0031-3998/89/2502-0209$02.00/0
PEDIATRIC RESEARCH

Vol. 25, No. 2, 1989
Printed in U.S.A.

Impaired Formation of the Second Messenger cAMP in Mononuclear Blood Cells of Children with Pertussis

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ABSTRACT. To determine the pathophysiologic significance of pertussis toxin (PT) on the human β-adrenergic system, β-adrenoceptor (β-R)-density and cyclic AMP response of peripheral mononuclear blood cells (MN leukocytes) were studied in children during the paroxysmal stage of natural pertussis infection, after vaccination with pertussis monovaccine, and in controls. Isoprenaline-induced cAMP accumulation, measured in a protein-binding assay, was significantly reduced to about 25–50% in children during the first week of paroxysmal pertussis, compared with controls. In contrast, cAMP accumulation after stimulation of the adenylyl cyclase by forskolin was unaffected. The density and affinity of β-R, estimated by ^{125}I-cyanopindolol-binding studies, were not significantly altered. The suggestion that PT might impair the coupling of the β-R signals to the adenylyl cyclase was confirmed by parallel *in vitro* studies on MN leukocytes from adults. These cells, when incubated with purified PT, showed a significantly diminished cAMP response to isoprenaline, whereas that to forskolin remained unaffected. As cAMP accumulation in response to prostaglandin E_1 and hydrocortisone was also reduced, it appears that PT may directly affect G-proteins serving as signal transducers for several stimulatory receptors. In contrast to the actual disease, in children treated with pertussis monovaccine, cAMP accumulation as well as the β-R were unaffected. It is concluded that in MN leukocytes obtained from children during the natural course of pertussis, as well as after incubation of normal MN leukocytes with PT, the stimulatory signal-transducing system for cAMP generation is inhibited. (*Pediatr Res* 25:209–213, 1989)

Abbreviations

β-R, β-adrenoceptor
IPN, isoprenaline
MN leukocytes, mononuclear white blood cells
PT, pertussis toxin
^{125}ICYP, ^{125}I-cyanopindolol
PGE_1, prostaglandin E_1

It is generally believed that pertussis infection is a toxin-mediated disease (1). The causative agent of whooping cough in humans, *Bordetella pertussis*, produces at least four distinct toxins, heat-labile toxin, lipopolysaccharide endotoxin, tracheal cytotoxin, and PT (2, 3).

Received June 28, 1988; accepted October 13, 1988.
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Supported by Grant Re 509/4-1 from the Deutsche Forschungsgemeinschaft.
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The major virulence determinant is PT, which is assumed to be responsible for some of the clinical manifestations of the disease (2), in particular lymphocytosis (4). Various biologic activities and factors, formerly considered to be independent entities, have PT as their common substrate. These include the histamine sensitizing factor (5, 6), lymphocytosis-promoting factor (4), islet-activating protein (7, 8), adjuvant effect (9), and mitogenic effect (10). Although well characterized in animal and cell culture models, almost nothing is known about the relevance of these factors and their mechanisms of action within the human system under *in vivo* conditions.

PT affects regulatory G-proteins of the membranous adenylyl cyclase system via ADP-ribosylation (7). Within the adrenergic system, PT has been shown to inhibit the inhibitory G-protein of α_2-adrenoceptors (11). However, whereas much information has accumulated concerning the effect of PT on inhibitory receptor systems linked to adenylyl cyclase in various cell types, insufficient information is available as to whether PT might also influence processes involving G-proteins that are stimulatory coupled to adenylyl cyclase. As β_2-R-mediated activation of the adenylyl cyclase in MN leukocytes involves the action of stimulatory G-proteins, the present investigation was performed on these cells which are readily available and have been shown to serve as an appropriate model to explore the effect of a disease on adrenergic mechanisms (12). cAMP accumulation as well as β-R density and affinity were studied in MN leukocytes from children during natural pertussis, after pertussis vaccination and in healthy controls. To correlate possible *in vivo* effects of pertussis to action of PT, the influence of purified PT on cAMP generation of isolated MN leukocytes from adult volunteers was also investigated. The cells were stimulated with β-mimetics and prostaglandin E_1 that are thought to act on specific cell surface receptors, coupled via G-proteins to the adenylyl cyclase and with forskolin, a diterpene acting directly at the catalytic subunit of the adenylyl cyclase.

MATERIALS AND METHODS

Patients. Pertussis was diagnosed on the basis of typical clinical features: whooping cough (100%), history of contact with a known case (98%), leukocytosis (> 12000/μl in 65%), absolute lymphocytosis, increased specific serum IgA, IgG, and IgM antibodies (ELISA) (36%). All cases occurred during a local pertussis endemic from February to October 1987. As adenoviruses (types 1, 2, 3, and 5) have also been associated with an illness that is indistinguishable from that caused by *B. pertussis*, complement fixation titers against adenoviruses were investigated, and children with titers higher than 1:20 were excluded. A total of 63 children was studied.

For determination of cAMP levels in the pertussis group, blood samples were taken from 15 children (3.7 y ± 0.4; range 0.8 to 5.7). The children were divided into two groups according to the

duration of the disease. One consisted of six children having the convulsive stage of pertussis for less than 7 days (age 3.6 y ± 0.5; range 1.2–5.6), and the other of nine children suffering from the disease for more than 7 days (age 3.8 y ± 0.9; 0.8 to 5.1).

In seven healthy children (1.3 y ± 0.4; range 0.4–2.7), cAMP levels of MN leukocytes were investigated 1–3 days after vaccination with *B. pertussis* monovaccine (Serum-und Impfinstitut, Berne, Switzerland; 2×10^{10} *B. pertussis*/dose; third or fourth vaccination during a routine vaccination course of four injections). The control group consisted of 11 children; six served as controls for pertussis-infected children (4.1 y ± 0.7; range 0.5–6), five for vaccinated children (1.6 y ± 1.0, range 0.4–3).

^{125}I-cyanopindolol binding was studied in eight control children (3.4 ± 1.0 y; range 0.7–9.5), in 16 children with pertussis (3.3 y ± 0.3; range 1.0–5.5), and six young children after vaccination (0.8 y ± 0.2; range 0.5–1.6).

Preparation of MN leukocytes. After obtaining consent from the parents and/or the patients themselves, an additional sample of 6–8 ml·EDTA anticoagulant-treated blood was taken along with routine laboratory samples between 0800 and 0900. For *in vitro* experiments, 30 ml of EDTA anticoagulant-treated blood samples from healthy, adult volunteers (aged 23–30 y), who had suffered from whooping cough 20 or more years before, were used. After 10 min of centrifugation at 400 × *g*, platelet-rich plasma was removed, the pellet was diluted with an equal vol of buffer A (50 mM Tris, 120 mM NaCl, 10 mM $MgCl_2$, pH 7.4), 5-ml aliquots of the mixture were layered on 7 ml of Ficoll (Pharmacia, Freiburg, FRG) and centrifuged (400 × *g*, 25 min, 20°C) (13). The MN cell layer was carefully removed, washed twice with equal vol of buffer A (1000 × *g*, 20 min) and resuspended in 2 ml of buffer A. In case of cAMP experiments, MN cells were washed using buffer C (136 mM NaCl; 2.7 mM KCl; 8 mM Na_2HPO_4; 1.4 mM KH_2PO_4; 0.9 mM $CaCl_2$; 1 mM $MgCl_2$, pH, 7.2). The isolated cells were counted in a Neubauer chamber and diluted further so that the final assay vol contained 2.5×10^5 cells/250 μl (radioligand binding studies), and 2×10^6 cells/300 μl (cAMP assays). Morphologic analysis of the MN leukocytes showed mainly lymphocytes (80–95%), nonspecific esterase-positive monocytes (5–20%), and, rarely, erythrocytes and thrombocytes.

β-binding assay on MN leukocytes. Aliquots of the cell suspension (50 μl) were incubated for 60 min (37°C) with different concentrations (0–160 pM) of 50 μl of the radioligand (−)^{125}ICYP (2200 Ci/mmol, Amersham Buchler, Braunschweig, FRG), 150 μl of buffer B (containing buffer A and 0.05 mM phentolamine, 0.05 mM chloroquine, 0.3 mM catechol) to reduce nonspecific binding, and 1 mM ascorbic acid (antioxidant) as described earlier (14). Specific binding was defined as total binding minus unspecific binding and amounted to 65% (at 10–80 pM ICYP) and to 40–50% (at 160 pM ICYP). After stopping the ICYP binding reaction by dilution with 5 ml of buffer B (37°C), the MN cells were collected by vacuum on Whatman GF/C filters and washed twice with 5 ml buffer B. Radioactivity on dried filters was counted in a Beckman 5500 γ-counter (Beckman Instruments, Inc., Munich, FRG) with 71% efficiency.

Cyclic AMP content of MN leukocytes. Isolated MN cells were suspended in buffer D (containing buffer C, 0.25 mg/ml BSA (Behring, Marburg, FRG) and 0.1 mM theophylline (Serva, Heidelberg, FRG)) and stimulated at 37°C for 15 min with forskolin (0.1 mM) or various concentrations of IPN (10 nM-10mM). Basal levels (after 15 min without stimulation) were also determined. After stopping the reaction at 95°C for 7.5 min and cooling on ice, centrifuged aliquots were stored at −20°C until determination of the cAMP content by a competitive protein binding assay using [^{3}H]cAMP (15).

cAMP content after pretreatment of isolated MN leukocytes with PT. Isolated MN cells were suspended in buffer D and preincubated at 37°C for 90 min with or without PT (List Biological Laboratories, Campfield, CA), final concentration 100 ng/ml in a final vol of 300 μl. Thereafter, 30 μl of buffer with or without test compounds to stimulate adenylyl cyclase added at 37°C for 15 min. The compounds included IPN, PGE_1, hydrocortisone (all from Sigma Chemical Co, Deisenhofen, FRG) and forskolin (Calbiochem, Frankfurt, FRG) at the final concentrations indicated. After the reaction had been stopped as described above, aliquots of the supernatant were stored at −20°C for determination of the cAMP content (see above). All sets of *in vitro* experiments with and without PT were performed simultaneously from a single preparation of MN cells.

Statistical methods. Data are expressed as arithmetic means ± SEM of *n* experiments. Linear regression analysis was used in radioligand binding studies. Values of maximum binding sites were normally distributed, whereas cAMP values followed a log-normal distribution and were transformed to normality before statistical analysis (16). One-way analysis of variance and Student's unpaired *t* test were performed for statistical comparison of data obtained from controls and children with pertussis. Results from *in vitro* studies with or without PT were compared using Student's paired *t* test.

RESULTS

cAMP content of MN leukocytes in children with pertussis and controls. Basal cAMP levels of MN cells were similar in all groups studied (Fig. 1). The same was true for cAMP accumulation after direct stimulation of adenylyl cyclase with 10^{-4} M forskolin (Fig. 1). IPN-stimulated cAMP accumulation was dose-dependent within the range of 10^{-8} to 10^{-3} M, showed maximal response between 10^{-4} and 10^{-3} M, and lower response at 10 mM or higher concentrations (Fig. 1).

MN leukocytes of children with pertussis during the 1st wk of the convulsive stage showed a significantly reduced cAMP accumulation after IPN-stimulation of adenylyl cyclase in comparison with controls (Fig. 1*A*). The same was true when these children were compared with those who had suffered from the convulsive stage of pertussis for longer than one week (Fig. 1*B*). In contrast, however, maximum cAMP levels after forskolin stimulation of adenylyl cyclase did not differ between the groups.

cAMP content of MN leukocytes after vaccination with pertussis monovaccine. To look for generalized systemic, PT-mediated impairments of adenylyl cyclase in children not infected but vaccinated with *B. pertussis*, MN leukocytes of children after application of pertussis monovaccine were investigated. In general, cAMP levels were lower in children younger than 2 y. There were no differences in the basal levels nor in cAMP levels after specific IPN stimulation or direct forskolin stimulation of adenylyl cyclase (Fig. 2).

cAMP content of MN leukocytes after in vitro incubation with PT. In vitro studies of isolated MN cells after addition of PT were performed to compare *in vivo* and *in vitro* PT effects directly and to investigate the mechanisms of action of PT further. Basal cAMP levels in MN cells after 90 min of preincubation with PT (100 ng/ml) were significantly reduced, to about 50% (Table 1). An effect like this could not be shown on MN cells of children during the natural course of pertussis. A diminished cAMP accumulation of MN cells pretreated with PT was demonstrated after specific stimulation of the adenylyl cyclase using IPN or PGE_1 in our *in vitro* assay. This correlates well with the findings in pertussis-infected children. Additionally, hydrocortisone-induced rise of cAMP was blocked by PT treatment of MN cells. Forskolin is classified as a potent direct activator of the catalytic subunit of the adenylyl cyclase that bypasses membrane receptors and G-proteins (17). Regardless of PT pretreatment, stimulation of the cells with forskolin caused similar cAMP accumulation. This demonstrates that PT treatment does not affect the stimulatory interaction between forskolin and adenylyl cyclase, indicating an unimpaired catalytic subunit.

^{125}ICYP-binding sites on MN leukocytes. Specific binding of ^{125}ICYP to intact MN cells of all children investigated showed saturation at about 150 pM ^{125}ICYP (Fig. 3). In a Scatchard

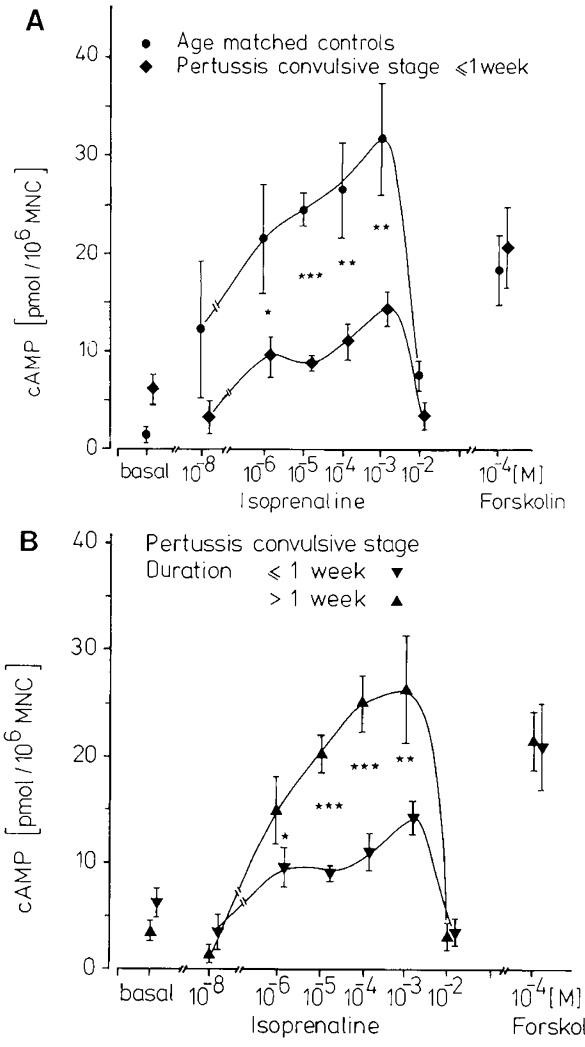

Fig. 1. Levels of cAMP in MN leukocytes isolated from peripheral blood, without (basal) or after IPN resp. forskolin stimulation (15 min, 37°C; for details, see "Materials and Methods") from children suffering from pertussis convulsive stage for 1 wk or less (n = 6), compared to controls (n = 6, A) and to children having convulsive stage for a longer period (n = 9, B). Mean values ± SEM, $p < 0.05$ (★), $p < 0.01$ (★★), $p < 0.001$ (★★★).

analysis of the data, the linear regression lines indicated a single class of receptor sites (Fig. 3, *inset*). Maximum number of binding sites and K_D were within the range observed in previous studies for the age group investigated (14). Although children during the early convulsive stage had, on an average, slightly lower maximum numbers of binding sites per cell, a significant dependency of the β-R number on the duration of the pertussis convulsive stage could not be established. Therefore, the whole pertussis group was directly compared to age-matched controls. The maximum number of binding sites (408.4 ± 52.9 sites per cell, n = 16 *versus* 510.9 ± 82.3 sites per cell, n = 8), and dissociation constants (52.5 ± 5.5 pM *versus* 47.2 ± 4.7 pM) did not differ between children with pertussis and controls. Vaccinated children (527.5 ± 56 sites per cell, n = 6; 55.3 ± 9.4 pM) had similar binding characteristics, too.

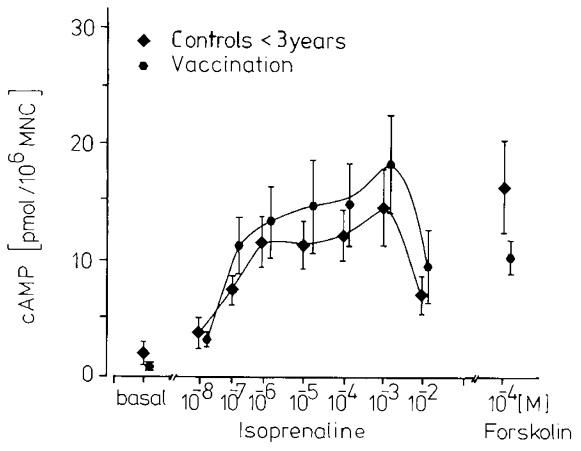

Fig. 2. Basal and stimulated levels of cAMP in MN leukocytes from children 1–3 days after vaccination with live pertussis monovaccine (n = 7) and age-matched controls (n = 5).

Table 1. *Effect of pertussis toxin on formation of cAMP in MN leukocytes**

	Without PT	With PT	n
Basal	2.5 ± 0.3	1.2 ± 0.2†	20
IPN (M)			
10^{-8}	2.9 ± 1.3	0.5 ± 0.05	4
10^{-7}	10.9 ± 4.0	2.3 ± 0.7†	4
10^{-6}	14.8 ± 5.1	4.8 ± 1.5†	5
10^{-5}	17.3 ± 5.2	6.0 ± 1.5‡	5
10^{-4}	17.6 ± 3.3	5.7 ± 1.1†	6
10^{-3}	21.3 ± 4.7	5.9 ± 1.2§	5
10^{-2}	7.2 ± 1.7	4.7 ± 0.7	4
PGE_1 (M)			
10^{-8}	0	0.03	1
10^{-7}	1.9 ± 0.9	0.7 ± 0.2	5
10^{-6}	21.7 ± 4.8	9.7 ± 1.8†	6
10^{-5}	41.2 ± 6.8	20.1 ± 3.8‡	6
10^{-4}	57.5 ± 8.3	35.4 ± 5.4‡	6
10^{-3}	4.5 ± 1.1	6.8 ± 2.7	4
Forskolin (M)			
10^{-8}	0.2 ± 0.1	0.3 ± 0.07	2
10^{-7}	0	0.4	1
10^{-6}	0	0.2	1
10^{-5}	1.3 ± 0.4	1.2 ± 0.2	3
10^{-4}	12.7 ± 0.7	12.4 ± 1.0	3
10^{-3}	1.0 ± 0.7	1.9 ± 0.7	3
10^{-4}‖	16.3 ± 2.4	12.6 ± 1.5	16
Hydrocortisone (M)			
10^{-6}	0.11 ± 0.09	0.04 ± 0.035	3
10^{-5}	0.24 ± 0.03	0.14 ± 0.015	4
10^{-4}	5.2 ± 0.7	2.7 ± 0.25§	6
10^{-3}	0.66 ± 0.57	1.2 ± 1	4

* Isolated MN cells from adult volunteers were preincubated for 90 min with PT (100 ng/ml) or without. Then cAMP-content (pmol/10^6 MN cells) was measured after 15 min without (basal) or with stimulation of adenylyl cyclase with different drugs at indicated concentrations. Values are mean ± SEM.

† Effect of PT: $p < 0.001$.

‡ Effect of PT: $p < 0.01$.

§ Effect of PT: $p < 0.05$.

‖ Obtained from experiments with IPN, PGE_1, or hydrocortisone, where 10^{-4} M forskolin was used as a positive control.

DISCUSSION

The present study demonstrates a marked reduction of β-R mediated IPN stimulation of cAMP generation in peripheral MN leukocytes during the 1st wk of the convulsive stage of pertussis in children, but not if the convulsive stage had lasted for more than 1 wk. Basal cAMP content did not differ between control and pertussis-infected children. Furthermore, forskolin, which stimulates adenylyl cyclase directly, produced identical cAMP accumulation in MN cells from children within the 1st wk of the

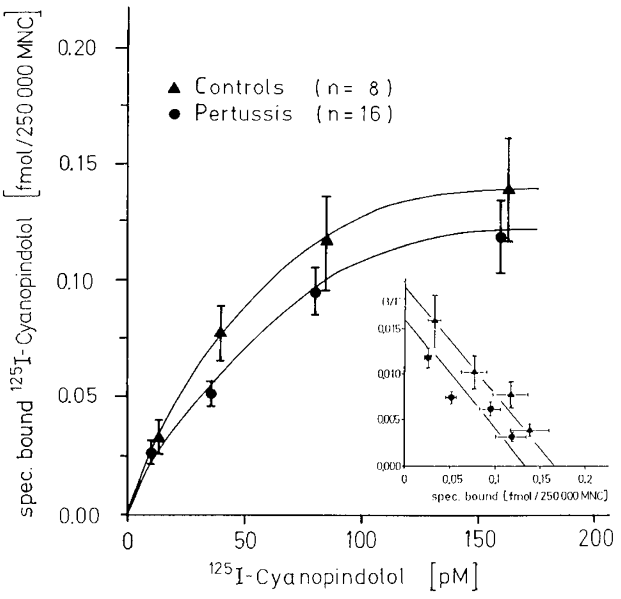

Fig. 3. Specific binding of various concentrations of ^{125}I-cyanopindolol to the β-adrenergic receptor on MN leukocytes obtained from control children and children suffering from convulsive stage of pertussis. *Inset,* Scatchard plots of the data. Binding characteristics were not different in the two groups (mean ± SEM).

convulsive stage and their healthy age-matched controls. Therefore, pertussis may selectively affect the β-adrenergic system. Because β-R density and affinity, as determined by a β-R antagonist, are not changed, it can be concluded that the signal transduction from the receptor to the adenylyl cyclase is impaired.

One hypothetical explanation for different cAMP responses to β-R-stimulation in children with or without pertussis infection might be the predominant selection of one subtype of MN leukocytes by "lymphocytosis-promoting factor" in children with pertussis. This is unlikely, however, as lymphocyte subpopulations remain unchanged in pertussis (18, 19). In addition, as B and T lymphocyte subsets have different densities of β-R (20), disproportionate changes in the subset ratio would probably result in different β-R densities on nonseparated MN cells. One further objection against changes of the distribution of lymphocyte subpopulations results from our *in vitro* studies, where changes in MN cell subpopulation from the experimental set are impossible for technical reasons.

The data from both our *in vivo* and *in vitro* studies correlate well with those of Parker and Morse (21) who observed decreased intracellular cAMP levels in response to IPN in lymphocytes that had been incubated with semipurified preparations of pertussis culture fractions.

As studies in animals have shown an impairment of β-R-mediated relaxation of tracheal smooth muscles after vaccination with *B. pertussis* (22–24), one might assume that PT impairs the β-R-mediated function of several organ systems. However, our study in vaccinated children has not indicated a generalized impairment of either β-R density and affinity or of cAMP response to IPN, within the 1st 3 days after vaccination with live *B. pertussis* monovaccine.

Several peculiarities, *e.g.* lymphocytosis, attenuation of hyperglycemic response to adrenalin (25), and paroxysmal supraventricular tachycardia (26) occurring during natural pertussis infection have been assumed to be PT-mediated and subject to extensive *in vitro* and *in vivo* investigations. PT seriously affects lymphocyte recirculation in mice (4, 8) and lymphocyte chemotaxis *in vitro* (27). Moreover, *in vitro* lymphocyte random motility and directed migration of mouse lymphocytes were inhibited in a dose-dependent fashion by PT (26, 27). As cAMP responses to stimulatory signals in that system had the tendency to show decreasing values, it was suggested that an impairment of the coupled signal transducing system might exist. Whether cAMP is directly involved in the lymphocytosis-promoting effect of PT at all, and which of the different mechanisms responsible might be linked to the diminished cAMP responses observed in our system, remains to be investigated further.

The present results may be taken as an indication that PT also impairs stimulatory signal transduction from β-R to the adenylyl cyclase. An impairment of inhibitory α_2-receptor cAMP coupling has been clearly established. Thus, PT abolishes α_2-receptor-induced hormonal inhibition of adenylyl cyclase via ADP-ribosylation of a 41 kD protein, identified as the a subunit of G_i (28). This results in effective decoupling of the inhibitory effects of G_i on the active site of the adenylyl cyclase (29, 30), thus leading to increased intracellular levels of cAMP in most investigated cell systems.

Our data of diminished cAMP accumulation in MN leukocytes after exposure to PT indicate that the effect of PT on peripheral white blood cell cAMP metabolism are quite different from that observed in most other investigated cell systems, where mostly supraoptimal cAMP responses after PT treatment were obtained. Either receptor-mediated stimulation of cAMP accumulation was potentiated (pancreatic islet cells, C6 glioma cells), or, as in most other cell systems, receptor-mediated inhibition (heart cells, neuroblastoma-glioma hybrid cells, platelets, fat cells, S 49 lymphoma cells) was abolished (7). PT, however, is able to modify functions of other G-proteins by ADP-ribosylation. These include G_o (31), transducin (28) and probably additional, so far only immunologically identified, substrates (10, 32, 33). As we were able to show that PT also affected PGE_1-mediated cAMP response, it might be assumed that PT affects further stimulatory proteins. However, the exact target of PT in MN leukocytes, mediating an inhibition of stimulatory signal transduction, cannot be determined by the present data.

This study shows that during the 1st wk of convulsive stage of natural pertussis infection, in contrast to the later course and to children that were vaccinated using live *B. pertussis*, the generation of the second messenger cAMP in MN leukocytes is affected in a similar way as could be demonstrated in *in vitro* experiments with pure PT. Whether other biologic activities of PT that have been obtained in cell culture and animal models also find their clinical correlates has to be substantiated by further investigations.

Acknowledgments. The authors thank Dr. A. Ernst for her untiring support and Mrs. I. Bergfeld and Mrs. M. Kempf for excellent technical assistance.

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