RESEARCH

GENE REPLACEMENT IN *BORDETELLA PERTUSSIS* BY TRANSFORMATION WITH LINEAR DNA

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We replaced the wild-type *TOX* operon of *Bordetella pertussis* with *in vitro* mutated, detoxified alleles by electroporetic transformation using unmarked linear DNA. Uptake of DNA was selected by transient ampicillin resistance and two simultaneous recombination events resulted in gene-replacement at the natural locus with no integration of heterologous DNA. *TOX* alleles were stable without selection and recombinant strains secreted non-toxic, fully assembled, protective pertussis toxin (PT) analogues with kinetics similar to the parental vaccine strain under production-scale fermentation conditions. Strains generated in this way are suitable for the production of recombinant whole-cell or component whooping cough vaccines that require no chemical modification of PT.

Whooping cough is a disease of the upper respiratory tract caused by *Bordetella pertussis*. The use of a whole-cell vaccine has greatly decreased the incidence of the disease[1]. However, concerns over the reactogenicity of this vaccine[2] and that it protects against disease rather than infection[1] have resulted in the development of acellular vaccines containing pertussis toxin (PT), filamentous haemagglutinin (FHA) and the 69 kD protein[3].

PT, a 105 kD exotoxin, is thought to be involved in the paroxysmal phase of whooping cough[4]. It consists of five polypeptide subunits (S1 to S5) and has two functional domains, A and B[5]. The NAD-dependent ADP-ribosyltransferase (ADPR) activity is located within the A subunit (S1), whilst the cell-binding and translocation activities are due to the B oligomer (S2, S3, S4, S5). ADP-ribosylation of the membrane-bound guanine nucleotide-binding negative regulatory protein of adenylate cyclase (G_i) by PT produces an increase in cAMP synthesis and is believed to account for the toxicity of the molecule. PT is however an important antigen in whooping cough vaccines and induces protective antibodies in animal models[6]. It is typically toxoided by chemical treatment that reduces its immunogenicity. Furthermore, the rare undesirable side-effects of whole-cell pertussis immunization may be due to reversion of detoxified PT to an active form. The gene(s) encoding PT *(TOX)* from several *B. pertussis* strains have been cloned and sequenced[7–9]. The glutamic acid at position 129 in the S1 subunit was identified as an active residue in NAD binding or hydrolysis by photocrosslinking of S1 to [^{14}C]-NAD[10]. Mutagenesis studies confirmed the key role of Glu^{129} and identified other residues involved in the enzymatic and toxic activities of S1[11–17].

The preferred form of PT in a whooping cough vaccine is a fully-assembled, highly immunogenic but non-toxic analogue[14–17]. Here, we describe the construction of derivatives of the Connaught vaccine production strain 10536, in which the endogenous *TOX* operon has been replaced by alleles mutated in the S1 gene by electroporetic transformation with linear DNA. Recombinant strains contained the mutated *TOX* operons integrated stably at the natural locus without the co-integration of heterologous DNA. Such strains are suitable for the production of either a whole-cell or component vaccine containing a genetically detoxified PT analogue.

RESULTS

Non-selective allelic exchange of the *B. pertussis TOX* operon. Replacement of the wild-type *TOX* operon of *B. pertussis* by mutated alleles was achieved by transformation with linear DNA and selection for two simultaneous homologous recombination events. The wild-type *TOX* allele was first replaced by a gene cartridge consisting of tetracycline resistance Tc^R and *S12* genes to provide positive and negative selection, and this cartridge was

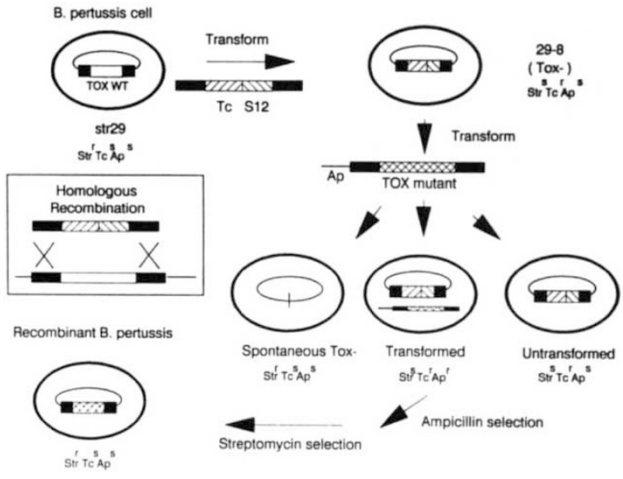

FIGURE 1 Allelic exchange in *Bordetella pertussis* by linear transformation. *B. pertussis* str29 was transformed with a linear DNA fragment to generate the *TOX* deleted Tc^r Str^s strain 29-8 by two simultaneous recombination events. *B. pertussis* 29-8 was subsequently transformed and cells that had taken up the transforming DNA selected by transient expression of the Ap^R gene present on the linear DNA fragment. Replacement of the Tc^R *S12* gene cartridge by the mutant *TOX* allele was selected by streptomycin resistance to generate a recombinant strain.

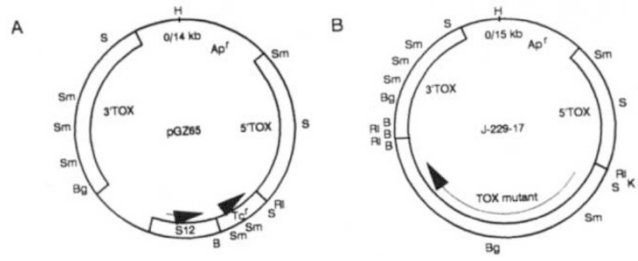

FIGURE 2 Restriction maps of plasmids for deletion of the *TOX* operon from *Bordetella pertussis* (A) and for integration of *in vitro* mutated *TOX* alleles (B). Restriction sites are: B, BamHI; Bg, BglII; H, HindIII; K, KpnI; RI, EcoRI; S, SalI; Sm, SmaI. 3'TOX and 5'TOX represent non-coding flanking sequences of the *TOX* operon. S12 represents the *E. coli* wild-type ribosomal *S12* gene.

subsequently replaced by mutated *TOX* alleles. The gene replacement strategy is illustrated in Figure 1.

Replacement of the *TOX* operon of *B. pertussis* 10536 with an *S12-tet* gene cartridge. *B. pertussis* str29 is a streptomycin-resistant derivative of the Connaught production strain, *B. pertussis* 10536. Plasmid pGZ65 (Fig. 2A) was designed for the deletion of the wild-type *TOX* operon. It is a colE1-based plasmid and thus cannot replicate in *B. pertussis*. It contains the Tc^R gene from plasmid pRK404[18] and the *E. coli S12* gene from plasmid pNO1523[19] sandwiched between 5'- and 3'-flanking sequences of the *TOX* operon. Restriction with HindIII provided a linear DNA fragment for transformation. Tetracycline-resistant (Tc^r) transformants were obtained at a frequency of about 2 per microgram of linear DNA. Transformants were sensitive to streptomycin (Str^s) because of expression of the *E. coli S12* gene[20]. They did not produce PT but continued to produce other *B. pertussis*-specific antigens FHA, fimbrial agglutinogens and the 69 kD protein. One Tox^-, Tc^r, Str^s strain (*B. pertussis* 29-8) was used for expression of mutated *TOX* alleles. Excision of the *TOX* operon from this strain was demonstrated by Southern analysis (Fig. 3). When *B. pertussis* 10536 chromosomal DNA was restricted with EcoRI and hybridized with a nick-translated probe for the entire *TOX* operon, a 4.7 kb band was detected (Fig. 3, Lane 1). This *TOX*-specific hybridization fragment was absent from the *TOX*-deleted strain *B. pertussis* 29-8 (Lane 6). Chromosomal DNA from 29-8 was restricted with SalI and hybridized with *S12* and Tc^R gene-specific probes. The 1.2 and 7.1 kb hybridization fragments obtained with the Tc^R gene probe (Lane 13) and the 7.1 kb fragment obtained with the *S12* probe (Lane 16) confirmed the integration of the selectable cartridge into the *B. pertussis* 29-8 (Fig. 3).

Integration of mutant *TOX* alleles into strain *B. pertussis* 29-8. Plasmids were constructed with *TOX* alleles containing detoxifying mutations sandwiched between the 5'- and 3'-*TOX* flanking sequences (Fig. 2B). These were linearized with HindIII and used to replace the Tc^RS12 cartridge of *B. pertussis* 29-8 by the mutated *TOX* operons. This strategy was designed to produce *B. pertussis* strains with a Str^r, Tc^s phenotype that secrete PT analogues with greatly reduced enzymatic and toxic activities[17].

Our first attempts to replace the Tc^RS12 gene cartridge by mutated *TOX* alleles were unsuccessful. This was due to the instability of the gene cartridge, which was spontaneously excised 10,000 times more frequently than the expected frequency of gene replacement. To distinguish between such spontaneous Str^r clones and true transformants, use was made of the ampicillin resistance (Ap^R) gene at the 5'-end of the transforming DNA (Fig. 1). Thus, three types of cells were obtained following transformation: untransformed cells, which retained the Tc_R-*S12* gene cartridge and could be readily excluded since they were Str^s; untransformed cells, which had spontaneously lost the selectable cartridge and were Tox^-, Str^r; and transformed cells containing the linearized transforming plasmid not yet integrated into the chromosome. The Ap^R gene in transformants could be used to select against the second class of untransformed cells of phenotype TOX^-, Tc^s, Str^r, Ap^s. Once the *TOX* allele was integrated by homologous recombination the Ap^R gene was lost and the cells became Ap^s. However, there was an interim period during which transformed cells containing the non-integrated plasmid were transiently resistant to ampicillin. Thus, by pre-selection with ampicillin (50 μg/ml) for 15–24 hours and then imposing streptomycin selection, the frequency of spontaneous Str^r, Tox^- clones was reduced about 1,000 fold. Transformants were selected on BG plates containing streptomycin, grown in liquid medium to determine the level of PT analogue secretion and analysed by colony hybridization to confirm their TOX^+, Tc^s, Str^s phenotype. The selection for temporary acquisition of ampicillin resistance resulted in about 50% of the clones examined being TOX^+ due to allelic exchange at the *TOX* locus.

Analysis of the integrated *TOX* operon. Replacement of the wild-type *TOX* operon by mutated alleles was demonstrated by Southern hybridization. Chromosomal DNA was isolated, restricted with EcoRI, BglII, SalI, SmaI and EcoRV and probed with a radiolabeled EcoRI *TOX* fragment that represents the entire coding sequence. Results for the *B. pertussis* strain 689-169 that produces the PT Gly^{129} analogue are shown in Figure 3. The hybridization pattern is identical to *B. pertussis* 10536 except for the appearance of a new fragment following digestion with EcoRV (Lanes 5 and 11), which is characteristic of the *TOX* Gly^{129} (GAA→GGA) mutation. DNA from *B. pertussis* strains 10536 and 689-169 was also probed with *S12* and Tc^R gene-specific probes and indicated that the *TOX* Gly^{129} allele was recombined at the *TOX* locus without integration of heterologous DNA.

Mutated regions of the *TOX* operon were amplified using the polymerase chain reaction, cloned into pBluescript SK and sequenced to confirm the S1 mutations.

Stability of re-integrated *TOX* alleles. The stability of mutant *TOX* alleles was determined in two ways. Firstly, 100 individual clones were isolated from a 10 liter fermentation. They were probed for the *TOX* operon and cultured to determine the level of PT analogue production. All clones were TOX^+ and secreted the PT analogue at wild-type levels. Secondly, a recombinant strain was maintained in exponential growth for 70 generations in liquid medium by serial-transfer. At each transfer, the level of PT analogue secretion remained constant and 100% of the cells retained the *TOX* operon.

Kinetics of growth and antigen production. Recombinant *B. pertussis* strains were grown in 14L, 35L, or 300L bioreactors. The rates of bacterial growth and antigen production were equivalent to those of *B. pertussis* 10536 and the production of PT analogues was about 10–20 mg/l.

Isolation and analysis of PT analogues from recombinant *B. pertussis* strains. PT analogues were purified from culture supernatants of recombinant *B. pertussis* strains and analysed by electrophoresis on SDS-polyacrylamide gels and reverse-phase HPLC. Analogues contained the five PT subunits S1 to S5 and were indistinguishable from wild-type toxin except for the HPLC retention time of subunit S1, which was influenced by the

amino acid substitutions introduced. A comparison of the PT analogue $Lys^{9}Gly^{129}$ with wild-type PT is shown in Figure 4.

We previously reported the properties of PT analogues containing mutations in subunit S1[14,17]. This demonstrated that ADP-ribosyltransferase activity was particularly sensitive to mutations at amino acids 9, 58 and 129. We therefore constructed strains of *B. pertussis* with simultaneous substitutions at two of these positions as indicated in Table 1. The ADP-ribosyltransferase activity of the PT analogues $Lys^{9}Gly^{129}$, $Glu^{58}Gly^{129}$ and $Glu^{58}Ser^{129}$ was less than ten millionths that of wild-type PT. They were virtually non-toxic in mice at doses as high as 40–50 μg, as measured by histamine sensitization and leukocytosis stimulation assays (Table 1). The mutant $Lys^{9}Gly^{129}$ is especially noteworthy, as previously discussed by ourselves and others[14–17], since it has no detectable toxicity at doses of 50 μg per mouse, and its enzymatic activity is less than one millionth that of wild-type PT. The dose for protection of mice against intracerebral challenge with virulent *B. pertussis* 18323 for chemically detoxified PT is about 10 μg and all PT analogues were protective at significantly lower doses (2–4 μg).

DISCUSSION

In this report, we describe the construction of recombinant *B. pertussis* strains that secrete fully assembled analogues of pertussis toxin. These strains were derived from the Connaught vaccine strain 10536 by the site-specific replacement of the wild-type *TOX* operon by *in vitro* mutated alleles, following electroporetic transformation with linear DNA fragments.

There are many methods for the generation of specific mutations in cloned genes. However, the substitution of *in vitro* mutated alleles for wild-type chromosomal genes by *in vivo* homologous recombination is often necessary to determine the effect of mutations in a homologous genetic background. In *E. coli* this has been achieved by Hfr mating[21], by the generation and resolution of unstable plasmid co-integrates[22], by P1 transduction[23] and by transformation with linear DNA fragments[24]. To minimize the enzymatic degradation of linear DNA, host strains with the mutations *recBC*, *sbcB* and *recD* are used. Although such mutations are not available for *B. pertussis*, transformation with linear DNA provides a simple and direct method of gene replacement. Clearly, the efficiency of transformation and the amount of DNA used must be high enough to overcome any degradation of incoming DNA. The combination of high-frequency transformation[25] and pre-selection of DNA uptake by transient expression of an antibiotic resistance gene may be critical to the success in gene-replacement described here.

Stibitz et al. described allelic exchange in *B. pertussis* that involved the introduction of a non-replicating plasmid by conjugation, the generation of a co-integrate and the resolution of this structure by recombination[20]. Transformation with linear DNA provides an alternative means of gene replacement. Many bacteria are transformed by electroporation[26] and it is probable that the introduction of large linear DNA fragments obtained with *B. pertussis* will also be achieved in other species.

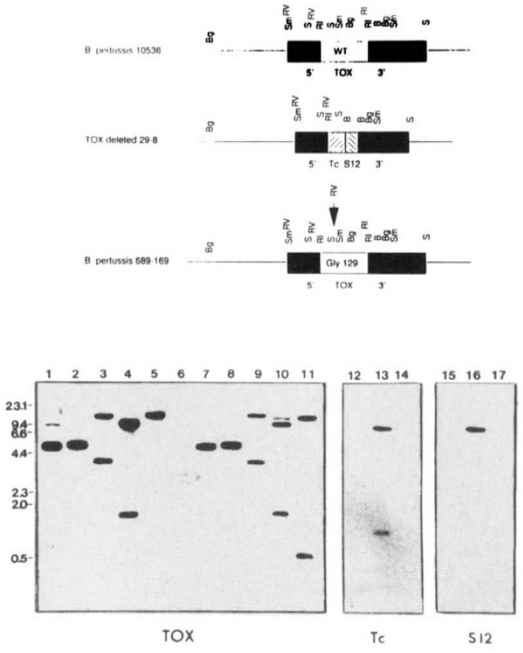

FIGURE 3 Southern analysis of a recombinant *Bordetella pertussis* strain and a representation of the genes present at the *TOX* locus. Strain 10536 (wild-type), lanes 1–6 and 12; 29-8 (*TOX* deleted strain) lanes 7 and 13; and 689–169 (Gly^{129} recombinant) lanes 7–11 and 17. Chromosomal DNA was isolated and digested with the restriction enzymes EcoRI (lanes 1, 6, 7), SmaI (lanes 2, 8), BglII (lanes 3, 9), SalI (lanes 4, 10, 12–17) and EcoRV (lanes 5, 11). The fragments were separated on a 0.8% agarose gel, transferred and probed with *TOX*, *S12* and Tc^{R} gene-specific probes as shown. The *TOX* Gly^{129} mutation resulted in the generation of a unique EcoRV site, shown by the appearance of an extra *TOX*-specific restriction fragment (lane 11) and indicated by an arrow.

TABLE 1 Biological characterization of PT analogues obtained from recombinant *Bordetella pertussis* strains.

PT Analogue	Expression (mg/l)[a]	Relative CHO Cell Clustering Activity (Percent)	Relative ADPR Activity (Percent)	Leukocytosis (ED μg)[b]	Histamine Sensitization (LD_{50} μg)[c]	Mouse Protection (ED_{50} μg)[d]
Wild-Type	20	100	100	0.02	0.05	—
Gly^{129}	10	0.2	0.2	3	5	4
$Lys^{9}Gly^{129}$	20	≤0.0005	<0.0001	>50[e]	>50[e]	4
$Glu^{58}Gly^{129}$	10	≤0.0005	0.0002	>50[e]	>40[f]	2
$Glu^{58}Ser^{129}$	10	≤0.0005	0.0004	50	>40[f]	2

[a] Expression of pertussis toxin and analogues was determined by PT-specific ELISA in supernatants from 10-300 L bioreactors.
[b] The effective dose (ED) is the amount of PT or analogue resulting in a doubling in the number of circulating lymphocytes.
[c] The LD_{50} is the dose required to kill 50% of the mice following challenge with histamine acid phosphate (1mg/10g body weight).
[d] The ED_{50} is the dose that protects 50% of the mice from a lethal challenge with *B. pertussis* 18323. This value cannot be determined for wild-type PT because of its toxicity.
[e] No activity was detected at the dose indicated.
[f] Dose resulted in two deaths out of 16 mice.

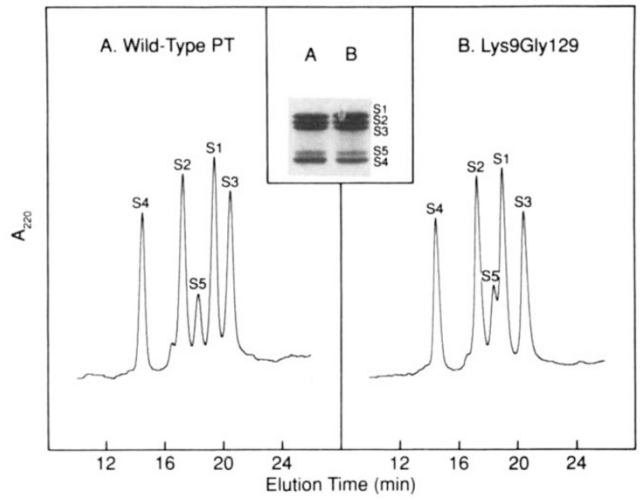

FIGURE 4 Subunit analysis of $Lys^{9}Gly^{129}$ pertussis toxin analogue by reverse-phase HPLC and SDS-gel electrophoresis. A and B, wild-type PT and $Lys^{9}Gly^{129}$ analogue, respectively.

Pertussis toxin is likely to be an essential component of any pertussis vaccine and ideally this should be detoxified by genetic means. By combining detoxifying mutations at amino acids 9, 58, or 129 in the S1 subunit of PT, three highly detoxified, immunogenic PT analogues were produced. These PT analogues, $Lys^{9}Gly^{129}$, $Glu^{58}Gly^{129}$ and $Glu^{58}Ser^{129}$ were all protective in the mouse intracerebral challenge assay and represent appropriate antigens for inclusion in a genetically detoxified whole-cell or component pertussis vaccine.

EXPERIMENTAL PROTOCOL

Bacterial strains and media. *B. pertussis* 10536 is a Connaught vaccine strain and str29 is a spontaneous Str^{r} derivative of 10536. Liquid cultures were grown in media supplemented with heptakis (2,6-di-O-methyl)-β-cyclodextrin[27] and plate cultures on Bordet Gengou (BG) medium (BBL) with 20% defibrinated sheep blood (Bockneck) containing streptomycin and tetracycline at concentrations of 100 and 10 μg/ml respectively.

Recombinant DNA techniques. DNA-modifying enzymes were from BRL or Boehringer Mannheim and were used as recommended. Plasmid DNA was isolated from *E. coli* JM109 (*recA1, endA1, gyr96, thi, hsdR17m, supE44, relA1* Δ*(lac-proAB), [F⁻traD36 proAB⁺ laqI^q ZΔM15]*) as described by Ish-Horowicz and Burke[28]. Chromosomal DNA was isolated by the method of Yacoob and Zealey[29]. Southern blot and colony hybridizations were performed as described by Maniatis et al[30]. The *TOX*-specific probe was a gel-purified 4.7 kb EcoRI restriction fragment that represents the entire *B. pertussis TOX* operon. Nick-translated pRK404 and pNO1523 plasmids were used to detect the *tet* and *S12* genes, respectively. Regions of the *TOX* operon were amplified by the polymerase chain reaction in a reaction mixture that consisted of 0.1–0.5 μg genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 100 μg/ml gelatin, 0.2 μM 20 bp oligonucleotide primers, 200 μM dNTPs and 25 units/ml of Taq polymerase for 25 cycles (denaturation, 94°C, 1 min; annealing, 45°C, 1 min.; extension, 72°C, 2 min.) using a Perkin Elmer Cetus DNA thermal cycler. Amplified fragments were cloned into pBluescript SK (Stratagene) and sequenced using an Applied Biosystems 370A DNA sequenator.

Transformation of *Bordetella pertussis* by electroporation. *B. pertussis* was grown in 1 L of medium to a density of about 5×10^{9} cells/ml and harvested by centrifugation (5000×g, 15 min., 4°C). The cells were washed twice in 500 ml of distilled water, once in 50 ml of 10% glycerol (BRL redistilled), resuspended in 10 ml of 10% glycerol and frozen at −70°C. For transformation, 200 μl of cells were combined with about 10 μg of linearized DNA and incubated on ice for 10 minutes. They were then subjected to a 650 V exponential decay pulse across a 0.8 mm electrode gap using a BTX Transfector 100 equipped with a Power Plus unit (Biotechnologies and Experimental Research, San Diego CA). One ml of medium was added and the cells incubated with shaking at 36°C. After 1 hour the culture was made 50 μg/ml with ampicillin and incubated at 36°C for 15–24 hours. Samples were removed after 16, 20 and 24 hours, plated onto BG medium containing streptomycin and transformants appeared after 3–5 days of incubation at 36°C.

Growth of recombinant *B. pertussis* strains in bioreactors. Fermentations in ChemAp bioreactors, inoculated to a starting A_{600} of about 0.1, were controlled for dO_2, pH and temperature. Cultures were grown to an A_{600} of about 5 (approximately 40 hours), and after cooling the cells were collected by centrifugation and the broth rendered cell-free by microfiltration across a 0.22 μM membrane.

Purification of PT analogues. PT analogues were purified from *B. pertussis* culture supernatants by two methods. Small quantities (1–5 mg) were isolated by affinity chromatography on fetuin-agarose. Supernatants were concentrated by ultrafiltration and applied to a fetuin-agarose column (Sigma, 2–4 ml per mg of PT) equilibrated with 10 mM potassium phosphate, 100 mM NaCl, pH 7.5 (Buffer A). The column was washed sequentially with Buffer A, Buffer A containing 0.5% Triton X-100 and 10× Buffer A, then eluted with 3 M potassium thiocyanate in 10× Buffer A. The eluate was dialysed against 50 mM Tris-HCl, 200 mM NaCl, 50% glycerol, pH 8.0, diluted five-fold with 50 mM Tris-HCl, pH 8.0 and applied to a hydroxylapatite column (Bio-Rad, 2–4 ml per mg of PT) equilibrated with 10 mM potassium phosphate pH 8.0 (Buffer B). The column was washed with Buffer B, 3× Buffer B, then eluted with 75 mM potassium phosphate, 225 mM NaCl, pH 8.0. The eluate was dialysed against 100 mM potassium phosphate, 50% glycerol, pH 8.0 and stored at −20°C. Larger amounts of analogues (5–30 mg) were isolated by adsorption to perlite (Filter-Aid)[31]. Concentrated culture supernatants were diluted five times with deionized water and applied to a perlite column (10 ml per mg of PT) equilibrated with 50 mM Tris-HCl, pH 8.0 (Buffer C). The column was washed with Buffer C, Buffer C containing 0.5% Triton X-100 and Buffer C again, then eluted with Buffer C containing 0.2 M NaCl. The eluate was further purified on a hydroxylapatite column as described above.

Characterization of PT analogues. PT analogues and other antigens were quantitated by specific ELISA; the former were also screened for enzymatic activity by the CHO cell clustering assay[32]. Purified PT analogues were analysed by SDS-15% polyacrylamide gel electrophoresis and by reverse-phase HPLC on a Vydac 214TP54 C_4 column (Separations group, 0.46×25 cm). Subunits were eluted using a linear gradient of 35–45% acetonitrile increasing at 0.4% per minute in 10 mM trifluoroacetic acid, with a flow rate 0.75 ml/min. ADP-ribosyltransferase activity was determined by the transfer of radiolabelled ADP-ribose from [adenylate-^{32}P]-NAD to bovine transducin[33]. Histamine sensitization and mouse protection activities were performed on Swiss-/Webster mice as described previously[17]. Leukocytosis-promoting activity was measured on groups of five female Swiss/Webster mice injected intravenously with PT or analogue in 200 μl of PBS. Proteins were assayed at three doses; 0.01–1 μg (wild-type), 1–10 μg (Gly^{129}), and 5–50 μg ($Lys^{9}Gly^{129}$, $Glu^{58}Gly^{129}$, $Glu^{58}Ser^{129}$). Four days later, 20 μl of blood were removed by tail-snipping and leukocytes counted by microscopic examination.

Acknowledgments

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