

THE EFFECT OF *BORDETELLA PERTUSSIS* ON LYMPHOCYTE CYCLIC AMP METABOLISM*

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In recent years *Bordetella pertussis* (or soluble products obtained from fluid cultures of this organism) has been shown to have potent biological activity in mice and rats. Thus, pertussis-treated animals exhibit the following: (a) marked leukocytosis (1, 2); (b) an increase in sensitivity to the lethal effects of histamine, acetylcholine, serotonin, bradykinin, endotoxin, and a variety of nonspecific stimuli such as cold stress (3); (c) potentiation of the immune response with selective stimulation of homocytotropic antibody synthesis (4, 5); (d) a decreased physiological and metabolic response to the intravenous injection of epinephrine (5, 6). The reduction in epinephrine responsiveness has been interpreted as being due to β adrenergic blockade, and has led to speculation that alterations in adrenergic responsiveness might underlie other biological effects of the organism (5).

Since adrenergic agents exert their effects on cells by producing changes in intracellular cyclic AMP concentrations, it would be desirable to study the effect of pertussis on cyclic AMP metabolism, preferably in a wholly in vitro system so that the complexities of in vivo metabolic studies could be avoided. A suitable tissue for these studies would be lymphocytes, since the ability of cells in the recirculating lymphocyte pool to emigrate from the blood is markedly decreased in pertussis-treated animals, which suggests a change in the surface properties of these cells (7). In the present report, evidence indicating that *B. pertussis* is able to alter cyclic AMP metabolism in human lymphocytes is presented.

Materials and Methods

Preparation of B. pertussis Culture Supernatant Fluid Fractions.—*B. pertussis* strain NIH 114 (3779B) was cultured in the medium and under the conditions described previously, except that the anion exchange resin was not utilized (2). After the addition of thimerosal to a

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final concentration of 1:5,000, the bacterial cells were removed by centrifugation. Fractionation of the culture supernatant fluids was achieved by the successive use of $(\text{NH}_4)_2\text{SO}_4$, density gradient centrifugation, and gel filtration on Sephadex G-150 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.). A typical scheme of fractionation and the designation of the products are summarized in Fig. 1.

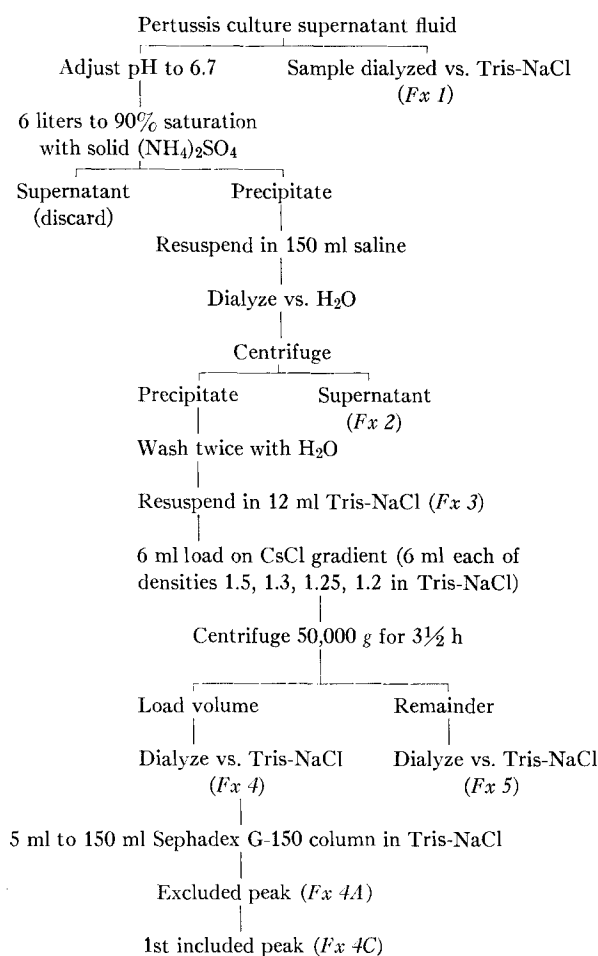


FIG. 1. Representative scheme for the preparation of fractions from *B. pertussis* culture supernatant fluids. Tris-NaCl is 0.1 M Tris-0.5 M NaCl, pH 10.0.

All fractions were dissolved in, or dialyzed against, 0.1 M Tris-0.5 M NaCl, pH 10 (Tris-NaCl), and diluted in the same buffer. The dilutions represent equivalent dilutions in terms of the volume of the original culture supernatant fluid. Thus, a fraction diluted 1:100 would contain in 1 ml an amount present in 0.01 ml of the culture supernatant fluid, assuming no loss. Alternatively, samples of the fractions were dialyzed exhaustively against distilled water, dried from the frozen state, and weighed.

Leukocytosis.—0.2 ml of dilutions of the fractions were injected intravenously into adult female Albany strain mice. Leukocyte counts were obtained 3 days later, utilizing a Model B Coulter Counter (Coulter Electronics, Inc., Fine Particle Group, Hialeah, Fla.).

Histamine Sensitivity.—After samples of blood for leukocyte counts had been obtained, 1 mg of histamine dichloride contained in 0.5 ml of isotonic saline was injected intraperitoneally. Deaths generally occurred within a few hours, but a 24-h period was used for scoring.

Lymphocyte Purification.—Heparinized peripheral blood was obtained from normal, healthy, human volunteers. Dextran (clinical grade H, Pharmachem Corp., Bethlehem, Pa.) was added to a final concentration of 0.6%, and the cells were separated by gravity sedimentation at 37°C for 45–60 min. Cells in the leukocyte-rich supernatant were washed with 0.15 M NaCl-0.01 M PO₄, pH 7.4 (PBS),¹ and then passed through nylon columns as described previously (8). The nonadherent cells were centrifuged twice at 100 g for 7 min and washed twice with PBS to remove platelets. The purified cell preparations contained >98% lymphocytes (200 nucleated cells counted). Lymphocyte recovery averaged approximately 25%.

Cell Incubation Experiments.—Sources and methods for preparing solutions of catecholamines and prostaglandins have been described previously. Methacholine (acetyl- β -methylcholine, Aldrich Chemical Co., Inc., Milwaukee, Wis.) and acetylcholine (Sigma Chemical Co., St. Louis, Mo.) were dissolved in 0.15 M NaCl just before use. Purified lymphocytes were suspended in Gey's solution at concentrations of 1–5 $\times 10^6$ cells/ml. Incubation volumes of 0.5 or 1.0 ml/tube were used. In the usual experiment, cells were preincubated with pertussis fractions for various time periods at 0 or 37°C, and 0.1 vol of PBS or PBS containing isoproterenol, prostaglandin E₁ (PGE₁), or a cholinergic agent was then added. After an additional 10 or 15 min at 37°C, the tubes were centrifuged at 2,500 rpm for 2 min at room temperature, the supernatants were decanted, and the cell pellets were frozen in ethanol-dry ice. Samples were stored at –70°C until assay. Cyclic AMP was determined by radioimmunoassay. Data on the specificity and sensitivity of the immunoassay have been presented in detail previously (8, 9).

RESULTS

Inhibition of Isoproterenol Responses by Crude Pertussis Culture Fluid.—Purified lymphocytes were incubated at 37°C with dialyzed crude pertussis culture supernatant (fraction [Fx] 1, see Fig. 1) for various time periods and then stimulated with 1 and 10 mM isoproterenol (Fig. 2). Preincubation of cells for 90 min in the presence of a 1:100 dilution of fraction 1 resulted in complete inhibition of the cyclic AMP response to 1 mM isoproterenol and nearly complete inhibition of the 10 mM isoproterenol response. At the lower isoproterenol concentration, partial inhibition was also observed after preincubation with the 1:10,000 dilution. When the preincubation period was shortened to 20 min, little or no inhibition was observed, even at the highest fraction 1 concentration. On the basis of these observations a preincubation period of 90 min at 37°C was selected as a suitable condition for further studies.

Inhibition of Isoproterenol Responses by Pertussis Fractions.—In an effort to characterize the isoproterenol-inhibiting activity further, partially purified *B. pertussis* culture fractions (see Fig. 1) were evaluated. Table I summarizes the

¹ Abbreviations used in this paper: Fx, fraction; PBS, phosphate-buffered saline (0.15 M NaCl-0.01 M PO₄, pH 7.4); PGE₁, prostaglandin E₁; PHA, phytohemagglutinin.

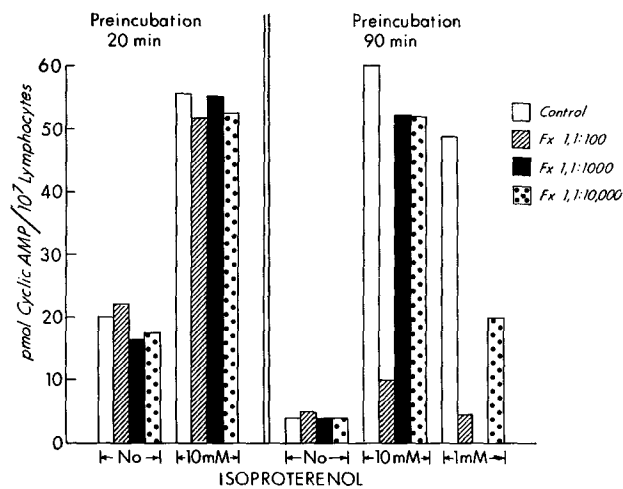


FIG. 2. Lymphocytes were preincubated for 20 and 90 min at 37°C with various dilutions of dialyzed culture supernatant fluid (fraction 1) or buffer. Isoproterenol or buffer was then added, and cellular cyclic AMP was measured after an additional 10 min at 37°C.

histamine- and leukocytosis-stimulating activities of these fractions. Leukocytosis was stimulated most effectively by fraction 4. Fractions 3 and 5 also had significant activity, particularly the former, whereas fraction 2 was essentially inactive. Considerable histamine-sensitizing activity was present in fractions 3, 4, and 5, fraction 4 again being the most potent.

The effect of the various fractions on the lymphocyte isoproterenol response is shown in Fig. 3. In comparison with the dialyzed supernatant (fraction 1), fraction 2 was almost completely inactive as an inhibitor of the cyclic AMP response to β adrenergic stimulation, whereas striking inhibitory activity was present in fractions 3 and 4, correlating well with the leukocytosis-producing activity of these fractions. This raises the possibility that leukocytosis induction and catecholamine inhibition have a common basis. However, it is necessary to explain why the total inhibitory activity in fractions 3 and 4 was actually greater than that in fraction 1 (e.g., there was >100% recovery of activity during purification).

This discrepancy may be due to the instability of the crude extract, which loses its leukocytosis-stimulating activity more readily than fractions 3 and 4. The cyclic AMP experiments were performed 5 days or more after the injection of animals for determination of leukocytosis-producing activity, and significant decay in fraction 1 activity might have occurred during this period. Alternatively, it may be that maximal activity was present, which could not be increased by the presence of more of the inhibitor; such a phenomenon has been described for the leukocytosis-promoting factor. An example is seen in Table I for fractions 3 and 4C. Similar explanations might account for the greater in-

TABLE I
*Representative Leukocytosis and Histamine-Sensitizing
 Activities of Various B. pertussis Fractions*

| Fraction | Weight injected | Leukocyte count* | Histamine‡ |
|----------|-----------------|------------------------------|------------|
| | μg | $\text{mm}^3 \times 10^{-3}$ | |
| 1 | 5 | 96.3 | 3/4 |
| | 1 | 48.6 | 1/4 |
| | 0.2 | 25.1 | 0/4 |
| 2 | 10 | 33.4 | 1/4 |
| | 2 | 17.0 | 0/4 |
| 3 | 5 | 87.6 | 4/4 |
| | 1 | 108.9 | 1/4 |
| | 0.2 | 71.6 | 1/4 |
| 4 | 1 | 135.8 | 3/4 |
| | 0.2 | 90.0 | 3/3 |
| 4A | 5 | 46.8 | 0/4 |
| | 1 | 23.7 | 0/4 |
| 4C | 1 | 108.1 | 2/4 |
| | 0.2 | 94.6 | 0/4 |
| 5 | 5 | 100.0 | 4/4 |
| | 1 | 48.2 | 2/4 |
| | 0.2 | 27.1 | 0/4 |

* The leukocyte counts were determined 3 days after injection.

‡ These same mice were injected intraperitoneally with 1 mg of histamine dihydrochloride after the leukocyte counts were done. The results are expressed as number dead/total number.

hibitory activity in fractions 4 and 5 (taken together) than in fraction 3, from which fractions 4 and 5 are derived.

Effect of Pertussis Fractions on PGE₁ and Cholinergic Stimulation.—Having established that pertussis culture fractions inhibit the cyclic AMP response to isoproterenol, it seemed desirable to evaluate the effect of other agents (PGE₁, cholinergic agents) that raise lymphocyte cyclic AMP concentrations. When PGE₁ was used to stimulate lymphocytes that had been exposed to various pertussis fractions, the same fractions that inhibited isoproterenol responsiveness reduced the PGE₁ response (Fig. 4). The response to cholinergic agents also was inhibited (see below). Thus, the inhibition of the cyclic AMP response by pertussis involves nonadrenergic as well as adrenergic stimuli.

Experiments with Known Quantities of Pertussis Fraction 4.—Inhibition of the cyclic AMP response by fraction 4 is considered in more detail in Table II.

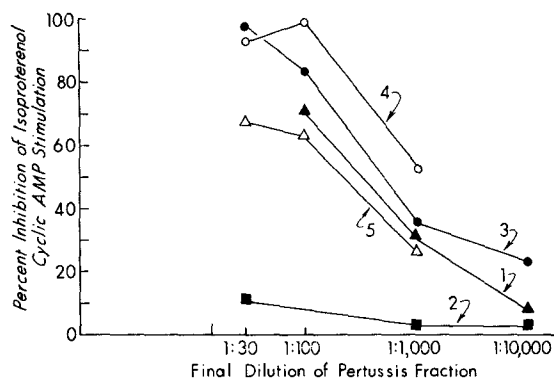


FIG. 3. 1.2×10^6 lymphocytes were preincubated with the indicated dilutions of *B. pertussis* fractions 1-5 for 90 min at 37°C, after which isoproterenol was added to a final concentration of 10 mM and the incubation continued for another 10 min. In control tubes 10 mM isoproterenol increased the cyclic AMP concentration from 20.8 to 124 pmol/ 10^7 cells. Fraction dilutions are expressed on the basis of the amount of dialyzed crude culture fluid supernatant (fraction 1) used as starting material. Thus 1 ml of fraction 4, undiluted, was obtained from 1 ml of fraction 1. With this particular culture preparation, the average mouse leukocyte responses (evaluated after 72 h) to 0.4 ml of fractions 2, 3, 4, and 5 were 51, 211, 191, and $131 \times 10^3/\text{mm}^3$, respectively; 0.2 ml of the parent fraction 1 produced a leukocytosis of $237 \times 10^3/\text{mm}^3$.

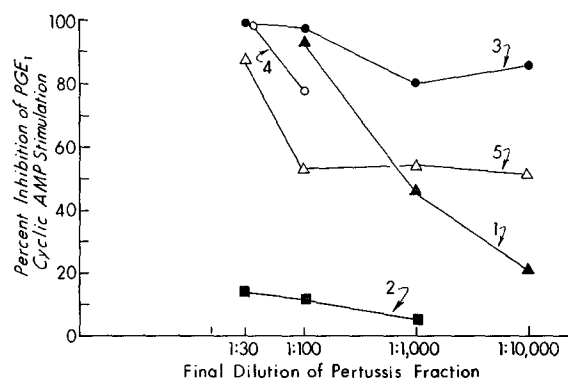


FIG. 4. 1.2×10^6 lymphocytes were preincubated with the indicated concentrations of *B. pertussis* fractions 1-5 for 90 min at 37°C, after which PGE₁ was added to a final concentration of 120 nM and the incubation continued for another 10 min. In control tubes PGE₁ increased the concentration of cyclic AMP from 20.8 to 48 pmol/ 10^7 cells. For an explanation of fraction dilutions, see legend for Fig. 3.

Responses to 0.1, 1.0, and 10 mM isoproterenol, 30 and 3 μM PGE₁, and 3 mM methacholine were all markedly inhibited at 500 ng/ml concentrations of fraction 4. The 30 μM PGE₁ and 1.0 mM isoproterenol cyclic AMP responses were also evaluated at fraction 4 concentrations of 50 and 5 ng/ml; 48% or more inhibition was obtained in each instance. Thus concentrations of fraction 4 as

TABLE II
*Effect of Fraction 4 on the Cyclic AMP Response of Human Lymphocytes**

| | Fraction 4 concentration | Cyclic AMP/ 10^7 cells | Inhibition |
|-----------------------------|--------------------------|--------------------------|------------|
| | ng/ml | pmol | % |
| Buffer | — | 9.0 | |
| Buffer | 500 | 13.0 | |
| 10.0 mM isoproterenol | — | 128.0 | |
| 10.0 mM isoproterenol | 500 | 14.0 | 96 |
| 1.0 mM isoproterenol | — | 30.0 | |
| 1.0 mM isoproterenol | 500 | 18.0 | 57 |
| 1.0 mM isoproterenol | 50 | 14.0 | 84 |
| 1.0 mM isoproterenol | 5 | 18.0 | 70 |
| 0.1 mM isoproterenol | — | 35.0 | |
| 0.1 mM isoproterenol | 500 | 15.0 | 73 |
| 30 μ M PGE ₁ | — | 112.0 | |
| 30 μ M PGE ₁ | 500 | 43.0 | 65 |
| 30 μ M PGE ₁ | 50 | 46.0 | 64 |
| 30 μ M PGE ₁ | 5 | 63.0 | 48 |
| 3 μ M PGE ₁ | — | 120.0 | |
| 3 μ M PGE ₁ | 500 | 33.0 | 78 |
| 3 mM methacholine | — | 15.0 | |
| 3 mM methacholine | 500 | 9.0 | 100 |

* 1.6×10^6 lymphocytes in a volume of 1.0 ml were preincubated with the indicated concentrations of fraction 4 for 90 min at 37°C, after which isoproterenol, PGE₁, methacholine, or buffer was added and the incubation continued for another 10 min at 37°C. Percent inhibition is based on a buffer control cell concentration of 9 pmol cyclic AMP/ 10^6 cells.

low as 5 ng/ml (3.1 ng/ 10^6 lymphocytes) inhibited the cyclic AMP response, and the possibility that inhibitory effects might occur at still lower concentrations was not excluded.

Further Characterization of the Activity in Fraction 4.—In an effort to better characterize the inhibitory activity in fraction 4, the fraction was subjected to Sephadex G-150 chromatography. Fraction 4A, which was excluded from the gel, did not produce marked leukocytosis and had little or no effect on the lymphocyte cyclic AMP response. Inhibition of the cyclic AMP response was obtained with fraction 4C, which was eluted just behind the exclusion volume (Table III). This fraction also contained the bulk of the leukocytosis-producing activity. However, in comparing fraction 4C with the parent fraction 4, recovery of leukocytosis-producing activity appeared to be greater than the recovery of cyclic AMP inhibitory activity.

Effect of Preincubation Temperature.—In view of the delayed onset of the inhibition of cyclic AMP responsiveness in time experiments, it seemed desirable

to evaluate the effect of preincubation temperature. When cells were preincubated with active pertussis fractions at 0°C for 90 min, there was little or no impairment of isoproterenol responsiveness. (A representative experiment with fraction 4 is shown in Table IV.) Thus there was temperature as well as time dependency for the inactivation.

Washing Experiments.—In a single experiment, cells were preincubated with

TABLE III
*Effect of Fraction 4 and Its Subfractions (4A and 4C)
on the Lymphocyte Cyclic AMP Response to PGE₁**

| Fraction | | Cyclic AMP | Inhibition |
|----------------------------------|--------------|------------------------------------|------------|
| | | $\mu\text{mol}/10^6 \text{ cells}$ | % |
| Buffer | | 10 | |
| PGE ₁ 3 μM | | 25 | |
| PGE ₁ 3 μM | 4, 1:100 | 13 | 80 |
| PGE ₁ 3 μM | 4, 1:1,000 | 9 | 100 |
| PGE ₁ 3 μM | 4, 1:10,000 | 11 | 94 |
| PGE ₁ 3 μM | 4, 1:100,000 | 26 | 0 |
| PGE ₁ 3 μM | 4C, 1:100 | 13 | 80 |
| PGE ₁ 3 μM | 4C, 1:1,000 | 23 | 13 |
| PGE ₁ 3 μM | 4C, 1:10,000 | 27 | 0 |
| PGE ₁ 3 μM | 4A, 1:100 | 27 | 0 |
| PGE ₁ 3 μM | 4A, 1:1,000 | 25 | 0 |
| PGE ₁ 3 μM | 4A, 1:10,000 | 25 | 0 |

* For incubation conditions and explanation of percent inhibition, see legend to Table II.

TABLE IV
*Effect of Preincubation Temperature on Fraction 4 Inhibition of the
Lymphocyte Cyclic AMP Response to Isoproterenol**

| Preincubation temperature | Condition | Cyclic AMP | Inhibition |
|---------------------------|----------------------------|-------------------------------------|------------|
| | | $\mu\text{mols}/10^6 \text{ cells}$ | % |
| 37°C | Buffer | 10.0 | |
| | 10 mM isoproterenol | 19.0 | |
| | 10 mM isoproterenol + Fx 4 | 11.0 | 89 |
| 0°C | Buffer | 9.0 | |
| | 10 mM isoproterenol | 25.0 | |
| | 10 mM isoproterenol + Fx 4 | 23.0 | 12 |

* 1.5×10^6 lymphocytes in 0.5 ml buffer were preincubated at 0 or 37°C with 500 ng/ml fraction 4 for 90 min, after which isoproterenol was added to a final concentration of 10 mM and the incubation was continued for another 10 min at 37°C.

fraction 1 at 37°C for 90 min, washed twice, and stimulated with isoproterenol in the usual way. Marked inhibition was still demonstrable, indicating that the inhibitory effects of *B. pertussis* were not readily reversed.

DISCUSSION

The results of these studies indicate that a macromolecular component of *B. pertussis* cultures is able to alter cyclic AMP metabolism in human lymphocytes in vitro. The major change is a reduction in the response of exposed cells to agents that normally raise lymphocyte cyclic AMP concentrations, including isoproterenol, PGE₁, acetylcholine, and methacholine. Since the inhibition is not limited to β adrenergic agonists, it cannot be considered to be a β adrenergic blocking effect, per se.

In contrast, on the basis of in vivo studies pertussis has generally been classified as a β adrenergic blocking agent (5, 6). However, the discrepancy may be more apparent than real since a careful review of data obtained in intact animals indicates that information on possible pertussis effects on responses to non-adrenergic hormones are actually very limited (5, 10). Moreover, there is ample evidence to indicate that pertussis and β adrenergic blocking agents do not produce identical physiological changes in vivo, although considerable similarity exists (11). Inasmuch as extra-adrenergic effects of pertussis are demonstrable in vitro, exact parallelism would not be expected since β antagonists would have a more limited spectrum of action than pertussis.

Pertussis is not the only microbial agent capable of altering cyclic AMP metabolism in mammalian cells. Endotoxins have recently been reported to potentiate the cyclic AMP response to epinephrine in mouse liver and spleen (12); and *Clostridia perfringens* neuraminidase raises cyclic AMP concentrations and potentiates the effect of epinephrine in frog erythrocytes (13). The enterotoxigenic exotoxin of *Vibrio cholera* increases cyclic AMP in intestinal mucosal cells, the change developing gradually over a period of 60–90 min (14). The alteration in cyclic AMP concentration appears to be a necessary event in the massive outpouring of intracellular electrolytes that characterizes cholera infection. Plant products that influence cyclic AMP metabolism include concanavalin A and *Phaseolus vulgaris* phytohemagglutinin (PHA), both of which initially raise cyclic AMP in human lymphocytes (9) (C. W. Parker, manuscript in preparation). It is interesting that with the exception of pertussis, all of the above agents raise cyclic AMP concentrations; however, PHA and concanavalin A can also produce a fall in cyclic AMP if incubation is continued for many hours (8) or if lymphocyte preparations depleted of immunoglobulin containing cells, erythrocytes, and platelets are studied.

The basis for the altered cyclic AMP response is not clear. The development of inhibition with the pertussis fractions is both time and temperature dependent, as might be the case if the inactivation involved an enzymatic action at the plasma membrane, an induced alteration in protein synthesis, or a need to

transport the inhibitory factor into the cell. It is of interest that there is a delay in the effect of *B. pertussis* on cyclic AMP metabolism in vitro whereas it takes 3–5 days to demonstrate maximal histamine sensitizing action in vivo (15). The delay in onset is in striking contrast to the β blocking effects of *d,l*-propranolol, which are demonstrable almost immediately, both in vivo and in vitro. Once the inactivation has occurred, it is not readily reversed, as indicated by the fact that washing the cells under conditions in which dissociation of the *B. pertussis* fraction from the cells is known to occur (A. Adler and S. I. Morse, unpublished observations) does not restore cyclic AMP responsiveness.

Although the presence of residual pertussis products on washed cells cannot be excluded, the long duration of pertussis-induced lymphocytosis in vivo (approximately 14 days) suggests that the hormonal unresponsiveness is corrected relatively slowly. The biochemical basis for the change in hormonal responsiveness is not known. The effect is apparently not at the level of cell permeability to cyclic AMP since studies of cell supernatants do not reveal large amounts of cyclic AMP in the medium. Nor is it likely that pertussis is enzymatically inactivating hormonal receptors, since simultaneous changes in prostaglandin, catecholamine, and cholinergic receptors would be required. In rat adipocytes, receptors for insulin and glucagon can be enzymatically degraded by trypsin with an associated loss of hormonal responsiveness under conditions in which catecholamine and other receptors are not altered (16, 17). Conceivably, the inhibition could involve changes in ATP concentration or alterations in adenylate cyclase or phosphodiesterase activity (18), either as a result of a direct action of pertussis or indirectly through secondary changes in protein synthesis. More experiments are needed to distinguish between these possibilities.

In view of the high dilution of pertussis fraction 4 capable of producing alterations in lymphocyte cyclic AMP metabolism in vitro, it seems likely that similar effects occur in vivo. In support of this possibility Ortez et al. (19) have observed reduced levels of cyclic AMP and a reduced response to epinephrine in the spleens of pertussis-treated mice. However, pertussis is known to produce marked changes in lymphocyte distribution; and the effects noted may have been secondary to shifts in the proportion of different cell populations in the spleen. Assuming that changes in lymphocyte cyclic AMP metabolism do occur in vivo, it is attractive to postulate that there might be a direct relationship between the alteration in cyclic AMP metabolism and the change in lymphocyte distribution. Unfortunately, final conclusions are not yet possible. In studies with purified *B. pertussis* fractions, leukocytosis-producing activity in mice and the ability to inhibit PGE₁ and isoproterenol stimulation in human lymphocytes seemed to parallel one another during purification until the final Sephadex G-150 step, at which the recovery of cyclic AMP-inhibiting activity in fraction 4C fell below the leukocytosis-producing activity. The failure to observe an exact parallel between the two activities could conceivably be due to the use of different lymphocytes for in vivo and in vitro studies or to a decreased stability

of the highly purified 4C fraction under the conditions chosen for the *in vitro* incubation.

The possibility that lymphocytosis and changes in cyclic AMP metabolism are produced by different macromolecules also must be considered. Work in progress on the effect of fraction 4C on mouse and rat lymphocyte cyclic AMP metabolism should help to clarify this question. Although it is still uncertain that the cyclic AMP-inhibiting activity is present in the major protein component of fraction 4C, it is clear that considerable purification has been achieved, since marked effects on lymphocyte cyclic AMP metabolism are obtained at fraction 4 concentrations as low as 5 ng/ml.

The availability of a bacterial product that markedly decreases hormonal responsiveness in at least one cell type may provide a new approach to the study of the role of cyclic AMP in complex cellular responses. Thus studies of the effect of pertussis on lymphocyte transformation and histamine release from mast cells, processes in which a modulating role for cyclic AMP has been suspected, will be of considerable interest.

SUMMARY

Bordetella pertussis culture fractions produce decreased metabolic responses to isoproterenol and epinephrine in mice and rats, suggesting the possibility of systemic β adrenergic blockade. The present study was undertaken to elucidate the mechanism of the alteration in adrenergic responsiveness and to clarify its relationship to other biological effects of the organism.

Lymphocytes were selected as a suitable tissue because of the marked alteration in lymphocyte distribution in pertussis-treated mice and rats, suggesting a change in the surface properties of these cells. Human peripheral blood lymphocytes, purified by nylon fiber chromatography, were studied. In short incubation experiments (20 min or less) *B. pertussis* did not alter the cyclic AMP response to isoproterenol, prostaglandin E (PGE_1), or methacholine. However, when cells were preincubated with *B. pertussis* for 90 min at 37°C, the responses to all three agents were markedly inhibited.

Although these observations provide direct confirmation of the ability of *B. pertussis* to inhibit catecholamine responsiveness, the fact that PGE_1 and methacholine responses were also inhibited suggests that blockade at the level of the β adrenergic receptor is doubtful. The inhibitory activity was localized in a nondialyzable, protein-rich fraction that is precipitated from *B. pertussis* culture fluid by ammonium sulfate at 90% of saturation. The bulk of the activity was obtained in the load volume after 50,000 *g* centrifugation in a cesium chloride gradient, density 1.2–1.5 (fraction 4). Fraction 4 produced a change in lymphocyte hormonal responsiveness at concentrations as low as 5 ng/ml.

The relationship between cyclic AMP inhibitory activity in isolated human cells and leukocytosis-producing activity in intact mice was studied. The two activities seemed to parallel one another quite closely until the final Sephadex

G-150 fractionation step, in which the two activities were obtained in the same column fraction, but a greater recovery of the leukocytosis-producing activity was obtained. Additional purification will be required to establish conclusively whether the same macromolecule is responsible for both activities.

The availability of a bacterial product that markedly inhibits cyclic AMP accumulation in purified lymphocytes may help to clarify the role of cyclic AMP in lymphocyte activation by antigen and nonspecific mitogens.

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