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Pertussis toxin potentiates T_h1 and T_h2 responses to co-injected antigen: adjuvant action is associated with enhanced regulatory cytokine production and expression of the co-stimulatory molecules B7-1, B7-2 and CD28

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Abstract

Pertussis toxin (PT) is a major virulence factor of *Bordetella pertussis* which exerts a range of effects on the immune system, including the enhancement of IgE, IgA and IgG production, delayed-type hypersensitivity reactions, and the induction of experimental autoimmune diseases. However, the mechanism by which PT mediates adjuvanticity remains to be defined. In this investigation we have shown that PT can potentiate antigen-specific T cell proliferation and the secretion of IFN-γ, IL-2, IL-4 and IL-5 when injected with foreign antigens. A chemically detoxified PT and a genetic mutant with substitutions/deletions in the S-1 and B oligomer components that abrogate enzymatic and binding activity displayed no adjuvant properties. In contrast, a non-toxic S-1 mutant devoid of enzymatic activity but still capable of receptor binding retained its adjuvanticity, augmenting the activation of both T_h1 and T_h2 subpopulations of T cells. In an attempt to address the mechanism of T cell activation, we found that PT stimulated the production of IFN-γ and IL-2 by naive T cells and IL-1 by macrophages. Therefore potentiation of distinct T cell subpopulations may have resulted in part from the positive influence of IFN-γ on the development of T_h1 cells and the co-stimulatory role of IL-1 for T_h2 cells. Furthermore, PT augmented expression of the co-stimulatory molecules B7-1 and B7-2 on macrophages and B cells, and CD28 on T cells, suggesting that the adjuvant effect may also be associated with facilitation of the second signal required for maximal T cell activation. This study demonstrates that the immunopotentiating properties of PT are largely independent of ADP-ribosyltransferase activity, but are dependent on receptor binding activity and appear to involve enhanced activation of T cells.

Introduction

Pertussis toxin (PT) is an exotoxin produced by *Bordetella pertussis* with a hexameric A–B structure similar to cholera toxin (CT) and *Escherichia coli* heat-labile toxin (LT) (1,2). The active A protomer of the toxin is composed of a single S-1 subunit with ADP-ribosyltransferase activity, which modifies GTP-binding regulatory proteins (G proteins), thus interfering with signal transduction in mammalian cells (2,3). The B oligomer component of the toxin has a pentameric structure which mediates the binding of the toxin to glycoprotein receptors on the surface of eukaryotic cells and induces polyclonal T cell activation (2,4).

It has been reported that PT, like CT and LT, possesses

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Table 1. Properties of toxin preparations used in study

Toxin	T cell mitogenicity	HA activity[a]	ADP ribosylation	CHO clustering	Leucocytosis promoting
Native PT	+	+	+	+	+
PT-9K/129G	+	+	–	–	–
PTX-RENK	–	NT	–	–	–
PTd	–	–	–	–	–

Summarized from references 4, 25, present study and unpublished observations.
[a]Haemagglutination (HA) activity against chicken erythrocytes; not tested (NT) for PTX-RENK, but this toxin was reported to have significantly reduced haptoglobin binding, compared with the native wild-type toxin.

adjuvant properties (5–14). PT has been shown to potentiate both local and systemic antibody responses, enhancing IgE, IgA and IgG production to co-injected antigens (9,10). PT has also been reported to augment delayed-type hypersensitivity (DTH) reactions and to exacerbate autoimmune diseases (11,12). Paradoxically PT appears to influence immune responses mediated by reciprocally regulated subpopulations of $CD4^+$ T_h cells that secrete IFN-γ and IL-2 or IL-4 and IL-5, termed T_h1 and T_h2 cells respectively. Recent studies have shown that the adjuvant effect of PT for IgE responses is associated with augmented production of IL-4 (13). Furthermore, the potentiation of DTH reactions by PT is associated with enhanced antigen-driven IFN-γ production by sensitized lymphoid cells (14). These findings suggest that the adjuvanticity of PT may be associated with enhanced production of both T_h1 and T_h2 cytokines.

As well being a major virulence factor, PT is considered to be a key component of the protective immune response to *B. pertussis* (2,15). PT-specific T cell and antibody responses are generated following recovery from *B. pertussis* infection and immunization with the whole cell pertussis vaccine (16–19). Indeed it has been suggested that the protective efficacy of the whole cell vaccine may in part be related to residual active PT, which has escaped the inactivation process (15,20). Furthermore, PT in a chemically or genetically detoxified form is a common component of each of the highly effective new generation acellular pertussis vaccines (21,22). Although chemical treatment can eliminate the undesirable toxic effects of PT, it has been shown to affect its antigenicity and immunogenicity (23,24) and may also alter adjuvant activities for immune responses against the toxin itself and against other antigenic components of the acellular vaccine. In contrast, recombinant PT molecules with mutations in the S-1 subunit that abrogate ADP-ribosyltransferase activity are non-toxic and highly immunogenic (2,4,25), and may also retain beneficial immunopotentiating activities of the native toxin that are lost through chemical toxoiding.

In this study we have investigated the ability of PT to enhance T cell responses following co-injection with filamentous haemagglutinin (FHA), another putative protective antigen of *B. pertussis*, or with a third party antigen, keyhole limpet haemocyanin (KLH). The use of chemically inactivated PT and recombinant PT molecules with mutations in either the S-1 or S-1 and B oligomer components of the toxin has enabled us to examine the relevance of ADP-ribosyltransferase activity and receptor binding on the adjuvant properties of PT. We have demonstrated that PT potentiates both T_h1- and T_h2-type responses to co-injected antigens. We also provide evidence that a non-toxic PT molecule that lacks ADP-ribosyltransferase activity, but is still capable of receptor binding, retains the ability to potentiate T cell responses to co-administered antigens.

Methods

Mice

BALB/c (H-2^d), C57BL/6 (H-2^b), mutant 129/Sv/Ev mice deficient in the IFN-γ receptor (IFN-$\gamma R^{-/-}$) (26) and wild-type 129/Sv/Ev (IFN-$\gamma R^{+/+}$) mice were bred and maintained under the guidelines of the Irish Department of Health. Breeding pairs of IFN-$\gamma R^{-/-}$ mice were purchased from B & K Universal (Hull, UK) with permission from Michel Aguet. All mice were used at 8–12 weeks of age.

Antigens and toxins

KLH was purchased from Sigma (Poole, UK). PT and FHA were prepared from *B. pertussis* Tohama I strain, and were found to be free of other *B. pertussis* components following analysis on silver-stained SDS gels. Chemically detoxified PT (PTd) was prepared by treatment with 0.2–0.5% formaldehyde for 7 days followed by dialysis against PBS containing 0.01% formaldehyde. PT-9K/129G, with mutations in the S-1 subunit, was prepared as described (25). PTX-RENK, a genetic mutant with substitutions/deletions in the S-1 and B oligomer components (4), was provided by Carine Capiau and Yves Lobet (SmithKline Beecham, Rixensart, Belgium). Compared with the wild-type native toxin, the two mutant toxins PT-9K/129G and PTX-RENK are non-toxic and do not induce leucocytosis or clustering of CHO cells (Table 1). PTX-RENK has reduced binding to sialoglycoproteins and CHO cells, and is not mitogenic for T cells (4), whereas PT-9K/129G retains mitogenicity and the ability to bind to receptors on eukaryotic cells (25).

Immunization

Mice were immunized by the i.p. route with KLH (20 μg) or FHA (5 μg) alone or mixed with 0.1–5.0 μg of PT, PT-9K/129G, PTX-RENK or PTd. Mice were sacrificed 2 weeks after one or two (weeks 0 and 4) immunizations, and serum and spleen cells prepared for detection of immune responses.

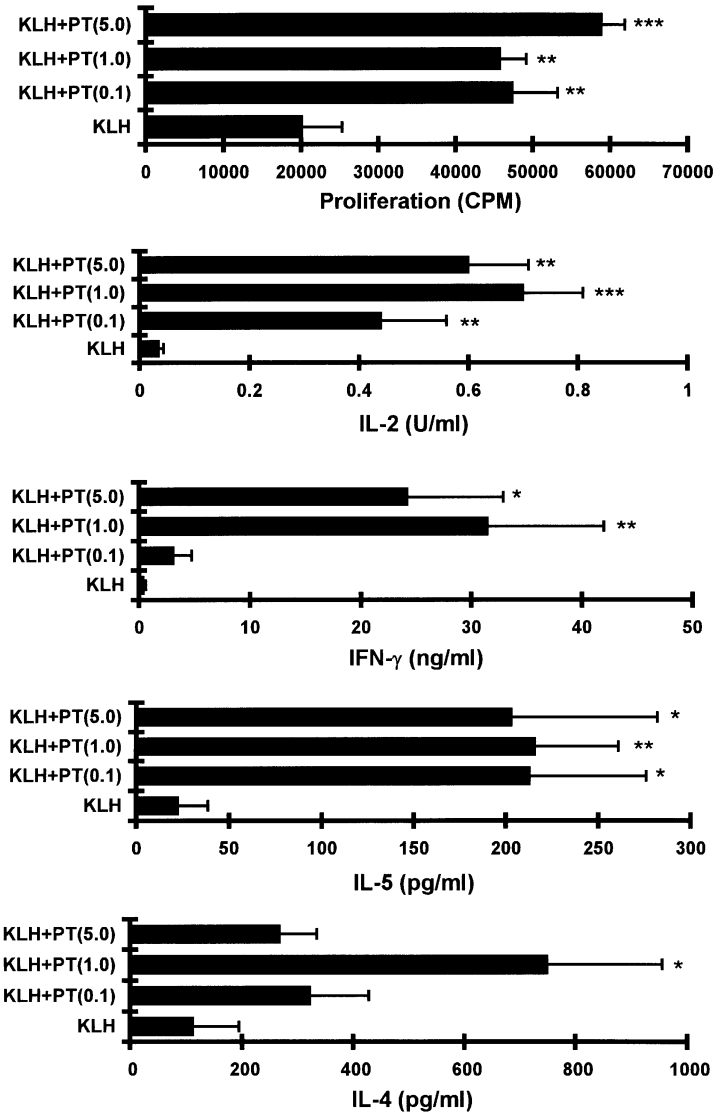

Fig. 1. PT potentiates T_h1 and T_h2 responses to co-injected antigens. Mice were immunized twice (0 and 4 weeks) with KLH alone or with 0.1, 1.0 or 5.0 μg of active PT, and at week 6 spleen cells were stimulated *in vitro* with KLH (20 μg/ml) and assessed for proliferation and cytokine secretion. Results are expressed as the mean (± SE) responses for spleen cells for five mice per group tested individually in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus KLH alone determined by Student's *t*-test.

Purification of T cells, B cells and macrophages

B cells and T cells were purified from spleen cells by a combination of negative and positive selection techniques as described (27). Monocytes and NK cells were first depleted using a Monoclear reagent (Immulan B cell accessory kit; Biotecx, Houston, TX). The depleted populations of cells were washed and filtered through a sterile fine nylon mesh to remove cell debris, and then passed over a separation column loaded with goat anti-mouse IgG-coated beads (Pierce & Warrnier, Chester, UK or Biotecx). B cells were retained by the column and were recovered by gentle agitation of the beads in a centrifuge tube containing HBSS supplemented with 1% FCS. T cells were recovered in the non-adherent fraction and passed through a fresh column. Flow cytometry analysis revealed that 92–95% of the non-adherent cells were $CD3^+$ T cells. Macrophages were prepared from spleen cells or peritoneal exudate cells (PEC; obtained by peritoneal lavage) by adherence to plastic.

T cell proliferation assays

Spleen cells (2×10^6/ml) or purified T cells (1×10^5/ml) and antigen-presenting cells (APC)/accessory cells (irradiated spleen cells, 2×10^6/ml) were suspended in RPMI medium (8% FCS) and cultured in triplicate 200 μl wells of 96-well microtiter plates with antigen (KLH; 0.8–20 μg/ml, FHA; 0.1–5.0 μg/ml, heat-inactivated PT; 0.1–5.0 μg/ml). Proliferation was assessed by $[^3H]$thymidine incorporation after 4 days as described (27). In an assessment of the mitogenic properties

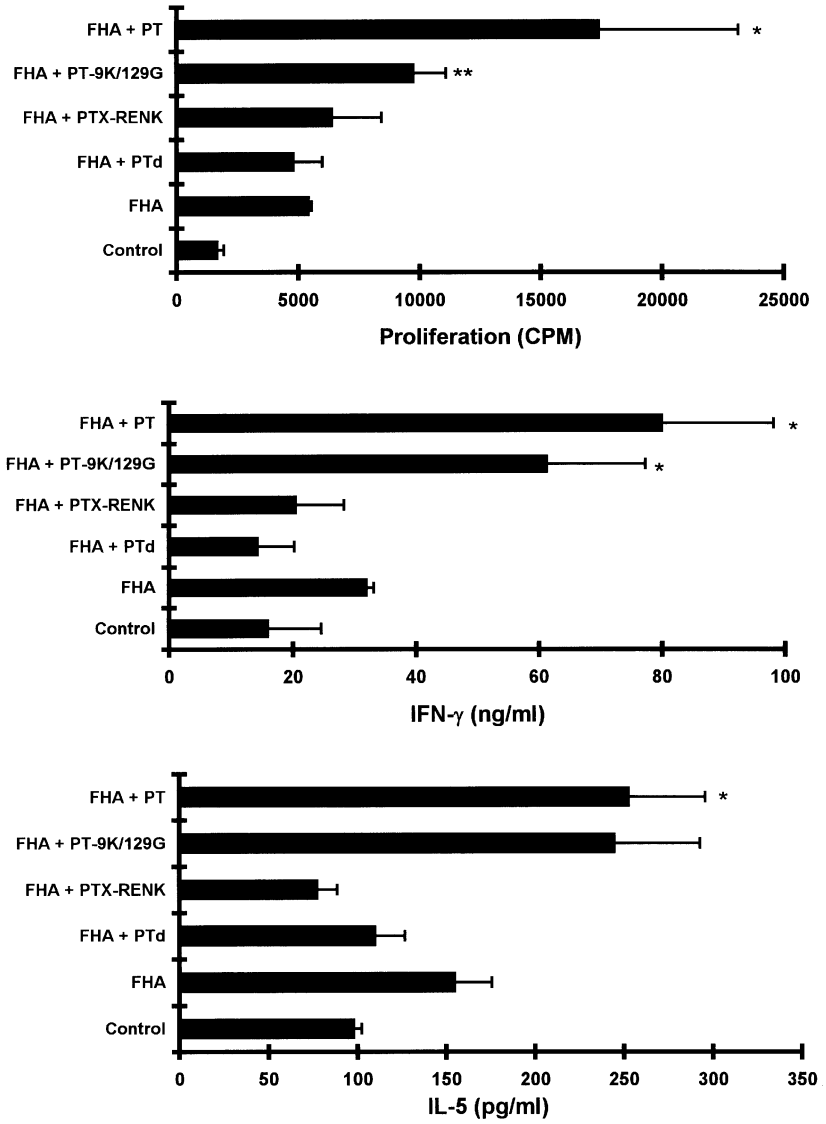

Fig. 2. A mutant pertussis toxin molecule PT-9K/129G that lacks ADP-ribosyltransferase activity retains adjuvant properties. Mice were immunized once with FHA (5 μg) alone or with 5 μg of PT, PT-9K/129G, PTX-RENK or PTd, and 14 days later spleen cells were stimulated *in vitro* with FHA (5.0 μg/ml) and assessed for proliferation and cytokine production. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus FHA alone determined by Student's *t*-test.

of PT, spleen cells or purified T cells and irradiated accessory cells were stimulated with native, recombinant or detoxified PT (0.08–5.0 μg/ml) and proliferation was tested after 3 days.

Cytokine assays

T cell cytokine production was assessed by culturing unseparated spleen cells or purified T cells in the presence of irradiated spleen cells with the different PT preparations or with antigens as described for the proliferation assay. Supernatants were removed after 24 h for detection of IL-2, and after 72 h for IFN-γ, IL-4 and IL-5. IL-2 levels were assessed by measuring the proliferation of an IL-2-dependent CTLL cell line in the presence of a neutralizing anti-IL-4 antibody 11B11. IFN-γ, IL-5 and IL-4 were measured by immunoassay using commercially available mAb (PharMingen, San Diego, CA) as previously described (28). Cytokine concentrations derived from standard curves prepared with recombinant cytokines of known concentrations.

The effect of PT on local and systemic cytokine production *in vivo* was assessed by injection of FHA alone or combined with 5 μg of PT into the footpad, and quantifying IFN-γ, IL-2 and IL-5 levels in the draining popliteal lymph nodes (homogenates) and serum 24 and 96 h later.

The ability of PT to stimulate IL-12 production was assessed using splenic or peritoneal macrophages. Macrophages were incubated with PT, lipopolysaccharide (positive control) or medium only (negative control) for 24 h and supernatants removed and assessed for IL-12 levels by immunoassay and bioassay as previously described (28). In the immunoassay commercially available (Genzyme Diagnostics, Cambridge,

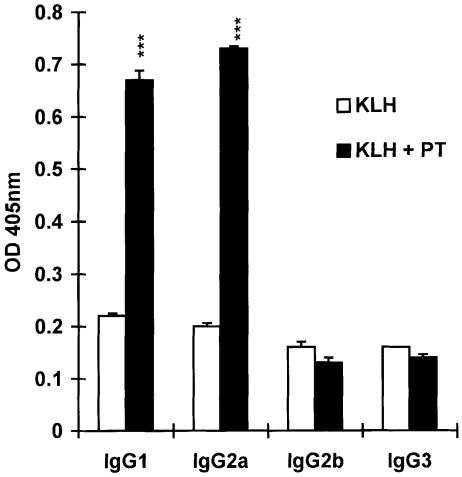

Fig. 3. PT potentiates IgG1 and IgG2a responses to co-injected antigens. Mice were immunized twice (0 and 4 weeks) with KLH alone or with 5.0 μg of active PT. Serum samples were prepared at week 6 and anti-KLH antibody responses were tested by ELISA. Results are mean OD values for serum samples (1/50 dilution) from five mice per group assayed in triplicate. $^{***}P < 0.001$ versus KLH alone determined by Student's *t*-test.

MA) anti-IL-12 mAb clone C17.8 (rat IgG2a) and clone C15.6 (rat IgG1), which recognize the p40 subunit of murine IL-12, either as monomer, homodimers or as part of the p70 heterodimer, were used for capture and detection respectively. Biologically active IL-12 concentrations were assessed by the ability of test supernatants to stimulate the production of IFN-γ by naive spleen cell preparations. To ensure that the production of IFN-γ was due to the presence of IL-12, test samples were also assayed in the presence and absence of a specific anti-IL-12 neutralizing antibody (2.5 μg/ml of protein G-purified sheep anti-murine IL-12, provided by Stanley Wolf, Genetics Institute, Cambridge, MA) which can completely neutralize up to 5 ng/ml of IL-12.

In order to assess the stimulation of IL-1 production, peritoneal macrophages were stimulated with different PT preparations for 24 h, and supernatants and pelleted cells recovered for the assessment of secreted and cell-associated IL-1. IL-1β concentrations were determined in supernatants by immunoassay using commercially available antibodies (Genzyme) as described for the T cell cytokine analysis. Cell associated IL-1 was assessed by immunoblotting; cell lysates were analysed by SDS–PAGE, transferred to nitrocellulose and probed with polyclonal sheep anti-mouse-IL-1 (kindly provided by Steve Poole, NIBSC, Hertfordshire, UK) which binds murine IL-1α and IL-1β.

Role of IL-1 as an accessory molecule in T cell activation

The role of IL-1 as an accessory molecule in the PT-enhanced activation of antigen-specific T_h1 or T_h2 response was assessed by *in vitro* stimulation of spleen cells with antigen in the presence or absence of the IL-1 receptor antagonist (IL-1ra). Spleen cells from mice immunized with KLH (20 μg) in the presence of PT (1 μg) were stimulated with KLH (1–15 μg/ml) alone or with 0.1 or 1.0 μg/ml IL-1ra. Supernatants were removed after 3 days, and tested for IL-4, IL-5 and IFN-γ production as described above.

Antibody isotype analysis

The levels of KLH-specific antibodies in serum were determined by ELISA using KLH (2 μg/ml) to coat the plates. Bound antibodies were detected using alkaline phosphatase-conjugated antibodies specific for mouse IgG1, IgG2a, IgG2b or IgG3 (PharMingen).

Expression of co-stimulatory molecules determined by flow cytometry

The effect of PT on co-stimulatory molecule expression *in vivo* was assessed on PEC from mice injected i.p. 24 h earlier with 1 μg of active PT or PBS alone. PEC were recovered by peritoneal lavage, washed and resuspended in PBS, and B7-1 and B7-2 expression was assessed using a phycoerythrin-conjugated hamster anti-mouse B7-1 (anti-CD80, clone 16–10AI) and FITC-conjugated rat anti-mouse B7-2 (anti-CD86, clone GL1) mAb respectively (PharMingen). After incubation for 30 min at 4°C, cells were washed and immunofluorescence analysis was performed on a FACScan (Becton Dickinson, San Jose, CA) and analysed using Lysys II.1 software. The instrument was calibrated with commercially prepared fluorescent beads (CaliBRITE beads; Becton Dickinson) using Autocomp software.

In order to test the effect of PT on co-stimulatory molecule expression *in vitro,* purified splenic T cells, splenic B cells or peritoneal macrophages from naive mice were cultured for 24 h at 37°C with PT or with genetically or chemically detoxified forms of the toxin in the concentration range of 0.1–5.0 μg/ml. After 24 h incubation, cells were washed, and stained with anti-B7-1 and anti-B7-2 as described above or with biotin-conjugated hamster anti-mouse CD28 mAb (clone 37.51; PharMingen) and FITC-conjugated streptavidin (PharMingen).

Results

PT enhances T_h1 and T_h2 responses to co-injected antigens

Immunization of mice with KLH in the presence of a range of doses of PT enhanced KLH-specific T cell proliferative responses (Fig. 1). Cytokine analysis revealed that this proliferation was associated with the secretion of moderate to high levels of IL-2, IL-4, IL-5 and IFN-γ (Fig. 1). Doses as low as 100 ng of PT per mouse were capable of potentiating the activation of both T_h1 and T_h2 subpopulations of T cells. The data presented in Fig. 1 represent responses detected after two immunizations; however, PT also enhanced cytokine production after primary immunization (Fig. 2 and data not shown). Furthermore analysis of anti-KLH antibodies in serum from immunized mice demonstrated that PT augmented IgG1 and IgG2a production, providing further evidence for the enhancement of both T_h1 and T_h2 responses *in vivo* (Fig. 3).

An examination of systemic and local cytokines production following injection of PT suggested that the stimulatory capacity of PT for cytokine production was not solely an *in vitro* phenomenon, but also occurred *in vivo*. When compared with FHA (5 μg) alone, co-injection with active PT (5 μg) resulted in significantly enhanced IFN-γ levels detected in draining

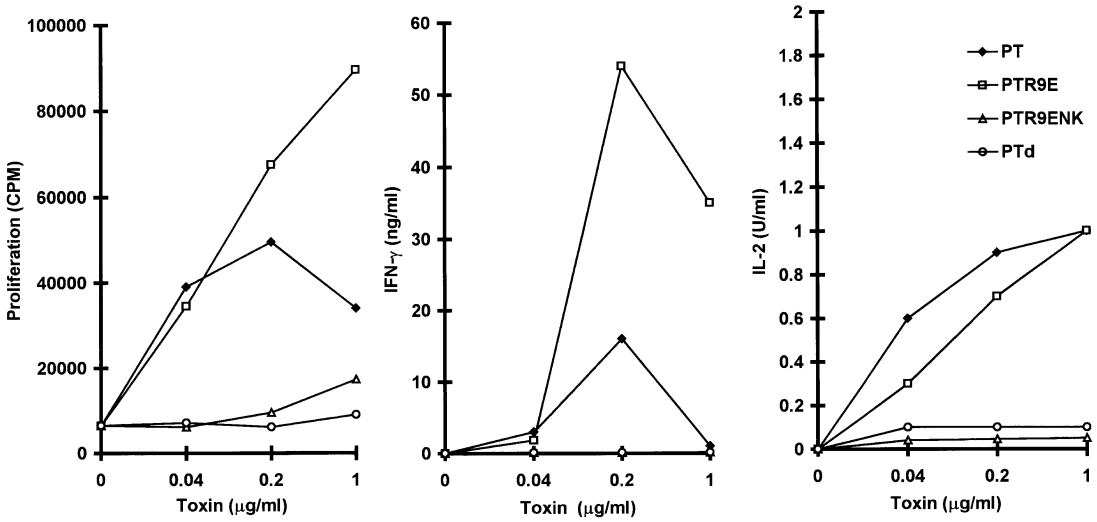

Fig. 4. PT stimulates proliferation and secretion of type 1 cytokines by spleen cells *in vitro*. Spleen cells from naive mice were cultured with a range of concentrations of PT, PT-9K/129G, PTX-RENK or PTd. Supernatants were removed after 1 day to test for IL-2 production, and after 3 days to test for IL-4, IL-5 and IFN-γ production. Proliferation was determined on a duplicate culture plate by [^{3}H]thymidine incorporation after 3 days. Results are expressed as the means for triplicate cultures of spleen cells from four to five mice and are representative of five independent experiments. The levels of IL-4 and IL-5 in all of the culture supernatants tested were <15 pg/ml.

lymph nodes (FHA only, 0.6 ng/ml; FHA + PT, 2.0 ng/ml) and in serum (FHA only, 4 ng/ml; FHA + PT, 10 ng/ml).

A genetically inactivated PT mutant PT-9K/129G retains its adjuvant properties

Previous studies had suggested that the superior efficacy of an acellular pertussis vaccine formulated with genetically rather than chemically detoxified PT may be related to adjuvant properties of the non-toxic PT mutant (29). Therefore we examined the adjuvanticity of genetically and chemically detoxified PT for the immune response to one of the components of the acellular vaccine, FHA. Active PT potentiated FHA-specific T cell proliferation and the secretion of IFN-γ, IL-2 and IL-5 (Fig. 2). IL-4 was below the limits of detection. However, we had previously reported that the detection of IL-4 following immunization with soluble *B. pertussis* antigens requires secondary re-stimulation of T cells *in vitro* (17, 18). A chemically detoxified form of the toxin (PTd) and the genetically detoxified mutant PTX-RENK, with substitutions/deletions in the S-1 subunit and B oligomer components that abrogate enzymatic activity and receptor binding, failed to augment antigen-specific T cell responses when co-administered with FHA (Fig. 2). In contrast, an S-1 mutant PT-9K/129G, devoid of enzymatic activity but still capable of receptor binding, did display adjuvant properties, potentiating the activation of both T_h1 and T_h2 cytokines (Fig. 2).

PT stimulates accessory cell-dependent production of T_h1 cytokines by naive T cells

A simple explanation for the adjuvant properties of PT was the enhanced production of cytokines that potentiate antigen presentation or T cell activation. We found that PT-induced proliferation of naive splenic T cells *in vitro* was accompanied by the secretion of high levels of IL-2 and IFN-γ, but not IL-4 or IL-5 (Fig. 4 and Table 2). However, moderate levels (~300 pg/ml) of IL-5 were detected following PT stimulation of spleen cells derived from IFN-γR$^{-/-}$ mice. The S-1 mutant PT-9K/129G retained the ability to induce the proliferation and the secretion of IL-2 and IFN-γ by naive spleen cells. Interestingly, PT-9K/129G appeared to induced higher levels of IFN-γ production than the native toxin (Fig. 4). In contrast, the chemically detoxified toxin and a genetically detoxified mutant PTX-RENK with substitutions/deletions in the S-1 subunit and the B oligomer did not induce T cell cytokine secretion over a range of concentrations (Fig. 4).

Using purified splenic T cells, PT stimulated IFN-γ and IL-2 production by naive T cells was found to be accessory cell dependent. Highly purified T cells did not produce cytokines in response to PT stimulation. However, co-culture with MHC-matched or mismatched irradiated spleen cells restored the toxin's ability to induce IFN-γ (Table 2).

The adjuvant properties of PT for T_h1 responses are not mediated by IL-12

The macrophage, dendritic cell and B cell derived cytokine IL-12 is a potent inducer and activator of T_h1 cells (30), and we have already reported that live and heat-inactivated *B. pertussis* can stimulate IL-12 production by macrophages (28). However, the results from an IL-12p40 immunoassay and an IL-12 bioassay demonstrated that PT alone (dose range 0.2–5.0 μg/ml) did not induce significant levels of IL-12 production by purified macrophages or unseparated spleen cells, whereas lipopolysaccharide (1.0 μg/ml) induced 400 pg/ml of IL-12 in the immunoassay and 50 ng/ml IFN-γ in the bioassay, which was completely inhibited by a neutralizing anti-IL-12 antibody (28 and data not shown).

Co-stimulatory signals by IL-1 may be involved in the potentiation of T_h2 responses by PT

IL-1 has been shown to function as a co-stimulatory molecule for the activation of antigen-induced proliferation and cytokine

Table 2. PT stimulates accessory cell-dependent IFN-γ production by naive T cells[a]

T cells	Accessory cells	IFN-γ (ng/ml)			IL-5 (pg/ml)		
PT (μg/ml)		0	1.0	5.0	0	1.0	5.0
Experiment 1							
BALB/c	BALB/c	<0.2	55	53	<15	<15	<15
BALB/c	–	<0.2	<0.2	<0.2	<15	<15	<15
Experiment 2							
BALB/c	BALB/c	<0.2	35.0		<15	<15	
BALB/c	C57BL/6	0.3	2.8		<15	<15	
BALB/c	–	<0.2	<0.2		<15	<15	
C57BL/6	BALB/c	3.0	28.0		<15	<15	
C57BL/6	C57BL/6	<0.2	9.0		<15	<15	
C57BL/6	–	<0.2	1.0		<15	<15	
–	BALB/c	<0.2	<0.2		<15	<15	
–	C57BL/6	<0.2	<0.2		<15	<15	

[a]T cells were purified from spleen cells of naive BALB/c (H-2^d) or C57BL/6 (H-2^b) mice by two passages through Pierce T cell isolation columns. T cells (1×10^5/ml) were stimulated *in vitro* with medium or PT (1.0–5.0 μg/ml) alone or in the presence of syngeneic or allogeneic accessory cells (2×10^6/ml). Supernatants were removed after 72 h and tested for IFN-γ and IL-5 by immunoassay. Results are mean values for triplicate cultures.

Table 3. IL-1β production by peritoneal macrophages stimulated with PT[a]

Toxin	IL-1β (pg/ml)			
Toxin concentration (μg/ml)	0	0.2	1.0	5.0
Medium	5.8 ± 2.0			
Active PT		10.1 ± 0.9	9.0 ± 0.9	250.0 ± 59
Heat-inactivated PT		7.5 ± 2	8.8 ± 5.4	6.4 ± 3.1
PT-9K/129G		15.5 ± 3.9	36.8 ± 11.5	287.7 ± 96
PTd	<5.0	7.8 ± 2	<5.0	

[a]Peritoneal macrophages (1×10^6/ml) were stimulated with medium only or increasing concentrations of the various toxin preparations. Supernatants were removed after 24 h and IL-1β concentrations were assessed by immunoassay. Results are mean (± SD) values from three experiments.

production by $CD4^+$ T cells, especially for the T_h2 subtype (31). Therefore we examined the possible role of IL-1 in the enhancement of antigen-specific T_h1 and T_h2 responses by PT. We found that active PT, or the S-1 mutant PT-9K/129G, but not heat-inactivated or chemically detoxified PT stimulated peritoneal macrophages to produce modest levels of IL-1β, detectable by immunoassay (Table 3). Cell-associated IL-1 was also detected by immunoblotting with a polyclonal sheep anti-rat IL-1 antibody which reacts with murine IL-1α and IL-1β (data not shown). We then examined the function of IL-1 in the activation of T_h1 and T_h2 cells primed with antigen in the presence of PT. IL-1ra inhibited IL-4 and IL-5, but not IFN-γ production by KLH-specific T cells from mice immunized with KLH in the presence of 1 μg of PT (Fig. 5), which suggests that the stimulation of IL-1 production by PT may have a role in augmenting T_h2, but not T_h1 responses to co-injected antigens.

PT enhances the expression of the co-stimulatory molecules B7-1 and B7-2 on macrophages and B cells and their ligand CD28 on T cells

We investigated whether PT could up-regulate the expression of the co-stimulatory molecules B7-1, B7-2 and CD28, and thereby enhance activation of T_h1 or T_h2 cells. Mice were injected i.p. with PT in PBS or with PBS alone, and PEC were isolated 24 h later and examined for B7-1 and B7-2 expression by flow cytometry. *In vivo* treatment with PT enhanced the expression of B7-1 and B7-2; the mean fluorescence intensity and the percentage of positive cells were significantly higher in PEC from mice injected with PT compared with control mice which received PBS alone (Table 4).

Flow cytometric analysis of purified splenic B cells or macrophages stimulated with PT and PT mutants *in vitro* demonstrated that the effect of PT on B7 expression was independent of the S-1 activity, but was dependent on the B oligomer binding activity of the toxin. Active PT or the recombinant non-toxic S-1 mutant PT-9K/129G enhanced B7-1 and B7-2 expression on B cells and macrophages, whereas PTX-RENK, with mutations/deletions in the S-1 subunit and B oligomer component, had no effect (Fig. 6). Although B7-2 expression was significantly up-regulated on B cells, the most dramatic effect was seen with macrophages. Here the numbers of cells expressing B7-2 increased from 34 to 62–64% and B7-1 from 16 to 37–38% with PT or PT-9K/129G. PT and the S-1 mutant PT-9K/129G also enhanced expression of CD28 on T cells. The percentage of $CD3^+$ T cells showing

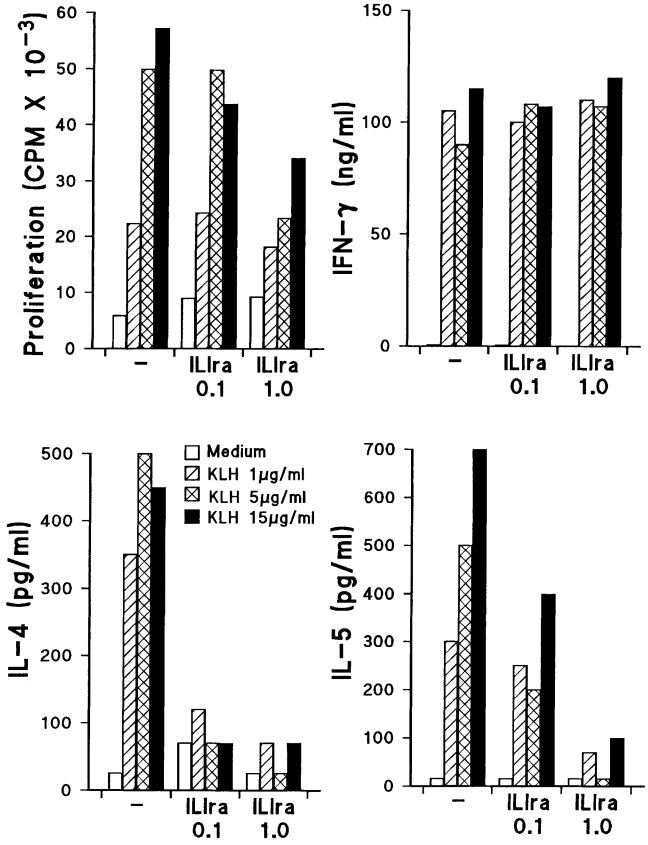

Fig. 5. Antigen-specific T_h2 responses augmented by PT are inhibited by IL-1ra. Spleen cells from mice immunized with KLH in the presence of 1.0 μg of PT were re-stimulated *in vitro* with KLH in the presence or absence of 0.1 or 1.0 μg/ml of IL-1ra and supernatants were removed after 72 h and tested for IFN-γ, IL-4 and IL-5. Results are means of triplicate assays.

Table 4. PT enhances B7-1 and B7-2 expression on peritoneal macrophages *in vivo*

Stimulus[a]	Percent positive cells (MFI)[b]	
	B7-1	B7-2
PBS	10 (75)	9 (88)
PT	66 (275)	74 (257)

[a]BALB/c mice were injected i.p. with PT (1.0μg) or PBS alone and PEC were isolated 24 h later.

[b]Expression of B7-1 and B7-2 was assessed by flow cytometry of cells labelled with anti-CD80 and CD86 respectively or with isotype-matched control antibodies (which labelled <3% positive cells for each sample). Results are expressed as percent positive cells and mean fluorescence intensities (MFI).

high density expression increased from 19 to 59 and 49% after 24 h incubation with active PT or the S-1 mutant respectively. In contrast PTX-RENK had a less dramatic effect, and was only observed at highest concentration tested (Fig. 6 and data not shown).

Discussion

T cells have been divided into distinct type 1 and type 2 subtypes of effector cells based on their function and the profile of cytokines they produce. The results of the present study demonstrate that PT can augment the induction of both of these populations of T cells *in vivo*. Co-injection of active PT with foreign antigens, even at relatively low doses, enhanced proliferation and secretion of IL-2, IFN-γ, IL-5 and in certain cases IL-4 by T cells specific for the antigen. Analysis of antibody responses against the foreign antigen demonstrated an enhancement of IgG1 and IgG2a sub-classes, thus providing further evidence that both T_h1 and T_h2 responses are augmented *in vivo*. Furthermore we demonstrated that the adjuvanticity of PT is dependent on receptor binding properties of the holotoxin, but that certain of the adjuvant effects can be dissociated from ADP-ribosyltransferase activity.

The mechanism of adjuvant action of PT and other bacterial toxins is poorly understood, but may involve potentiation of antigen presentation and T cell priming through increased regulatory or accessory cytokine production or co-stimulatory molecule expression. Previous studies have shown that PT induces proliferation of naive T cells and that the mitogenic activity of PT for T lymphocytes is dependent on non-T cells (32). The results of the present study have shown that active PT and an S-1 mutant stimulates IFN-γ and IL-2 production by T cells in the presence of MHC-matched or mis-matched accessory cells, but that a non-binding mutant lacks these effects. We have preliminary evidence that this property of

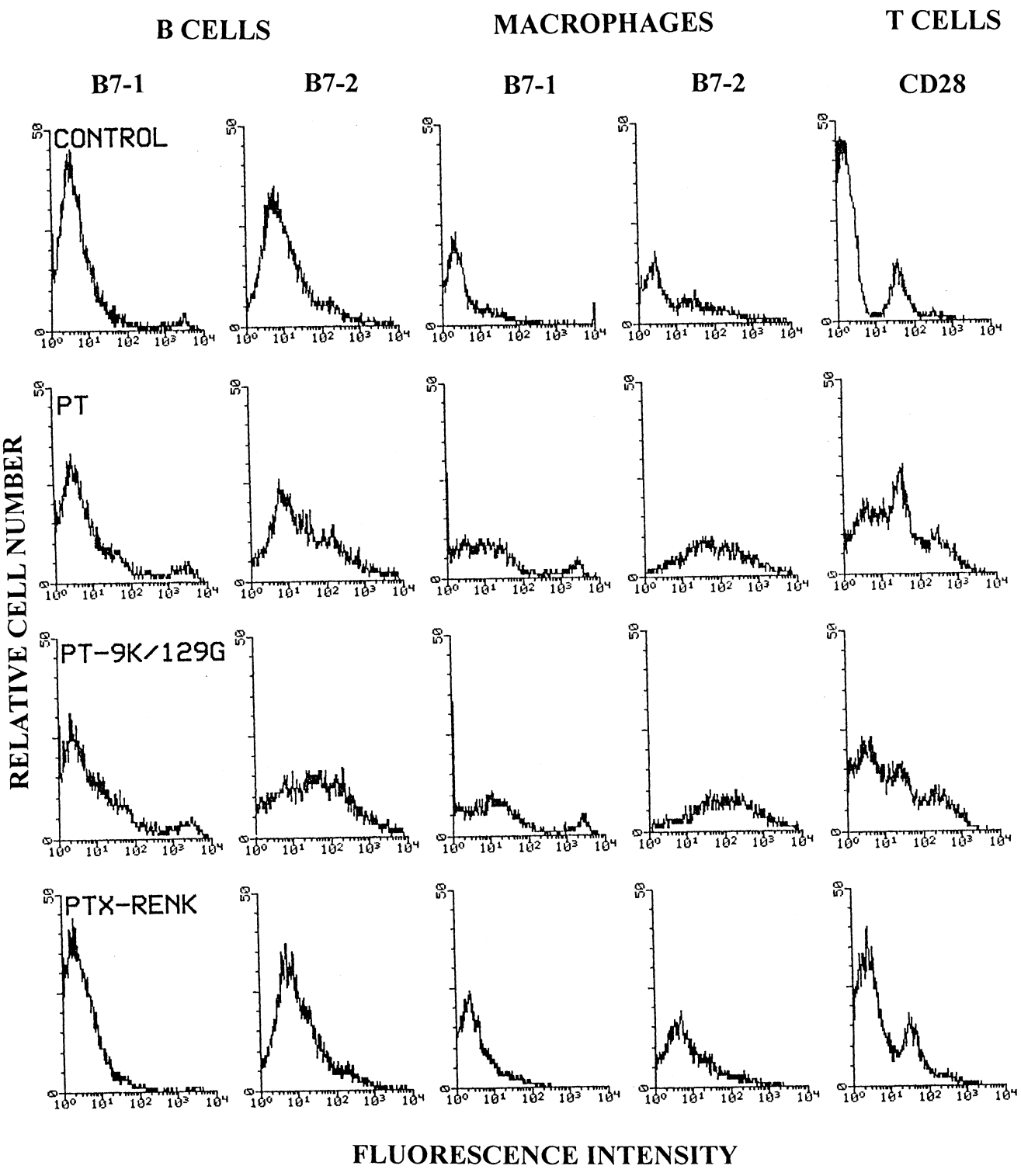

Fig. 6. PT and PT-9K/129G enhance the expression of B7-1 and B7-2 on B cells and macrophages and CD28 on T cells. Purified splenic B cells, T cells or macrophages from naive mice were stimulated *in vitro* with medium only (A) or 5 μg/ml of PT (B), PT-9K/129G (C) or PTX-RENK (D) and levels of B7-1, B7-2 and CD28 were assessed by flow cytometry after 24 h. Results are representative of four experiments.

the toxin may contribute to its adjuvanticity through the production of regulatory cytokines in the local lymphoid organ. Since IFN-γ enhances T_h1 development, polyclonal activation of T cells and local production of IFN-γ may selectively potentiate the induction of antigen-specific T_h1 cells *in vivo*.

Since IL-12 is a potent inducer of IFN-γ production by T

cells and selectively potentiates T_h1 responses to co-injected antigens (28,30,33), it is possible that the adjuvant effect of PT for T_h1 responses may be mediated by IL-12. However, we failed to detect bioactive IL-12 or IL-12p40 by immunoassay following stimulation of macrophages or spleen cells with PT. Furthermore, unlike *B. pertussis* lipopolysaccharide (28), PT-stimulated IFN-γ production by naive T cells was not IL-12 dependent, suggesting that IL-12 does not play a major role in the adjuvant properties of PT. However, since IFN-γ augments IL-12 production by macrophages, the synergistic effect of IFN-γ production by PT-stimulated T cells and IL-12 production by lipopolysaccharide-stimulated macrophages may explain the potent adjuvant properties of whole *B. pertussis* for T_h1-mediated responses, such as the induction of experimental autoimmune diseases. This is compatible with our previous reports that infection with *B. pertussis* or immunization with the whole cell pertussis vaccine is associated with potent activation of T_h1 cells in mice and in children (16,17,34).

We also provide evidence that the adjuvant effect of PT for T_h2 cells may in part be related to its ability to stimulate pro-inflammatory cytokine production. IL-1, which is secreted by macrophages in response to a range of stimuli, including endotoxin from Gram-negative bacteria, has been shown to have selective effects on T_h2 responses by acting as a accessory molecule in T cell activation (31). We found that PT stimulated IL-1 production by macrophages and blocking experiments with IL-1ra *in vitro* suggested that IL-1 may play a selective role in the enhanced T_h2 responses to antigens co-injected with PT.

Expression of the co-stimulatory molecules B7-1 and B7-2 on APC and their counter-receptor CD28 on T cells has a major regulatory influence on antigen presentation and T cell activation, especially for primary T cell responses (35–37). Macrophages and B cells have been shown to require activation signals to up-regulate expression of B7-1 and B7-2 in order to become competent APC for priming $CD4^+$ T cells (35,38); PT appears to provide such an activation signal. We have demonstrated that PT and the S-1 mutant up-regulates the expression of B7-1 and B7-2 on the surface of macrophages and B cells, and CD28 on T cells. In contrast, the S-1 and B oligomer mutated toxin PTX-RENK, which displayed no adjuvant properties, had little effect on co-stimulatory molecule expression. There have been conflicting reports on the roles of B7-1 and B7-2, and of distinct APC types in regulating the activation of the T_h1 and T_h2 developmental pathways. In certain systems it has been demonstrated that interaction between CD28 and B7-1 or B7-2 was selective for T_h1 and T_h2 activation respectively (36,37). However other studies have demonstrated that B7-1 and B7-2 may regulate both T_h1- and T_h2-mediated responses (35,39). The data from our study suggests that the capacity of PT to potentiate both T_h1- and T_h2-type responses may derive in part from the ability of the toxin to enhance the expression of B7-1 and B7-2 on both macrophages and B cells, which are considered to function as antigen-presenting cells for T_h1 and T_h2 cells respectively (40). Furthermore, enhancement of CD28 expression on T cells may play a role in the ability of PT to potentiate immune responses, not only by enhancing antigen presentation for proliferative expansion of antigen-activated T cells, but also by preventing their deletion by programmed cell death (41).

There have been conflicting reports on the role of ADP-ribosyltransferase activity in the adjuvant effects of PT, CT and LT (5–8,10). We have demonstrated that chemically detoxified PT and a genetic mutant with substitutions/deletions in the S-1 and B oligomer components that abrogate enzymatic and binding activity displayed no adjuvant properties. In contrast a non-toxic S-1 mutant devoid of enzymatic activity but still capable of receptor binding retained its adjuvanticity, augmenting the activation of T_h1 and T_h2 responses to co-injected antigens. However, there is indirect evidence to suggest that the B oligomer and the S-1 subunits may have distinct influences on T cell cytokine secretion through effects on alternative signal transduction pathways. It has been reported that cAMP up-regulates IL-4 and IL-5 production by activated $CD4^+$ T cells while down-regulating IL-2 production (42,43). The ADP-ribosyltransferase activity of the S-1 subunit augments cAMP production by inactivation of the inhibitory signalling to the adenylate cyclase complex (3). This component of the toxin may therefore have an additional affect on the activation of T_h2-type cells through enhanced IL-4 and IL-5 secretion, as has been reported for CT (7,43). However, a recent report has suggested that the adjuvanticity of CT for T_h2 responses can be dissociated from ADP-ribosyltransferase activity (5). Nevertheless, the role of the S-1 component of PT in the enhancement of T_h2 responses is consistent with the demonstration that enzymatically active PT is required to augment the production of IgE by human B cells *in vitro* (44). Conversely B oligomer-induced signal transduction through the inositol phosphate pathway results in the production of mRNA for IFN-γ and IL-2 (45), suggesting that this function of the B oligomer may have a selective effect in potentiating T_h1 responses.

Regardless of the role of the S-1 component, we have shown that receptor binding through the B oligomer is a critical activity in the adjuvant action of PT. Furthermore, the loss of adjuvant activity in chemical toxoided PT or genetic mutations in the B oligomer was associated with their failure to stimulate IL-2 and IFN-γ production by T cells, IL-1β production by macrophages, or to enhance B7-1 or B7-2 expression on B cells and macrophages. Since these properties were retained by the S-1 mutants, which also displayed immunomodulatory function, this provides indirect evidence that the adjuvant properties of PT may involve a combination of effects that result in enhanced antigen presentation and activation of T cells.

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Abbreviations

CT	cholera toxin
LT	*E. coli* heat-labile toxin

DTH	delayed-type hypersensitivity
FHA	filamentous haemagglutinin
IFN-γR$^{-/-}$	IFN-γ receptor disrupted (mice)
IL-1ra	IL-1 receptor antagonist
KLH	keyhole limpet haemocyanin
PEC	peritoneal exudate cells
PT	pertussis toxin
PTd	chemically detoxified pertussis toxin

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