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Co-stimulation of T cells via CD28 inhibits human IgE production; reversal by Pertussis toxin

C. T. M. VAN DER POUW-KRAAN, H. J. A. M. RENSINK, R. RAPPUOLI* & L. A. AARDEN
*CLB, Amsterdam, The Netherlands, and *IRIS, Siena, Italy*

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SUMMARY

In lymphocyte cultures, IgE production was achieved by stimulating T cells with anti-CD2 and IL-2. Here we show that anti-CD28, in the presence or absence of IL-2, reduces this IgE production approximately 10-fold. This inhibition of IgE production was almost completely reversed by Pertussis toxin (PT). PT had no effect on IgE production when the cells were stimulated in the absence of anti-CD28. No major effects of PT were found on IgM production. PT had no effect on purified B cells, stimulated with IL-4 and anti-CD40. In the presence of saturating amounts of rIL-4 similar results were obtained, albeit the absolute amounts of IgE produced were higher in all situations. Furthermore, PT-induced IgE production was still dependent on IL-4, as was evident from experiments in which anti-IL-4 was added to the culture. The IgE enhancing effect was dependent on the adenosine diphosphate (ADP)-ribosyltransferase activity of PT, because a mutant molecule lacking this activity was not able to restore anti-CD28-induced inhibition of IgE synthesis. Thus, we show that co-stimulation with anti-CD28 causes an inhibition of T cell-dependent IgE production by B cells, which inhibition can be specifically overcome by PT. An analysis of the molecular pathways underlying this phenomenon may contribute to our understanding of the regulation of IgE synthesis in (patho)physiological conditions.

Keywords Pertussis toxin IgE IL-4 CD28 CD2

INTRODUCTION

In vitro IgE production is dependent on the presence of IL-4 or IL-13 and B cell contact with activated T cells [1–3]. We analysed the effect of CD28 co-stimulation of T cells *in vitro*, an event very likely to occur *in vivo* as activated B cells are known to express several ligands for CD28 [4–6]. However, the effect of CD28 stimulation of T cells on IgE production has not been described. Here we show that co-activation of T cells with stimulating CD28 antibodies causes a strong inhibition of IgE production. This could be a mechanism to suppress IgE production *in vivo*, because under normal conditions IgE is found in extremely low concentrations compared with other subclasses of immunoglobulins. In mice and rats, the adjuvant effects for IgE production of the whole bacterium *Bordetella pertussis* (the etiological agent of whooping cough) or of the secreted Pertussis toxin (PT) have been well described [7–10]. As little as 0·1 ng PT increased IgE and IgG1 production to the antigen used for immunization [7]. The precise mechanism of action of PT on IgE production is not known.

PT consists of two functional subunits: the catalytic A promoter, S1, which catalyses transfer of the adenosine diphosphate(ADP)-ribose moiety of intracellular nicotinamide-adenine-dinucleotide (NAD) to host G proteins in several cell types [11–14], and the B oligomer, containing four different polypeptides, S2 to S5, mediating binding to the host tissue and allowing the A subunit to enter the cell [13]. On T lymphocytes two different mechanisms of action of PT can be distinguished: first, PT acts as a mitogen on murine and human T cells [11,15–17] and also on human B cells [15]. This activity can be achieved by the B subunit alone [11,15,16]; second, PT is able (via the activity of the A subunit) to ribosylate a 39–41-kD G protein in T cells [16–20]. The concentrations of PT required for the mitogenic and catalytic activities are separated by three orders of magnitude (μg/ml and ng/ml are required, respectively) [17].

In this study we show that PT also enhances *in vitro* human IgE production, but only in a condition where IgE production is suppressed, i.e. when T cells are activated through CD28. This finding allowed us to analyse the mechanism of PT on IgE production.

Correspondence: Lucien Aarden, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Plesmanlaan 125, 1066 AD, Amsterdam, The Netherlands.

MATERIALS AND METHODS

Lymphocyte purification

Lymphocytes were isolated from peripheral blood of normal

healthy volunteers by counterflow elutriation techniques [21] and contained <2% monocytes. B cells were purified from these lymphocytes by labelling the cells with a cocktail of MoAbs; anti-CD2 (CLBT11.2/1), anti-CD3 (CLB-T3/3), anti-CD4 (CLB-T4/1), anti-CD8 (CLB-T8/4), anti-CD16 (CLB-FcRgran1), anti-CD14 (CLB-CD14) followed by separation with immunomagnetic beads (Dynabeads-M450; Dynal A.S., Oslo, Norway) by means of a Dynal magnetic particle concentrator. Purity was tested functionally (Fig. 6) and by FACS analysis; purified B cells consisted of >90% $CD19^+$ cells and <2% $CD3^+$ cells.

Cytokines

Recombinant human IL-4 and IL-2, both *Escherichia coli*-derived, were gifts from Sandoz (Basel, Switzerland) and Hoffman-La Roche (Nutley, NY), respectively.

Culture conditions

Lymphocytes were preincubated with 0·1 μg/ml PT (Sigma, St Louis, MO) or PT-9K/129G, a mutant PT molecule, lacking the ADP-ribosyltransferase activity [22] at 2×10^6 cells/ml for 3 h at 37°C in culture medium: Iscove's modified Dulbecco's medium (IMDM), with 10% fetal calf serum (FCS) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 5×10^{-5} M 2-mercaptoethanol (2-ME) and 20 μg/ml human transferrin (Behringwerke, Marburg, Germany). Control cells were also kept at 37°C for 3 h. Thereafter lymphocytes were used for culturing without washing. In later experiments the preincubation step was replaced by addition of PT directly to the cell cultures at lower concentrations.

For immunoglobulin production, 5000 lymphocytes were cultured in the same culture medium, six-fold, in 200-μl round-bottomed wells (Greiner, Nuertingen, Germany) and stimulated with CD2 antibodies CLB-T11.1/1 plus CLB-T11.2/1 at 1 μg/ml, CD28 antibody CLB-CD28/1 at 5 μg/ml and rIL-2 at 50 U/ml. rIL-4 was used at 20–50 ng/ml, anti-IL-4 MoAb (CLB-IL4/6, clone 5B5) at 5 μg/ml. After 11 days of culture each well was tested for its immunoglobulin contents. For T cell-independent IgE production, purified B cells were stimulated with anti-CD40 (MoAb 89; a kind gift from J. Banchereau, Dardilly, France) at 1 μg/ml and rIL-4 (20 ng/ml). Supernatants of six-fold cultures were harvested at day 11 and each well was tested for its IgE contents.

Measurement of immunoglobulin production

IgE levels were determined, as described before [23], by a sandwich radioimmunoassay (RIA) using monoclonal CLB-IgE/1 and ^{125}I-labelled sheep anti-human IgE (CLB-SH/25). Purified human IgE (Behringwerke) was used as a standard. The detection limit of this assay is 100 pg/ml.

IgM production was measured using an IgM-specific one-step ELISA, as described before [24]. Microtitre plates (Dynatech) were coated with 1·25 μg/ml polyclonal anti-IgM (CLB-KH15). For detection, peroxidase-labelled monoclonal anti-IgM (CLB-MH15/HRP; 1 μg/ml) was used.

RESULTS

Inhibition of endogenous IL-4 dependent IgE production by anti-CD28

In human lymphocyte cultures, at low cell density, substantial amounts of IgE could be produced by stimulating the T cells with anti-CD2 and IL-2. When IL-2 was replaced by anti-CD28, about 10-fold less IgE was produced (Fig. 1a). This IgE production was dependent on endogenous IL-4 production, since it could be inhibited by anti-IL-4. The addition of rIL-4 enhanced IgE production, indicating that the amount of endogenous IL-4 was not optimal for IgE production. Either anti-CD2, anti-CD28, IL-2 or IL-4 alone did not stimulate immunoglobulin production (not shown). IgM production was three-to-four-fold less when IL-2 was replaced by anti-CD28 (Fig. 1b). rIL-4 had no effect on IgM production, but anti-IL-4 enhanced IgM production, indicating that the amount of endogenous IL-4, which was not sufficient for optimal IgE production, did maximally inhibit IgM production.

The low IgE production observed, when the cells were stimulated with anti-CD2, anti-CD28 and IL-4, was not due to a lack of IL-2, because IL-2 was sufficiently produced by the T cells under this stimulation condition (not shown), and the addition of rIL-2 did not alter IgE or IgM production (Fig. 1c).

PT reverses anti-CD28-induced inhibition of IgE production

The amount of IgE produced by peripheral blood lymphocytes (PBL) stimulated with anti-CD2 and IL-2 was not significantly altered when the cells were preincubated with PT (Fig. 2). In contrast, when IL-2 was replaced by anti-CD28, IgE production was about 10-fold less, and could now be enhanced by preincubating the cells with PT. This enhancement of IgE production was specific for isotype-switching because IgM production was not significantly altered by PT. When PT was added directly to the cultures, the amounts of IgE produced were comparable to the amounts of IgE found with the preincubation method. PT was found to enhance IgE production already at 0·8 ng/ml, and IgE production remained high up to 100 ng/ml of PT (not shown).

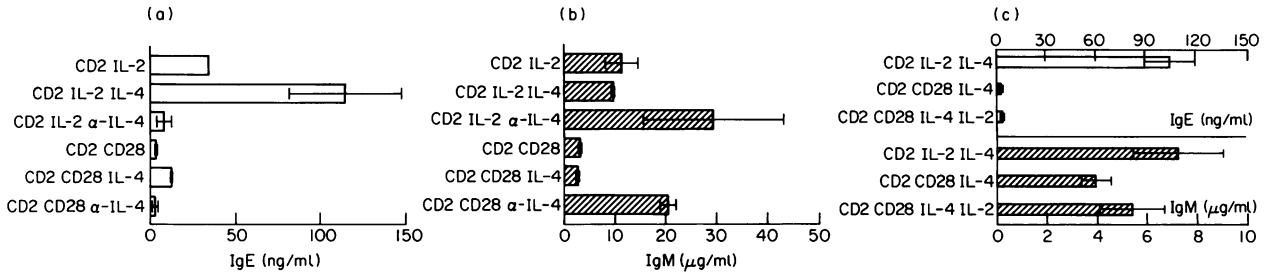

Fig. 1. IgE and IgM production by lymphocytes. Five thousand lymphocytes/200 μl well were stimulated as indicated. IL-4 was used at 50 ng/ml. Results are the mean production ± s.e.m. of IgE and IgM of three donors after 11 days of culture.

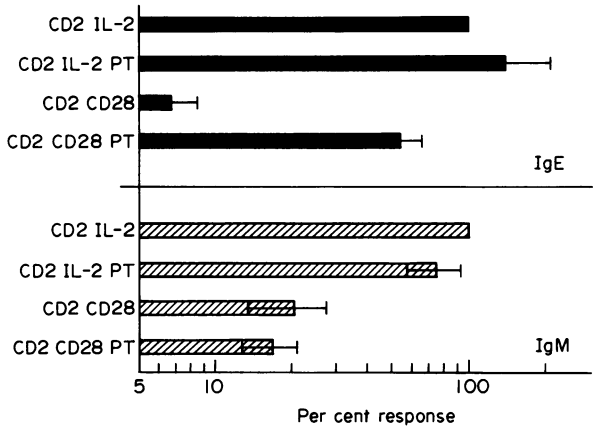

Fig. 2. Pertussis toxin (PT) enhances IgE production in absence of exogenous IL-4 by lymphocytes, stimulated with anti-CD2 and anti-CD28. Cells were preincubated with culture medium, 0·1 μg/ml PT, and thereafter stimulated with anti-CD2 and IL-2 or with anti-CD2 and anti-CD28. Mean per cent of IgE and IgM production of the anti-CD2 plus IL-2 stimulation is shown of four donors. Mean immunoglobulin production by lymphocytes, stimulated with anti-CD2 and IL-2, was 20·6 ng/ml IgE and 9·4 μg/ml IgM.

In the presence of saturating amounts of IL-4, PT was still able to reverse the anti-CD28 induced inhibition of IgE production. PT did not significantly alter cytokine production (IL-4, IL-2 and interferon-gamma (IFN-γ)) by lymphocytes that were stimulated with either anti-CD2 and IL-2 or with anti-CD2 plus anti-CD28 (not shown).

Because PT did not affect IL-4 production, we expected that PT would also enhance IgE production in the presence of saturating amounts of IL-4. This was indeed the case, as shown in Fig. 3. Although IgE production was about four times higher than in the situation without rIL-4 (compared with Fig. 2, see legend), the effect of PT was the same as in the situation without rIL-4; anti-CD28-induced inhibition of IgE

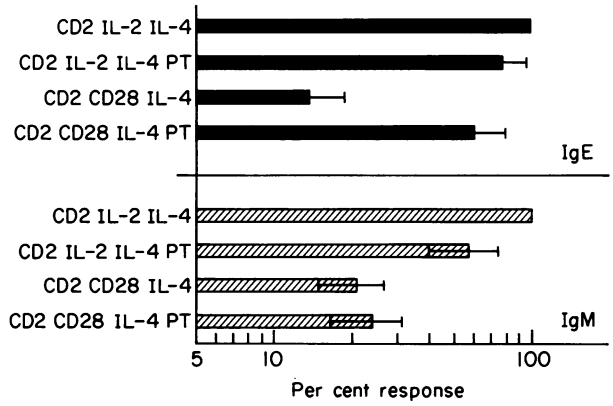

Fig. 3. IgE and IgM production in the presence of rhIL-4. Lymphocytes were preincubated with or without 0·1 μg/ml Pertussis toxin (PT) and thereafter stimulated with anti-CD2, IL-2 and IL-4, or anti-CD2, anti-CD28 and IL-4. Results obtained are from the same experiments as shown in Fig. 2. Mean per cent of IgE and IgM production of the anti-CD2, IL-2 and IL-4 stimulation is shown of four donors. Mean immunoglobulin production by lymphocytes, stimulated with anti-CD2, IL-2 and IL-4, was 80·6 ng/ml IgE and 9·5 μg/ml IgM, respectively.

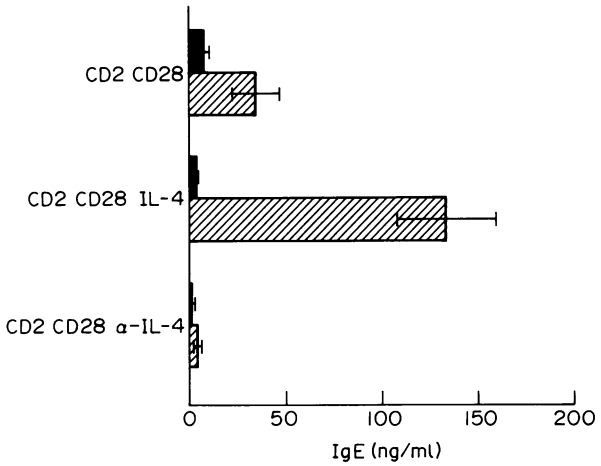

Fig. 4. Enhancement of IgE production by Pertussis toxin (PT) is dependent on IL-4. Lymphocytes were stimulated as indicated after preincubation with (▨) or without (■) 0·1 μg/ml PT. Anti-IL-4 was added at the start of the culture.

production was reversed by PT. IgM production was not altered by the addition of IL-4, and minor effects of PT were observed on IgM production.

IL-4 dependence of PT-induced enhancement of IgE production

To analyse further the role of IL-4 in PT-enhanced IgE production, we added blocking antibodies to IL-4 to lymphocytes stimulated with anti-CD2 and anti-CD28 (Fig. 4). Endogenous IL-4-induced IgE production was inhibited by anti-IL-4, also after preincubation with PT. Thus, the enhancing effect of PT on IgE production was totally dependent on the presence of IL-4.

The effect of PT on IgE production was not altered by the addition of IFN-γ or anti-IFN-γ (not shown). These results indicate that the enhancement of IgE production by PT was due neither to a decrease in IFN-γ production nor to interference with IFN-γ signalling.

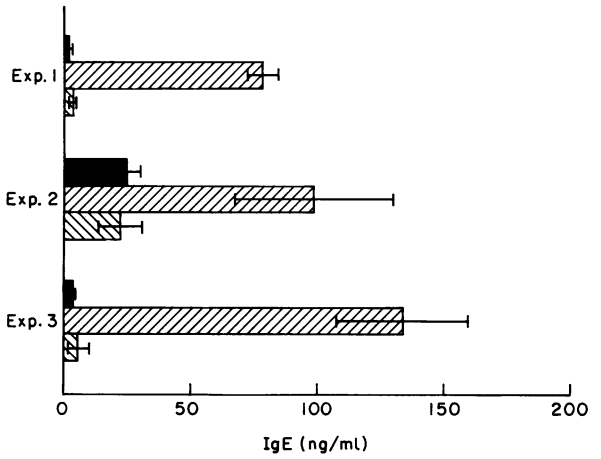

Fig. 5. Mutant PT-9K/129G does not enhance IgE production. Lymphocytes were stimulated with anti-CD2, anti-CD28 and IL-4. Pertussis toxin (PT) or PT-9K/129G (PTm) were used at 5 ng/ml (exp. 1 and exp. 2) or cells were preincubated with 0·1 μg/ml PT or PTm (exp. 3). ■, Without PT; ▨, PT; ▧, PTm.

ADP-ribosyl transferase activity is required for enhancement of IgE production

To dissect whether the ADP-ribosyl transferase activity of PT is necessary for the enhancement of IgE synthesis, we used a mutant PT molecule in which two amino acids have been altered in the S1 subunit [22]. As a consequence, the enzymatic activity of this molecule is lost, but the mitogenic activity is unimpaired [25]. In contrast to the wildtype molecule, this PT mutant was not able to enhance IgE production by lymphocytes stimulated with anti-CD2, anti-CD28 and IL-4 (Fig. 5). As expected, the mutant PT-9K/129G was capable to induce lymphocyte proliferation, in a manner comparable to the wildtype PT, but at a 1000-fold higher concentration than required for IgE enhancement (not shown).

PT has no effect on IgE production by purified B cells

Highly purified B cells stimulated with anti-CD40 and IL-4 were able to produce IgE in the absence of T cells (Fig. 6). To confirm that no T cells were present, the cells were also stimulated with anti-CD2, IL-2 and IL-4 or with anti-CD2, anti-CD28 and IL-4, which did not result in any IgE production. PT was not able to enhance IgE production by B cells stimulated with anti-CD40 and IL-4, but slightly inhibited IgE production in this system. The addition of anti-CD28 had no effect in this system.

DISCUSSION

Hitherto, the IgE-enhancing effect of PT has only been described *in vivo*, both for rodents [7–10] and for humans [26,27]. So far, attempts to show *in vitro* IgE-enhancing activity have not been reported. In this study we demonstrate that *in vitro* PT also enhances IgE production by human cells, which enabled us to study the effect of PT on human IgE production in more detail. Lymphocytes stimulated with anti-CD2 and IL-2 efficiently produced IgE. This production of IgE was inhibited by addition of anti-CD28. This held true whether

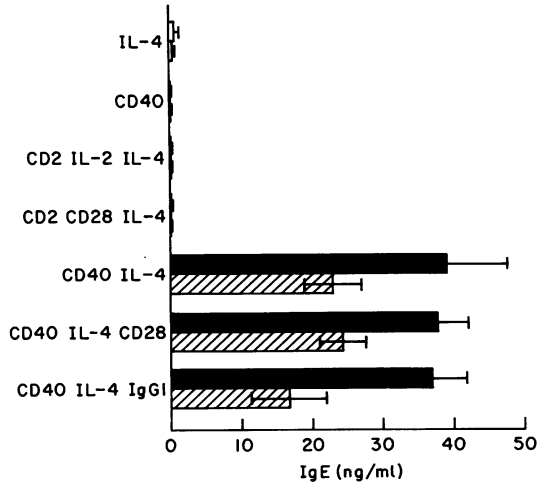

Fig. 6. Pertussis toxin (PT) does not enhance IgE production by highly purified B cells. B cells (5000 cells/200 μl well) were stimulated as indicated in the absence (■) or presence (▨) of PT (5 ng/ml). Anti-CD40 (MoAb 89) was used at 1 μg/ml, and the IgG1 control antibody at 5 μg/ml.

or not exogenous IL-4 was used, although in the presence of rIL-4, IgE production was higher in all stimulation conditions. PT only enhanced IgE production during CD28 co-stimulation. As activated B cells are known to express various ligands for CD28 [4–6], this effect of CD28 co-stimulation is likely to occur *in vivo*. Therefore we feel that inclusion of anti-CD28 better approaches the normal *in vivo* situation in which IgE production is suppressed. CD28 is present on plasma cells as well as T cells [28], but the fact that CD28 stimulation of purified B cells did not inhibit IgE production indicates that T cells are responsible for the inhibitory effects of CD28 stimulation on IgE production. At present, we do not know why CD28 co-stimulation inhibits IgE and to a lesser extent IgM production. Antibodies to CD28 have been reported to be potent co-stimulators for production of a variety of cytokines, such as IL-1, IL-2, IL-4, IL-6, tumour necrosis factor (TNF) and IFN-γ [29–34]. Possibly, the inhibition of immunoglobulin production is due to enhanced cytokine production, but involvement of an altered membrane protein expression on T or B cells cannot be ruled out. Interestingly, only the anti-CD28-induced inhibition of IgE and not of IgM production was reversed by PT. PT had no effect on IgE production in the absence of CD28 co-stimulation.

We observed some variation between different donors with respect to the degree of enhancement of IgE production by PT. In general, a strong inhibition of IgE production induced by CD28 co-stimulation also resulted in a strong enhancement of IgE production by PT during this stimulation.

The fact that PT only has an effect on IgE and not on IgM production raises the possibility that PT alters cytokine production that specifically affects IgE production. We therefore examined the influence of PT on IL-4, IL-2 and IFN-γ production. It has been shown previously that PT reduced IL-2 production by a concanavalin A (Con A)-stimulated T cell hybridoma [19]. In our hands, PT did not significantly alter IL-2, IL-4 or IFN-γ production, nor did PT alter proliferation induced by anti-CD2 and anti-CD28, or by anti-CD2 plus IL-2-stimulated lymphocytes (not shown). PT by itself was not able to induce cytokine production or proliferation at the concentrations used in our experiments. These results indicate that the reversal of inhibition of IgE production could not be ascribed to enhanced IL-4 or diminished IL-2 or IFN-γ production. Furthermore, in the presence of exogenous IL-4, IL-2 or IFN-γ, a similar enhancement of IgE production by PT was observed. Recently, it was described that the adjuvant effect of PT involved enhanced IL-4 production by spleen and lymph node cells 1 week after immunization of mice [9]. Possibly, in mice, PT inhibits the production or the signal transduction of cytokine(s) that diminish the development of IL-4-producing cells, analogous to IFN-γ [35], transforming growth factor-beta (TGF-β) [36], and IL-12 [37,38]. We observed the enhancing effect of PT on IgE production also in the presence of saturating amounts of IL-4, which suggests that the increased production of IL-4 *in vivo* is not the only mechanism of PT on IgE enhancement.

The target cells for PT might very well be T cells, because: (i) depending on the T cell stimulus, IgE production was enhanced by PT: only when CD28 co-stimulation was used was IgE production enhanced by PT; (ii) purified B cells stimulated with anti-CD40 and IL-4 with or without anti-CD28 did not show an enhancement, but rather an inhibition

of IgE production when PT was added. Removal of $CD8^+$ T cells or $CD16^+$ natural killer (NK) cells did not change the observed effects of anti-CD28 and PT (not shown). The fact that T cells would be the targets for PT fits with the observation that in nude rats there is no adjuvant effect of PT [10].

The lymphocyte receptor for PT has not been characterized. On Jurkat cells, a 43-kD protein has been identified which binds to PT [39]. Binding of PT to Jurkat cells was found to be diminished by sialidase treatment of the cells [40], indicating that PT binds to glycosylated residues on the cell surface.

We observed that PT was already active on IgE production at a concentration of 0·8 ng/ml, whereas the induction of lymphocyte proliferation required 1000-fold higher concentrations (not shown). Similar dose-dependent activities of PT were found for its enzymatic and mitogenic properties on T cells [17]. This suggests that the enzymatically active part of PT induces IgE enhancement. This suggestion was confirmed by our observation that an enzymatically inactive PT molecule PT-9K/129G [22] was unable to show an effect on IgE production. Wildtype PT and mutant PT-9K/129G were comparably mitogenic for lymphocytes (not shown). Also in mice, immunized with hen egg albumin, the adjuvant effect of PT on IgE and IgG1 production required amounts in the nanogram range [7,8]. Together with the results from this study, this suggests that the PT-induced enhancement of IgE, *in vivo*, will also depend on the enzymatic activity of PT.

In other cell types a 41-kD Giα protein, coupled to adenylate cyclase, has been shown to be the target for PT [11–14]. The ribosylation of this G protein results in abolition of its inhibitory activity on adenylate cyclase, and leads to increased cyclic adenosine-mono-phosphate (cAMP) production. Elevated levels of intracellular cAMP are known to inhibit T cell IL-2 and IFN-γ production [31,41]. Since we did not observe an inhibition of IL-2 and IFN-γ production, we have no indication that PT affects adenylate cyclase in T cells.

In conclusion, we describe a system in which PT is active at concentrations similar to those required *in vivo* [7,9]. The fact that PT reverses a CD28 co-stimulation-induced suppression in combination with the observed *in vivo* enhancement of IgE synthesis by PT suggests that under normal *in vivo* conditions IgE production is actively suppressed. Together with our observation that the enzymatic activity of PT is required for the IgE-enhancing effects this could provide a clue to dissect the mechanisms of IgE regulation under normal and pathological (allergy, parasitic infections) conditions.

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