaluated by adding to each well 10^4 freshly washed VDA2 cells suspended in 100 μl of complete medium containing 1% pyruvate, 1% nonessential amino acids, and 10% FCS. After a 24-h incubation, 1 μCi [^{3}H]thymidine (TRA120; Amersham Corp.) was added. 16–18 h later, the cells were harvested and the amount of internalized radioactivity was determined by liquid scintillation. The stimulation index was calculated as the ratio of the radioactivity incorporated in the presence of toxin to the radioactivity incorporated in the absence of toxin.

Immunogenicity. Groups of 10 mice (OF1; IFFA Credo, Lyon, France) were immunized subcutaneously with 1 μg of PTX-CRM adsorbed on aluminium hydroxide. 3 wk later, the animals were bled and given a second dose of 1 μg of PTX-CRM as previously. 1 wk after the booster dose, total bleeding was performed and individual sera were tested by ELISA to determine the titer of antitoxin antibodies. Titers were expressed in arbitrary ELISA units by comparison with a mouse reference serum. Pooled sera were used to evaluate the CHO neutralizing antibody titers.

Results

Rationale for the Design of B Oligomer Mutations. Nogimori et al. (36) have previously shown that chemical modification of lysine residues abolishes several but not all biological activities of PTX. The activities that are affected by the modification of lysines are those that involve dimer 2. Therefore, lysine residues in the S3 subunit not conserved in S2 may be located at its receptor-binding site. Three nonconserved lysine residues can be found in the sequence of the S3 subunit: Lys 10, Lys 93, and Lys 105 (22, 23). They are replaced by glutamine, asparagine, and asparagine, respectively, in the S2 subunit. It is therefore possible that one or several of these three lysine residues are involved in D2-specific binding of PTX.

Iodination of PTX severely reduces biological activities, such as hemagglutination and CHO cell cytotoxicity. Fetuin coupled to agarose can prevent inactivation of the toxin by iodination (37). Since iodination usually affects tyrosine residues, it is possible that tyrosine residues, conserved in S2 and S3, are close to their receptor-binding sites. Of the many tyrosines in S2 and S3, all but two in S3 are at conserved positions. Two of them (Tyr 102, and Tyr 103) are in close proximity to Lys 105 in S3, and one of them (Tyr 92) is close to Lys 93 in S3. This led us to examine the importance of Tyr 102, Tyr 103, and Asn 105 in S2, and Lys 10, Lys 93, Tyr 102, Tyr 103, and Lys 105 in S3 in B oligomer activities.

Table 1. *Haptoglobin-binding of Culture Supernatants of B. pertussis Strains Producing PTX or PTX-CRM*

	Haptoglobin–binding (percent residual binding)*
WT‡	100
S2:Y102Δ	50
S2:Y102/103ΔΔ	4
S2:N105Δ	<1
S3:Y102Δ	144
S3:K105Δ	102

* Calculated as 100 × (concentration of PTX evaluated in dimer 1-specific ELISA/total concentration of PTX).
‡ *B. pertussis* strain BPRA containing pLAFRII with either the wild-type *ptx* gene (WT) or mutant *ptx* genes resulting in the indicated alterations of the S2 or S3 subunit.

Activities of the PTX-CRM. To identify PTX-CRM which possess altered haptoglobin-binding activities, Tyr 102, Tyr 103, or Asn 105 in the S2 subunit were deleted by site-directed mutagenesis of the S2 gene, and each of the three mutant genes (Y102Δ, Y102Δ/Y103Δ, and N105Δ) was introduced into BPRA on a pLAFRII-derived plasmid. The resultant recombinant BPRA strains were then analyzed for the expression of the mutant *ptx* genes by ELISA using culture supernatants. All strains produced and secreted PTX-CRM, indicating that none of the mutations abolished expression, assembly, or secretion (data not shown). When haptoglobin was used in the model ELISA for dimer 1-specific binding, drastic differences in the haptoglobin-binding were observed among the different PTX-CRM analyzed (Table 1). Deletion of Tyr 102 only reduced haptoglobin-binding by about 50%. Deletion of both Tyr 102 and Tyr 103 reduced binding to 4% of residual activity. Deletion of Asn 105 resulted in no detectable haptoglobin-binding, and therefore to <1% residual activity. No effect on the haptoglobin-binding capacity was observed when Glu 129 of the S1 subunit was modified (not shown). In addition, the deletion of Tyr 102 or Lys 105 in the S3 subunit also had no effect on haptoglobin-binding. Therefore, haptoglobin-binding specifically involves the Asn 105 residue of the S2 subunit.

Table 2. *CHO-Cell-clustering Activity of Culture Supernatants of B. pertussis Strains Producing PTX or PTX-CRM*

	CHO cell clustering activity (dil)*
WT‡	64
S3:K10Δ	2
S3:Y92Δ	16
S3:K93Δ	4
S3:Y102Δ	64
S3:Y102/103ΔΔ	4
S3:K105Δ	<2

* Expressed as the reciprocal of the highest dilution at which clustering is observed.
‡ *B. pertussis* strain BPRA containing pLAFRII with either the wild-type *ptx* gene (WT) or mutant *ptx* genes resulting in the indicated alterations of the S2 or S3 subunit.

Since D2 was reported to specifically bind to CHO cell membrane receptors (18), several site-directed mutations were introduced in the S3 subunit gene and the resulting mutant proteins analyzed for their residual CHO cell cytotoxicity. As shown in Table 2, deletion of neither Tyr 92 nor of Tyr 102 had any significant effect on the CHO cell cytotoxicity. In contrast, CHO cell cytotoxicity was greatly reduced but detectable in PTX-CRM carrying the deletion of Lys 10, Lys 93 and the double deletion of Tyr 102 and Tyr 103 in the S3 subunit. No cytotoxicity was observed with PTX-CRM in which Lys 105 of S3 was deleted. ELISA using mAbs against individual subunits indicated that none of the described mutations affected the stoichiometry of the individual subunits, suggesting the structural integrity of correctly assembled PTX-CRM (data not shown).

These results indicate that asparagine 105 of subunit S2 and the lysine residues 10, 93, and especially 105 in S3 are involved in the biological activities of PTX, probably through their specific interactions with the toxin receptor. We chose the deletion of the two residues at position 105 for further analysis.

Construction of B. pertussis Strains BPRA-NK, BPRA-RE, and BPRA-RENK. To further investigate the effect of the deletion of residues 105 in S2 and S3 on the activities of PTX, both mutations were combined in the same *ptx* gene and introduced into the *B. pertussis* BPRA chromosome by homologous recombination. Both mutations were introduced into pRIT13070 to yield pPT-NK as described in Materials and Methods. This plasmid was then introduced into the PTX-deficient *B. pertussis* BPRA strain by electroporation. Integration of pPT-NK into the *B. pertussis* BPRA chromosome was obtained by homologous recombination involving the flanking regions of the *ptx* gene. In a similar way, PTX-CRM were constructed containing alterations in the S1 subunit to compare them with the B oligomer mutants with respect to the various biological activities. Substitutions of both Arg 13 and Glu 129 by, respectively, Leu and Gly were chosen because these changes affect either NAD binding or catalysis (9, 11, and Antoine, R., A. Tallett, S. van Heyningen, and C. Locht, manuscript in preparation). A combination of two mutations affecting the enzymatic activity of PTX results generally in undetectable levels of biological activities that are dependent of ADP-ribosyltransferase activity of the toxin (12–14). The *B. pertussis* strain containing these S1 mutations was named BPRA-RE. Finally, we combined all four mutations (mutations R13L and E129G in S1, deletions N105Δ and K105Δ in S2 and S3, respectively) in a single strain named BPRA-RENK.

Western blot and ELISA analyses on the culture supernatants indicated that the resulting strains were able to produce and secrete PTX-CRM, reactive with anti-PTX mAbs specific

Table 3. *Immunoprecipitation of PTX and PTX-RENK with Anti-PTX mAbs*

Antibody	(ELISA reactivity)	Immunoprecipitation	
		PTX	PT-RENK
5C2	(S1)*	+‡‡	+
64A3	(S1)*	+	+
85B4	(S1)*‡	+	+
F18C6	(D2)§	ND	+
27F9	(D1/D2)‖	+	+
86E1	(D1/D2)	+	+
F6D1	(D1)¶	ND	+
59F4	(D2)**	ND	+

* Recognizes S1 in Western blot.
‡ Neutralizes CHO cell clustering and NAD glycohydrolase activities of PTX.
§ Recognizes S3 in Western blot; neutralizes the CHO cell-clustering activity of PTX; and reduces the haptoglobin binding of PTX.
‖ Recognizes S4 weakly in Western blot.
¶ Neutralizes both the CHO cell-clustering activity and the haptoglobin binding of PTX.
** Neutralizes the CHO cell-clustering activity of PTX.
‡‡ Upon immunoprecipitation, all PTX subunits are detected on Western blot.

for S1 or the B oligomer (not shown). To verify its correct assembly, we immunoprecipitated PTX-RENK in parallel with wild-type PTX using mAbs recognizing the different subunits (Table 3). The results showed that all of the tested antibodies, directed either against S1 or the B oligomer, and able to inhibit the activities of PTX, such as NAD glycohydrolase, CHO cell cytotoxicity, and haptoglobin binding, were also able to recognize and precipitate both holotoxins. Taken together, these results indicated that the mutations in S1, S2, and S3 and their combination do not abolish production, assembly, and secretion of the PTX-CRM. The *B. pertussis* strains BPRA-RE, BPRA-NK, and BPRA-RENK were subsequently grown in fermentors and the PTX-CRM produced and secreted by these strains were purified near homogeneity.

Table 4. *NAD Glycohydrolase and CHO Cell-Clustering Activities of Purified PTX and PTX-CRM*

	NAD glycohydrolase (pmole min^{-1} μg PT^{-1})	CHO Cell Clustering (percent residual toxicity)*
PTX	0.36	100
PTX-RE	<0.03	≤0.0013
PTX-NK	0.26	7.5
PTX-RENK	ND	<0.0020

* Expressed as 100 × (highest dilution of tested PTX inducing no clustering/highest dilution of wild-type PTX inducing no clustering).

Activities of the Purified PTX-CRM. The enzymatic activities of the purified PTX-CRM were analyzed and compared with those of the wild-type holotoxin. As shown in Table 4, PTX-NK expressed NAD glycohydrolase activity at a level similar to purified PTX, whereas no significant activity was expressed by PTX-RE up to a concentration of 20 μg/ml, representing a reduction in activity of at least 10,000-fold. These results are consistent with the effect of the S1 alterations in isolated S1 analogs produced in *E. coli* (8–11) and confirm that the mutations in the B oligomer do not affect the enzymatic activities of S1.

The CHO cell-clustering assays showed that all three mutant holotoxins expressed diminished cytotoxic activity. As expected and consistent with previous results (12–14), no cytotoxic activity could be measured with PTX-RE and PTX-RENK, because of the effect of the S1 mutations on the enzymatic activities. PTX-NK showed low but detectable (7.5% of wild-type level) CHO cell-clustering activity (Table 4). The decrease of cytotoxicity observed with PTX-NK was independent of the enzymatic activity, supporting the implication of the B oligomer mutations in the receptor-binding activity of the toxin as the cause of the diminished toxicity.

To investigate whether the alterations in the B oligomer also affect the mitogenic activity of PTX for T cells, we compared the effect of the mutant holotoxins and of the wild-type PTX on spleen cells from naive mice. To specifically evaluate the effect on T cells, we measured the IL-2 secretion in the culture supernatant instead of proliferation. As shown in Fig. 1, no IL-2 secretion was detected for PTX-RENK and PTX-NK at all concentrations tested, whereas 1–3 μg/ml of PTX or PTX-RE clearly induced IL-2 secretion. This showed that the reduction of the binding properties of PTX-CRM also affects the induction of IL-2 secretion, and consequently its mitogenicity for T cells.

Immunogenicity of PT-RE and PT-RENK. It was interesting to investigate whether the reduction of the B oligomer-specific activities also reduces the immunogenicity of PTX-RENK. We therefore compared its immunogenicity to that of PTX-RE

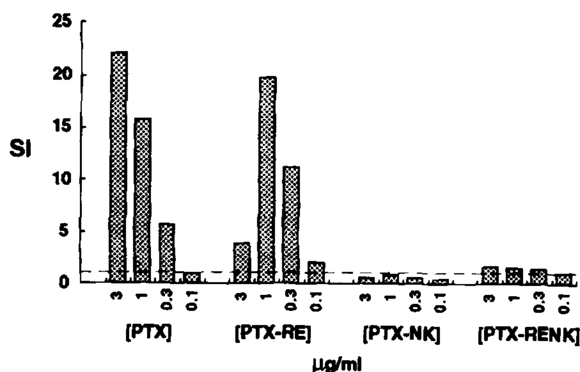

Figure 1. IL-2 secretion by mice spleen cells induced by various concentrations of PTX analogs. BALB/c spleen cells were incubated with indicated concentrations of various PTX-CRM for 24 h at 37°C. The IL-2 concentration in the culture supernatant was measured in a proliferation assay using VDA2 cells. The results are expressed as stimulation index (SI). (*Dotted line*) SI value of 1.

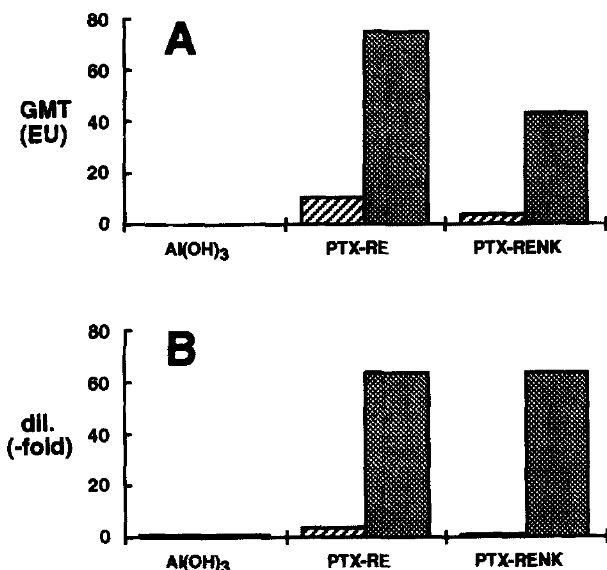

Figure 2. Immunogenicity of PTX-RE and PTX-RENK. Groups of 10 mice were immunized twice subcutaneously with 1 μg of PTX-CRM with a 3-wk interval. Sera were taken at the time of the injection of the second dose (▨) and 1 wk later (▩). (*A*) Total anti-PTX response expressed as the geometric mean titer of the individual sera. (*B*) CHO cell assay neutralization titers are shown as reciprocal of the highest dilution resulting in 100% inhibition in a CHO cell-clustering assay.

in outbred mice (Fig. 2). We did not include PTX-NK in this comparison because of its too high residual toxicity. 1 wk after the second immunization, no significant difference in the production of neutralizing antibodies could be observed between the two groups, and only slight differences were detected for total anti-PTX antibody titers, indicating that the B oligomer mutations do not significantly affect the immunogenicity of PTX-RENK.

Discussion

Specific binding to its receptors represents the initial step in the pathophysiologic action of a bacterial toxin. PTX, one of the most complex bacterial toxins known to date, contains several receptor-binding sites. Although the receptors for PTX have not yet been identified, several glycoproteins on cell surfaces and host tissues, as well as some serum glycoproteins, such as haptoglobin and fetuin, have been demonstrated to bind to PTX. The carbohydrate portion of these proteins appears to be essential for their interaction with PTX. Some of these glycoproteins are known to contain terminal sialyllactosamines which are important for toxin binding, since their removal abolishes binding to the toxin (17, 38). The nature of both the polypeptide and carbohydrate portions may vary among the various PTX-receptors, and this variation is responsible for the dimer 1- or dimer 2-specific binding of PTX (18). In this study, we show that Asn 105 of the S2 subunit is important for binding of the toxin to sialoglycoproteins such as haptoglobin, whereas the analogous Lys 105 of the S3 subunit is involved in the interaction with CHO cells. These results are in good agreement with previous observations showing that dimer 1 (S2-S4) preferably binds to haptoglobin (20, 21), and dimer 2 (S3-S4) to the CHO cell membrane receptor (18). Together, these observations suggest that haptoglobin-binding involves S2, and CHO cell receptor binding involves S3, and that Asn 105 and Lys 105, respectively, are important residues in these specific interactions.

Given the importance of the negatively charged sialic acid residues in PTX binding, it is tempting to speculate that the positively charged Lys 105 of S3, and the strongly polar Asn 105 of S2 may interact with the terminal sialyl groups on the receptor molecules. Consistent with this hypothesis is that sialyllactose markedly inhibits dimer 2 binding, but has less effect on dimer 1 binding (18), the electrostatic interactions being weaker. While this paper was in preparation, Saukkonen et al. (39) identified carbohydrate recognition domains on the S2 and S3 subunits produced in *E. coli* at the NH_2-terminal half of the subunits, some of which are reasonably close to residues 105 in the primary sequence. The amino acid residues interacting with the sugar moieties would be expected to lie in close proximity to those interacting with the sialyl groups. It is interesting that alterations of Lys 10 or Lys 93 of the S3 subunit, both located in the carbohydrate recognition domains proposed by Saukkonen et al. (39) also resulted in a decrease of CHO cell cytotoxicity, albeit to a lesser extent than the alteration of Lys 105 (Table 2).

Antibodies directed against various S2- or S3-specific peptides showed that those reacting with peptides corresponding to the regions located in the vicinity of residue 105 have the highest toxin-neutralizing activities, presumably because of their interaction with receptor binding (40). On the other hand, most toxin-neutralizing mAbs reactive with the B oligomer, including those presented in this study, appear to recognize conformational epitopes (41, 42), suggesting that the receptor-binding of PTX depends on the conformation of the B oligomer. The PTX-CRM presented in this study reacted with a panel of mAbs, most of which recognize conformational epitopes, including the neutralizing antibodies reactive with the B oligomer. As determined by immunoprecipitation, the antigenicity of PTX-RENK was indistinguishable from that of PTX-RE, indicating that the alterations in the B oligomer did not affect the conformation in an important way. This observation also suggests that the residues 105 may not be exposed at the surface of the toxin, but rather lie within a canyon or a pocket in the S2 and S3 subunits, similar to what has been proposed for the receptor-binding sites of many viruses (43).

Although many biological activities of PTX depend on its S1-catalyzed ADP-ribosyltransferase activity, some, such as mitogenicity, are independent of this enzymatic activity (15). In fact, reduction in enzymatic activity may sometimes result in increased mitogenic activity (16). The PTX-CRM with impaired receptor-binding abilities, however, presents an undetectable level of T cell mitogenicity, as indicated by its inability to stimulate IL-2 secretion by spleen cells exposed to the holotoxin, regardless of the toxin's ability to catalyze ADP-ribosylation. This demonstrates that reduction in receptor-binding not only reduces the ADP-ribosyltransferase–

dependent, but also the ADP-ribosyltransferase–independent biological activities of PTX. It is interesting that toxin molecules used in current acellular vaccine formulations are often inactivated by treatments with formaldehyde or glutaraldehyde (4, 44), chemicals that primarily affect lysine residues in proteins. Since the S1 subunit does not contain lysines, the chemical treatment is likely to affect the B oligomer-specific activities.

In vivo, PTX-stimulated IL-2 secretion may have adjuvant effects (45). As shown in Fig. 2, immunogenicity of PTX-RENK was slightly lower than that of PTX-RE with respect to the production of total anti-PTX antibodies, but no difference was observed between the two PTX-CRM when toxin-neutralizing antibodies were compared. This shows that if the B oligomer alterations affecting IL-2-secretion induced slightly decreased total anti-PTX antibodies, they did not significantly affect the production of physiologically important anti-PTX antibodies in the mouse.

The identification and subsequent alteration of the receptor-binding sites on toxins is expected to help in the design of genetically detoxified molecules devoid of any detectable activity but structurally nearly identical to the original molecule. This approach is particularly useful for the development of new generation vaccines against infectious diseases mediated by bacterial toxins. Pertussis or whooping cough is such a toxin-mediated disease against which a new generation vaccine is highly desirable. A recent important clinical trial confirmed that PTX, either alone or in conjunction with other *B. pertussis* antigens, was highly protective in children, and should therefore be included in the new vaccines against pertussis (5). However, complete detoxification of this molecule would be an absolute prerequisite before a PTX-based vaccine can be routinely used. The genetic modifications of the PTX gene presented in this study clearly demonstrate that alterations of the receptor-binding sites strongly affect the biological activities of PTX. The reported alterations not only affected the activities that are dependent on the S1 subunit-catalyzed ADP-ribosylation, but also those that are independent of this enzymatic activity, in particular, the PTX-mediated mitogenicity. Despite the reduction of both ADP-ribosyltransferase–dependent and –independent activities, the PTX-RENK was able to elicit high titers of toxin-neutralizing antibodies, and therefore a PTX-CRM with alterations in the S1 subunit as well as in the B oligomer, such as the one described here, may be considered a useful candidate for the development of a new generation vaccine against whooping cough.

We thank N. Pepin and C. Troonbeeckx for technical assistance, M. Francotte and S. Min for characterizing the mAbs; P. Desmons and M. Van Handenhove for the purification of PTX and the PTX-CRMs. We also thank J. Cohen and T. Cabezòn for the critical reading of the manuscript. The oligonucleotides were synthesized by B. Colau.

This work was supported in part by a grant of the Walloon Region.

Address correspondence to Dr. Yves Lobet, Research Department, SmithKline Beecham Biologicals, Rue de l'Institut 89, B-1330 Rixensart, Belgium.

Received for publication 5 October 1992.

References

1. Weiss, A.A., and E.L. Hewlett. 1986. Virulence factors of *Bordetella pertussis*. *Annu. Rev. Microbiol.* 40:661.
2. Munoz, J.J., H. Arai, R.K. Bergman, and P.L. Sadowski. 1981. Biological activities of crystalline pertussigen from *Bordetella pertussis*. *Infect. Immun.* 33:820.
3. Munoz, J.J., H. Arai, and R.L. Cole. 1981. Mouse-protecting and histamine-sensitizing activities of pertussigen and fimbrial hemagglutinin from *Bordetella pertussis*. *Infect. Immun.* 32:243.
4. Sato, Y., M. Kimura, and H. Fukumi. 1984. Development of a pertussis component vaccine in Japan. *Lancet.* i:124.
5. *Ad hoc* group for the study of pertussis vaccines. 1988. Placebo-controlled trial of two acellular pertussis vaccines in Sweden-protective efficacy and adverse events. *Lancet.* ii:955.
6. 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.
7. Tamura, M., K. Nogimori, S. Murai, M. Yajima, K. Ito, T. Katada, M. Ui, and S. Ishii. 1982. A role of the B-oligomer moiety of islet-activating protein, pertussis toxin, in development of the biological effects on intact cells. *Biochemistry.* 21:5516.
8. Barbieri, J.T., and G. Cortina. 1988. ADP-ribosyltransferase mutations in the catalytic S-1 subunit of pertussis toxin. *Infect. Immun.* 56:1934.
9. Pizza, M., A. Bartolini, A. Prugnola, S. Silvestri, and R. Rappuoli. 1988. Subunit S1 of pertussis toxin: mapping of the regions essential for ADP-ribosyltransferase activity. *Proc. Natl. Acad. Sci. USA.* 85:7521.
10. Burnette, W.N., W. Cieplak, V.L. Mar, K.T. Kaljot, H. Sato, and J.M. Keith. 1988. Pertussis toxin S1 mutant with reduced enzyme activity and a conserved protective epitope. *Science (Wash. DC).* 242:72.
11. Locht, C., C. Capiau, and C. Feron. 1989. Identification of

amino acid residues essential for the enzymatic activities of pertussis toxin. *Proc. Natl. Acad. Sci. USA.* 86:3075.

12. Pizza, M., A. Covacci, A. Bartoloni, M. Perugini, L. Nencioni, M.T. De Magistris, L. Villa, D. Nucci, R. Manetti, M. Bugnoli, et al. 1989. Mutants of pertussis toxin suitable for vaccine development. *Science (Wash. DC).* 246:497.
13. Loosemore, 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.-L. 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.
14. Locht, C., R. Antoine, C. Feron, G. Dequesne, M. Francotte, and M. De Wilde. 1990. Cross-reactive pertussis toxin mutant proteins containing alterations in the S1 subunit. *Vaccines (Cold Spring Harbor).* 90:435.
15. Ui, M. 1988. The multiple biological activities of pertussis toxin. *In* Pathogenesis and Immunity in Pertussis. A.C. Wardlaw and R. Parton, editors. John Wiley & Sons Ltd., Chichester, UK. 121–145.
16. Kimura, A., K.T. Mountzouros, P.A. Schad, W. Cieplak, and J.L. Cowell. 1990. Pertussis toxin analog with reduced enzymatic and biological activities is a protective immunogen. *Infect. Immun.* 58:3337.
17. 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.
18. Witvliet, M.H., D.L. Burns, M.J. Brennan, J.T. Poolman, and C.R. Manclark. 1989. Binding of pertussis toxin to eucaryotic cells and glycoproteins. *Infect. Immun.* 57:3324.
19. Clark, C.G., and G.D. Armstrong. 1990. Lymphocyte receptors for pertussis toxin. *Infect. Immun.* 58:3840.
20. 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 cholera* and *Escherichia coli* and identifies S2 as the haptoglobin-binding subunit. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 204:336.
21. Francotte, M., C. Locht, C. Feron, C. Capiau, and M. De Wilde. 1989. Monoclonal antibodies specific for pertussis toxin subunits and identification of the haptoglobin-binding site. *Vaccines (Cold Spring Harbor).* 89:243.
22. Locht, C., and J.M. Keith. 1986. Pertussis toxin gene: nucleotide sequence and genetic organization. *Science (Wash. DC).* 232:1258.
23. 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.
24. Antoine, R., and C. Locht. 1990. Roles of the disulfide bond and the carboxy-terminal region of the S1 subunit in the assembly and biosynthesis of pertussis toxin. *Infect. Immun.* 58:1518.
25. Simon, R., U. Priefer, and A. Puhler. 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. *BioTechnology.* 1:784.
26. Friedman, A.M., S.R. Long, S.E. Brown, W.J. Bulkema, and F.M. Ausubel. 1982. Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. *Gene.* 18:289.
27. Locht, C., P.A. Barstad, J.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.
28. Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 546 pp.
29. Ruuskanen, O., A. Noel, A. Putto-Laurila, J. Petre, C. Capiau, A. Delem, D. Vandevoorde, E. Simoen, D.E. Teuwen, H. Bogaerts, and F.E. André. 1991. Development of an acellular pertussis vaccine and its administration as booster in healthy adults. *Vaccine.* 9:117.
30. Stainer, D.W., and M.J. Scholte. 1971. A simple chemically defined medium for the production of phase I *Bordetella pertussis*. *J. Gen. Microbiol.* 63:211.
31. Goyard, S., J. Pidoux, and A. Ullman. 1991. An *Escherichia coli* insertion element (IS2) provides a functional promoter in *Bordetella pertussis*. *Res. Microbiol.* 142:633.
32. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680.
33. Hewlett, E.L., K.T. Sauer, G.A. Myers, J.L. Cowell, and R.L. Guerrant. 1983. Induction of a novel morphological response in Chinese hamster ovary cells by pertussis toxin. *Infect. Immun.* 40:1198.
34. Locht, C., W. Cieplak, K.S. Marchitto, H. Sato, and J.M. Keith. 1987. Activities of complete and truncated forms of pertussis toxin subunits S1 and S2 synthesized by *Escherichia coli*. *Infect. Immun.* 55:2546.
35. Locht, C., Y. Lobet, C. Feron, W. Cieplak, and J.M. Keith. 1990. The role of cysteine 41 in the enzymatic activities of the pertussis toxin S1 subunit as investigated by site-directed mutagenesis. *J. Biol. Chem.* 265:4552.
36. Nogimori, K., M. Tamura, M. Yajima, N. Hashimura, S.-I. Ishii, and M. Ui. 1986. Structure-function relationship of islet-activating protein, pertussis toxin: biological activities of hybrid toxins reconstituted from native and methylated subunits. *Biochemistry.* 25:1355.
37. Armstrong, G.D., and M.S. Peppler. 1987. Maintenance of biological activity of pertussis toxin radioiodinated while bound to fetuin-agarose. *Infect. Immun.* 55:1294.
38. Armstrong, G.D., L.A. Howard, and M.S. Peppler. 1988. Use of galactosyltransferases to restore pertussis toxin receptor activity to asialogalactofetuin. *J. Biol. Chem.* 263:8677.
39. Saukkonen, K., W.N. Burnette, V.L. Mar, H.R. Masure, and E.I. Tuomanen. 1992. Pertussis toxin has eukaryotic-like carbohydrate recognition domains. *Proc. Natl. Acad. Sci. USA.* 89:118.
40. Walker, M.J., J. Wehland, K.N. Timmis, B. Raupach, and M.A. Schmidt. 1991. Characterization of murine monoclonal antibodies that recognize defined epitopes of pertussis toxin and neutralize its toxic effect on Chinese hamster ovary cells. *Infect. Immun.* 59:4249.
41. 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.
42. Sato, H., and Y. Sato. 1990. Protective activities in mice of monoclonal antibodies against pertussis toxin. *Infect. Immun.* 58:3369.
43. Lentz, T.L. 1990. The recognition event between virus and host cell receptor: a target for antiviral agents. *J. Gen. Virol.* 71:751.
44. Gupta, R.K., S.N. Saxena, S.B. Sharma, and S. Ahuja. 1988.

Studies on the optimal conditions for inactivation of *Bordetella pertussis* organisms with glutaraldehyde for preparation of a safe and potent pertussis vaccine. *Vaccine.* 6:491.
45. Weinberg, A., and T.C. Merigan. 1988. Recombinant interleukin 2 as an adjuvant for vaccine-induced protection. Immunization of guinea pigs with herpes simplex virus subunit vaccines. *J. Immunol.* 140:294.