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Interaction of Pertussis Toxin with Human T Lymphocytes

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The binding of pertussis toxin (PT) to the human T-cell line Jurkat was examined by using flow cytometry. Fluorescein isothiocyanate (FITC)-labeled PT bound rapidly to the cells in a specific manner as determined by blocking experiments with unlabeled toxin, B oligomer, and the S2-S4 and S3-S4 dimers. Monoclonal antibodies against the S3 subunit of the toxin also significantly inhibited the binding of FITC-PT. Sialidase treatment of the cells resulted in decreased binding of FITC-PT, indicating that sialic acid residues are involved in the binding process. In addition, we studied the effect of PT binding on the expression of cell surface molecules. On binding of PT to the cell surface, a rapid down-regulation of the T-cell receptor (TCR)-CD3 complex was observed. The modulation of the TCR-CD3 complex was independent of the toxin's enzymatic activity, as the B oligomer and a nonenzymatic toxin mutant induced modulation comparable to that caused by the native holotoxin. Isolated dimers did not cause down-regulation. Stimulation of the TCR-CD3 complex, leading to reduced cell surface expression of this complex, provides a possible explanation for the second messenger production associated with the interaction of PT or B oligomer with T lymphocytes. We therefore conclude that PT activates T cells by divalent binding to the TCR-CD3 complex itself or by binding a structure closely associated with it.

Pertussis toxin (PT), one of several toxins produced by *Bordetella pertussis*, the etiological agent of whooping cough, has the A-B structure characteristic of other bacterial toxins (25). The toxin comprises an enzymatically active A subunit (S1) and a B oligomer, made up of five subunits (S2-S4 and S3-S4 dimers connected by S5), which is responsible for binding to the target cell (25, 26). Both dimers have been implicated in the binding process (26), and there is evidence of different binding specificities (12, 31).

PT shows a variety of biological effects which results from binding to cells, internalization, and subsequent ADP-ribosylation of a family of GTP-binding regulatory proteins (G proteins). The effects of the toxin on cells of the immune system are multiple and include induction of lymphocytosis, inhibition of macrophage migration, adjuvant activity, and T-cell mitogenicity (10). The mitogenic action of the toxin appears to be independent of the enzymatic subunit, as the B oligomer is able to mimic the effects of the holotoxin (26).

The interaction of PT or B oligomer with T lymphocytes results in the rapid intracellular accumulation of Ca^{2+}, an increase in the levels of inositol triphosphate and diacylglycerol, and activation of protein kinase C and a tyrosine protein kinase (6, 17, 24, 27).

In this study, we have examined the interaction of PT with human T lymphocytes, using the transformed human T-cell line Jurkat and flow cytometry in order to better understand which parts of the toxin are involved in binding. We also studied the effect of PT binding on the expression of T-cell surface markers that are involved in T-cell activation.

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MATERIALS AND METHODS

Purification of PT, B oligomer, and dimers. PT was purified from the culture supernatant of fermentor-grown *B. pertussis* by Affi-Gel blue affinity chromatography and then by fetuin-Sepharose affinity chromatography (19). B oligomer was prepared as described previously (2). S2-S4 and S3-S4 dimers were purified from the native holotoxin on a CM-Sepharose column in a 5 M urea buffer as described previously (25). PT-9K/129G, a nonenzymatic mutant (13), was kindly provided by Rino Rappuoli, Sclavo Research Center, Siena, Italy.

FITC labeling of PT. While bound to fetuin, PT was labeled with fluorescein isothiocyanate (FITC) in a manner similar to the method used for radioiodination of the toxin (3). Briefly, PT (3 mg) bound to 4.5 ml (bed volume) of fetuin-Sepharose 4B in 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)–1 M NaCl (pH 8.5) was incubated with a 400-fold molar excess (12 μmol) of FITC (BBL Microbiology Systems, Cockeysville, Md.) for 2 h at room temperature. The fetuin-Sepharose suspension was washed four times with the reaction buffer, and FITC-PT was eluted with 50 mM Tris–4 M $MgCl_2$ (pH 7.8) and dialyzed extensively against 50 mM Tris–0.5 M NaCl (pH 7.8). FITC incorporation was determined by measurement of the A_{492} of the FITC-PT preparation and was found to be 2.9/1 (FITC/PT) on a molar basis. The biological activity of the labeled toxin was tested in the CHO assay (7), and its ability to induce T-lymphocyte proliferation was determined as described previously (26).

MAb. Monoclonal antibodies (MAb) produced against PT subunits have been described previously (14). The MAb used in this study were 151-C1, anti-S1; 2B2, anti-S2; 170-C4, anti-S3; and 227, anti-S4. Additional anti-PT MAb (18C9-H3, anti-S2; and 2131G7, anti-S3) were prepared in the same manner (8a). Immunoglobulin G (IgG) was purified from ascites by Na_2SO_4 precipitation and by protein A-Sepharose (Pharmacia, Uppsala, Sweden) chromatography ac-

cording to the manufacturer's procedure. Purified IgG of the CD28 MAb CLB.CD28/1 (28) was a gift from Rene van Lier, Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands. Anti-T cell receptor (TCR) (WT31), FITC-labeled anti-TCR, CD3 (Leu-4), phycoerythrin (PE)-labeled CD3, CD2 (Leu-5b), FITC-labeled CD2, CD5 (Leu-1), PE-labeled CD5, CD45 (Hle-1), and FITC-labeled CD45 were purchased from Becton Dickinson (Mountain View, Calif.).

Cells. The human T-cell leukemia cell line Jurkat was maintained in Iscove's modified Dulbecco's medium supplemented with 5% (vol/vol) heat-inactivated pooled human AB serum, 40 μM 2-ME, 100 IU of benzylpenicillin per ml, and 100 μg of streptomycin per ml in a 37°C, 5% CO_2-humidified incubator. Unless stated otherwise, Jurkat cells were fixed in 1% paraformaldehyde in Iscove's medium and stored at 4°C before being used in experiments. Peripheral blood lymphocytes from healthy human volunteers were isolated on a Ficoll-Hypaque gradient and contained an average of 95% T lymphocytes.

PT-binding assay. Jurkat cells (5×10^5) were incubated with FITC-PT in Iscove's medium with 0.5% bovine serum albumin (BSA) in a total volume of 0.5 ml. The cells were washed twice in ice-cold medium and phosphate-buffered saline (PBS), and flow cytometry analysis was performed.

Alternatively, cells were incubated with unlabeled PT or B oligomer and then with a rabbit anti-PT serum and FITC-labeled goat anti-rabbit Ig (Becton Dickinson) to detect binding.

Competition assay. Jurkat cells (5×10^5 per sample) were preincubated with unlabeled PT, PT-9K/129G, B oligomer, or dimers in Iscove's modified medium containing 0.5% BSA for 30 min at 37°C or on ice. FITC-PT (1 μg/ml) was then added, and the cells were incubated for an additional 15 min at 37°C or on ice. Cells were washed, and fluorescence was determined as described above.

Inhibition assay. FITC-PT (1 μg/ml) was incubated with increasing concentrations of purified anti-PT MAb for 1 h at 37°C, Jurkat cells (5×10^5) were added, and incubation was continued for an additional 30 min at 37°C. Cells were washed twice, and FITC-PT binding was determined.

Sialidase treatment of cells. Jurkat cells (5×10^6) were incubated with 2.5 U of sialidase type V (Sigma Chemical Co., St. Louis, Mo.) in 5 ml of PBS–1 mM $CaCl_2$ for 30 min, washed, and then incubated with FITC-PT for an additional 30 min. Control cells were treated identically, but no sialidase was added. Cells were washed again, and FITC-PT binding was determined.

Modulation of cell surface antigens. Unfixed Jurkat cells (10^6/ml) were incubated with PT, B oligomer, or dimers at a concentration of 2 μg/ml for various times in Iscove's medium containing 0.5% BSA at 37°C. Cells were washed two times in ice-cold medium, and FITC- or PE-labeled anti-T cell surface antigen MAb were added. Cells were incubated for 30 min on ice and washed twice, and expression of the surface antigens was determined. In those instances in which no directly labeled MAb was available, FITC-labeled goat anti-mouse Ig (Becton Dickinson) was added in a second step to detect binding of the MAb.

Flow cytometry. Cell suspensions were analyzed by using FACScan (Becton Dickinson) equipped with a 15 mW argon ion laser (488 nm). Fluorescence intensity was measured on a logarithmic scale. Data were analyzed with the Consort 30 and LYSIS software packages (Becton Dickinson). Fluorescence was expressed as the mean fluorescence value of 10^4 cells or as the percent binding of FITC-PT, calculated as $[(A - bg)/(B - bg)] \times 100\%$, where A is the mean fluorescence of cells incubated with blocking agents and FITC-PT, B is the mean fluorescence of cells incubated with FITC-PT only, and bg is the background fluorescence of the cells. All experiments were performed at least in duplicate with standard deviations below 10%. Representatives of two or more experiments are shown.

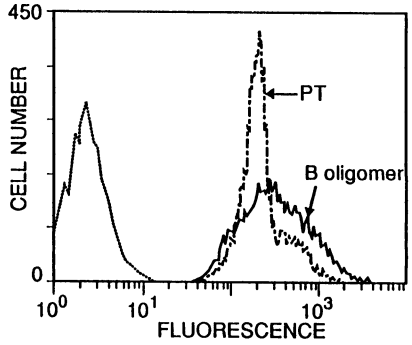

FIG. 1. Binding of PT and B oligomer to peripheral blood lymphocytes. Unfixed peripheral blood lymphocytes (10^6/ml) were incubated with PT or B oligomer (both at a concentration of 1 μg/ml) for 45 min on ice. After the cells were washed, binding of PT was determined by flow cytometry after incubation with an anti-PT serum and a FITC-labeled second antibody as described in Materials and Methods.

RESULTS

Binding of FITC-PT to Jurkat cells. We were able to detect binding of PT and B oligomer to human T cells by using flow cytometry (Fig. 1). To facilitate inhibition and competition experiments, PT was directly labeled with FITC. In order to maintain optimal biological activity of PT after FITC labeling, the toxin was labeled while bound to the model receptor fetuin. This procedure yielded FITC-PT which retained 25 to 50% of CHO cell clustering and mitogenic potencies. PT labeled without fetuin protection, however, kept only 6.25% of its biological activity, as measured by those two assays, and was not used further. Throughout our experiments, PT was used in the concentration range of 0.5 to 5 μg/ml, concentrations at which the toxin is a mitogen and causes rapid second messenger production in T cells (6, 17, 24, 27).

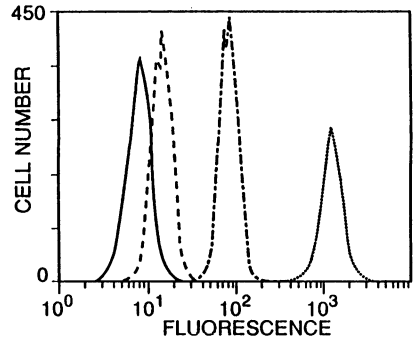

FIG. 2. Binding of FITC-PT to Jurkat cells. Unfixed Jurkat cells (10^6/ml) were incubated with FITC-PT at concentrations of 0.05 (– – –), 0.5 (— · —), and 5.0 (. . . . .) μg/ml for 30 min on ice. The cells were washed, and FITC-PT binding was detected with a fluorescence-activated cell sorter (FACS) as described in Materials and Methods. ——, negative control.

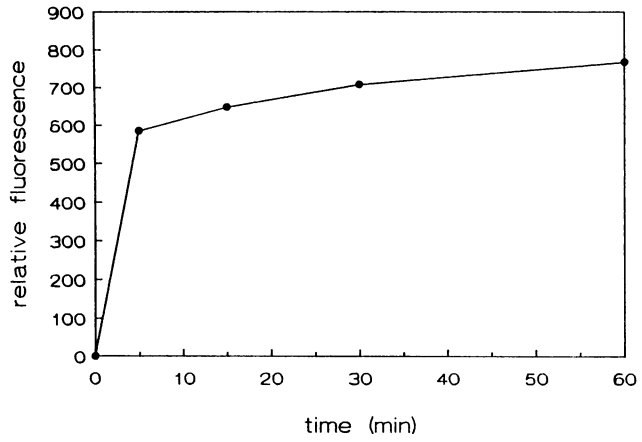

FIG. 3. Time course of FITC-PT binding to Jurkat cells. Paraformaldehyde-fixed Jurkat cells (10^6/ml) were incubated with FITC-PT (1 μg/ml) for the indicated times at 37°C. After the cells were washed, the binding of FITC-PT was determined by flow cytometry analysis as described in Materials and Methods.

At those concentrations, FITC-PT bound efficiently to the human T-cell line Jurkat (Fig. 2). Concentrations above 10 μg/ml caused aggregation of the cells, making flow cytometry analysis impossible. FITC-PT bound rapidly to Jurkat cells, and near maximal binding at a 1-μg/ml concentration was obtained within 15 min of incubation (Fig. 3).

To determine whether the binding of FITC-PT was specific, competition experiments were performed with unlabeled PT, B oligomer, S2-S4 and S3-S4 dimers, and PT-9K/129G, a genetically inactivated PT mutant lacking ADP-ribosyltransferase activity. As shown in Fig. 4, binding of FITC-PT to Jurkat cells could be blocked by incubation of the cells with PT, B oligomer, and PT-9K/129G prior to the addition of FITC-PT, both at 37°C and on ice. The dimers showed substantial blocking of FITC-PT binding only when the experiment was performed on ice.

TABLE 1. Inhibition of FITC-PT binding to Jurkat cells by anti-PT MAb[a]

MAb (specificity)	Binding of FITC-PT (%) at MAb concn (μg/ml) of:		
	1	5	10
151C1 (S1)	97.9	94.7	89.0
2B2 (S2)	91.3	86.6	69.8
18C9H3 (S2)	100.5	101.5	95.5
170C4 (S3)	83.6	31.2	23.5
2131G7 (S3)	75.0	47.8	39.2
227 (S4)	98.7	97.3	105.8

[a] FITC-PT (1 μg/ml) was incubated with anti-PT MAb for 1 h at 37°C before being added to fixed Jurkat cells. After 30 min at 37°C, the cells were washed, and FITC-PT binding was determined by flow cytometry analysis.

Inhibition of FITC-PT binding to Jurkat cells with anti-PT MAb. We also tested anti-PT MAb for their ability to interfere with FITC-PT binding to Jurkat cells. The two MAb against the S3 subunit (170C4 and 2131G7) inhibited the binding of FITC-PT to Jurkat cells by 76.5 and 60.8%, respectively, at the highest concentration tested (Table 1). At that concentration, the only other MAb to cause substantial inhibition was 2B2 (anti-S2), which blocked FITC-PT binding by 30.2%. The MAb that do not inhibit the binding of FITC-PT to Jurkat cells also do not interfere with other activities of the toxin, whereas 170C4 and 2131G7 block PT-induced clustering of CHO cells and 2B2, 170C4, and 2131G7 inhibit agglutination of goose erythrocytes by PT (reference 30 and unpublished observations).

Influence of sialidase treatment of Jurkat cells on FITC-PT binding. PT is known to have lectin-like properties since it can agglutinate erythrocytes and can bind to glycoproteins (1, 19, 25, 31). In order to determine whether the binding of PT to the Jurkat cell line is dependent on lectin-like binding, we incubated the cells for 30 min with sialidase. As shown in Fig. 5, the binding of FITC-PT to Jurkat cells was strongly reduced after sialidase treatment, indicating that sialic acid

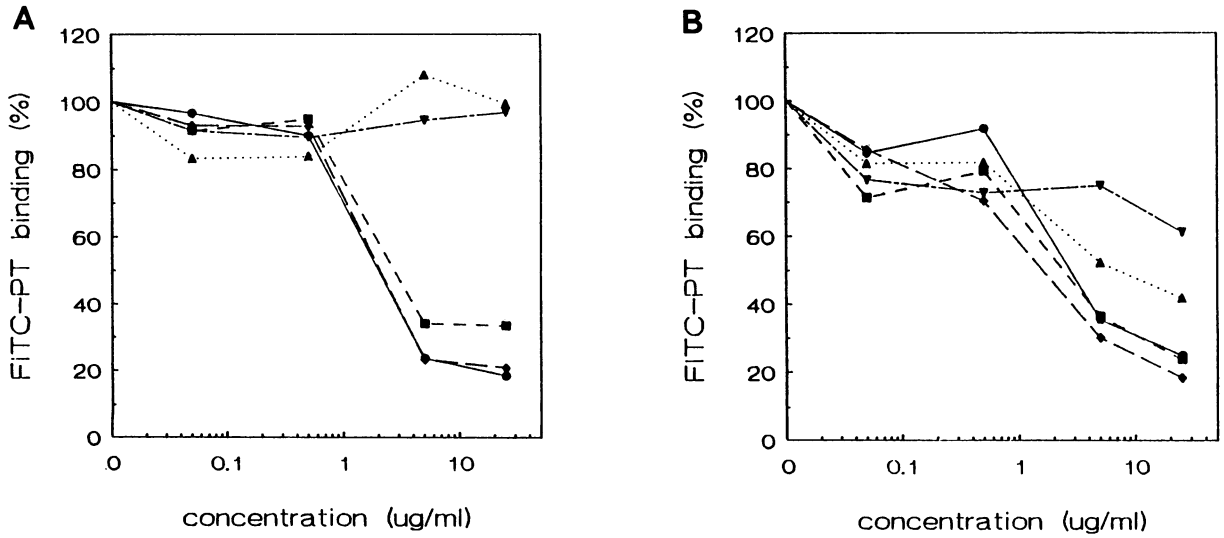

FIG. 4. Blocking of FITC-PT binding to Jurkat cells at 37°C (A) or on ice (B). Fixed Jurkat cells were preincubated with unlabeled PT, PT-9K/129G, B oligomer, or dimers for 30 min, FITC-PT (final concentration, 1 μg/ml) was added, and incubation was done for an additional 15 min. The binding of FITC-PT was determined by FACS analysis after the cells were washed. ●, PT, ■, PT-9K/129G; ◆, B oligomer; ▲, S2-S4; ▼, S3-S4.

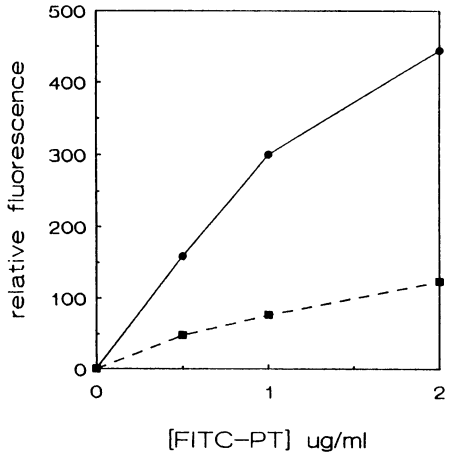

FIG. 5. Influence of sialidase treatment on the binding of FITC-PT to Jurkat cells. Jurkat cells (5×10^6) were incubated with 2.5 U of sialidase for 30 min. Sialidase treated and control cells were then incubated with FITC-PT for an additional 30 min, and binding of FITC-PT was analyzed with an FACS. ●, control; ■, sialidase.

residues on cell surface macromolecules are needed for the binding of PT to Jurkat cells.

Influence of PT on the cell surface expression of the TCR-CD3 complex. Treatment of unfixed Jurkat cells with PT or the B oligomer at a 2-μg/ml concentration caused a reduction in the expression of the TCR-CD3 complex (Fig. 6). After a 60-min incubation with PT, expression of the complex was reduced to approximately 60% (values ranging between 48.7 and 64.8% in two independent experiments performed in duplicate).

Incubation with the B oligomer for the same period of time resulted in a reduction to approximately 40% (range, 27.9 to 45.8%). It must be noted, however, that the B oligomer was used at a higher molar concentration in those experiments. The down-regulation of the TCR-CD3 complex by PT and B oligomer was comparable to that observed with the anti-TCR MAb at a 0.5-μg/ml concentration and could be observed after just 1 min of PT treatment (data not shown). The expression of CD2 remained around 100% with both PT and B oligomer. Incubation with a combination of the S2-S4 and S3-S4 dimers at equal concentrations induced only a slight decrease in the expression of TCR and CD3. Incubation of cells with the individual dimers, even at a concentration of 10 μg/ml, had no effect on the expression of the TCR and CD3 (data not shown). To rule out that the observed decrease in the binding of anti-TCR and anti-CD3 MAb to the cells was caused by steric hindrance as a result of PT binding rather than by the loss of surface expression of the TCR-CD3 complex, we incubated formaldehyde-fixed Jurkat cells with PT at the same concentration (2 μg/ml) and then with the anti-TCR and anti-CD3 MAb. No reduction in the binding of the anti-TCR and anti-CD3 MAb compared with that of control cells was measured after prior incubation with PT (data not shown), demonstrating that with unfixed cells the observed effect on TCR and CD3 binding by MAb was indeed caused by down-regulation. We also analyzed the influence of PT on the surface expression of other T-cell antigens. After 30 min of treatment with PT or PT-9K/129G, little or no decrease in the expression of CD4, CD5, CD28, and CD45 was observed (Table 2). Results similar to those seen with the Jurkat cell line were obtained with freshly isolated peripheral blood lymphocytes (data not shown).

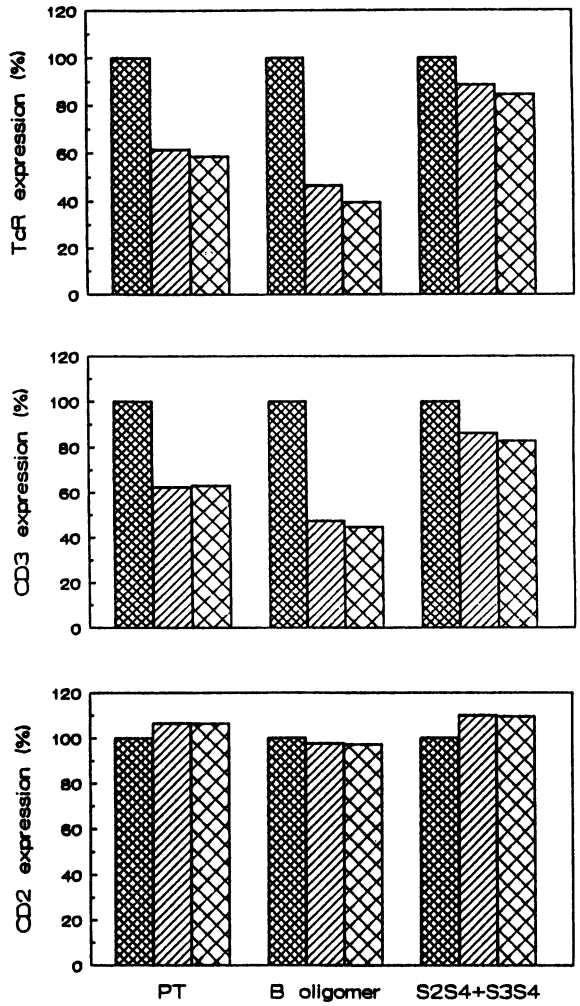

FIG. 6. Influence of PT, B oligomer, and dimers on the expression of TCR-CD3 and CD2 on Jurkat cells. Jurkat cells were incubated with PT, B oligomer, or a combination of the S2-S4 and S3-S4 dimers (each at a concentration of 2 μg/ml) for 0 (▩), 15 (▨), or 60 (⊠) min at 37°C. Cells were washed, and the expression of the indicated antigen on the cell surface was determined by flow cytometry analysis. Results are given as the percentage of expression compared with that of untreated cells.

DISCUSSION

In this study, we have investigated the binding of PT to the human T-cell line Jurkat. The binding of FITC-PT was specific in that unlabeled toxin and B oligomer blocked binding in a dose-dependent manner at both 4 and 37°C. The S2-S4 and S3-S4 dimers, constituents of the B oligomer, showed significant blocking at 4°C. Binding sites on both dimers therefore seem to play a role in the interaction of PT with T lymphocytes, thereby providing divalent binding of the toxin to the cell surface. Recently, it has been shown that both S2 and S3 contain eucaryotic-like carbohydrate recognition domains (18). Both subunits were also found to be involved in the binding of PT to human macrophages (29).

Of the anti-PT MAb tested, the two directed against the S3 subunit showed the strongest inhibition of FITC-PT binding, emphasizing the role of this subunit in the binding to T lymphocytes. These two MAb also inhibit the toxin's mitogenic action (8a), and MAb 170C4 is able to protect neonatal

TABLE 2. Influence of incubation of Jurkat cell line with PT on expression of cell surface markers[a]

Surface antigen	Expression[b] of surface molecules after incubation with:	
	PT	PT-9K/129G
TCR	60	54
CD2	113	116
CD3	66	62
CD4	103	ND[c]
CD5	108	103
CD28	89	ND
CD45	101	ND

[a] Jurkat cells (10^6/ml) were incubated with PT or PT-9K/129G (both at 1 μg/ml) for 30 min at 37°C. Cells were washed, and the expression of the indicated antigens was determined by flow cytometry as described in Materials and Methods.

[b] Percentage of untreated cells.

[c] ND, not done.

mice from a lethal respiratory challenge with *B. pertussis* (20). Additional evidence for the role of this subunit in the interaction with lymphocytes comes from chemical modification studies by Nogimori et al. (12), who showed that methylation of lysine residues in the S3-S4 dimer abolished the toxin's mitogenic, adjuvant, and lymphocytosis-promoting activities. MAb 2B2 (anti-S2) inhibited FITC-PT binding by 30.2% at the highest MAb concentration tested, compared with up to 76.5% inhibition by anti-S3 MAb.

Treatment of Jurkat cells with sialidase caused a strong reduction in FITC-PT binding, suggesting that the receptor for PT is a glycoconjugate. This is consistent with the previous result that sialidase treatment abolished binding of PT to its putative receptor on CHO cells (4). Also, PT was unable to ADP-ribosylate G proteins in variant CHO cell lines exhibiting sialic acid deficiencies on their cell surface macromolecules, indicating that sialic acid residues are essential for optimal functional binding of PT (31). Sialic acid has also been shown to block the S3-mediated binding of PT to human macrophages (29).

The observed rapid internalization of the TCR-CD3 complex can be the result of direct interaction of PT with the complex or a secondary effect from the toxin binding to other cell surface molecules closely associated with it. Modulation of the TCR-CD3 complex cannot be the result of the toxin's enzymatic activity since the isolated B oligomer and PT-9K/129G, an enzymatically inactive PT mutant, gave down-regulation comparable to that observed with the native toxin. Divalent binding seems to be essential, since the individual dimers were unable to mimic the effect of the holotoxin. The finding that the combination of dimers was able to induce some modulation may be explained by the forming of multimeric complexes of S2-S4 and S3-S4 even in the absence of the S5 subunit.

Cholera toxin, another bacterial ADP-ribosyltransferase that interferes with normal T-cell functioning (8), can also modulate the TCR-CD3 complex (22). In contrast to the B oligomer of PT, the binding subunit of cholera toxin alone is not able to induce down-regulation of the TCR-CD3 complex, indicating that cholera toxin's enzymatic activity is required (22). Furthermore, a 60-min lag between the addition of cholera toxin and the initiation of down-regulation was observed (21).

The down-regulation caused by the toxin is similar to that caused by anti-TCR MAb, and the rapid second messenger production in Jurkat cells on interaction with PT has been reported to be indistinguishable from that induced by direct stimulation of the TCR-CD3 complex with MAb (6, 17). An argument against direct binding of PT to the TCR-CD3 complex on Jurkat cells comes from two receptor isolation studies with photoaffinity-labeling reagents. Clark and Armstrong (5) isolated a 70-kDa protein, whereas Rogers et al. (16) detected binding of PT to a 43-kDa protein. The 43-kDa protein was apparently not one of the TCR chains, as binding to this protein was also observed in a TCR-CD3$^-$ Jurkat cell line. Our observation that the S2-S4 dimer is also involved in binding to lymphocytes is supported by the finding of the latter group, who reported binding of the S2-S4 dimer to the 43-kDa protein. The differences in the receptors isolated by the two groups may be explained by differences in labeling techniques, and the possibility that both proteins are part of the same multimeric complex cannot be excluded. If they are indeed functional receptors for PT, they must be closely associated with the TCR-CD3 complex in order to explain its down-regulation following PT binding. CD2, CD5, CD28, and CD45, other cell surface molecules associated with activation, do not seem to be targets for PT as they are not rapidly modulated after binding of PT to the cells.

Although activation of the TCR-CD3 complex offers an explanation for the rapid second messenger production observed upon incubation of T lymphocytes with PT and for the in vitro mitogenicity of the toxin, the relevance of the observed rapid modulation of the TCR-CD3 complex for the effects of PT on cells of the immune system in vivo awaits further study.

Lymphocytosis, the increase in peripheral blood lymphocyte count, a characteristic sign of pertussis disease, does not seem to be caused by proliferation of T lymphocytes but rather by effects of the toxin on T-cell mobilization (9, 23). Furthermore, the ADP-ribosyltransferase activity of the toxin is required for the induction of lymphocytosis, since the enzymatically deficient PT-9K/129G mutant is unable to cause lymphocytosis in mice (13). PT-9K/129G, however, is still as potent a mitogen as the native toxin (11) and is also able to modulate the TCR-CD3 complex (this study). Aggregation of the TCR-CD3 complex on the cell surface is required for the activation of the signal transduction apparatus in T cells (15). PT could fulfill this requirement by binding different sites on the complex or molecules closely associated with it, as we know that the S2-S4 and S3-S4 dimers differ in binding properties (31).

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