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J Clin Invest. 1973;52(3):691-697. <https://doi.org/10.1172/JCI107230>.

Research Article

Cholera enterotoxin inhibits the antigen-induced, IgE-mediated release of histamine from human leukocytes and the lysis of allogeneic mastocytoma cells by splenic lymphocytes from specifically immunized mice. This effect requires a prolonged preincubation time of the toxin with the lymphocyte/leukocyte preparations: a demonstrable inhibition requires about 30 min of pre-incubation and the toxin activity is still increasing at 90-180 min. Cholera enterotoxin also stimulates adenyl cyclase and leads to increased levels of cyclic AMP in the lymphocyte/leukocyte preparations. The concentration of toxin required for both cyclic AMP accumulation and inhibition of the biologic responses is about the same (ca. 1 ng/ml), and the time course of cyclic AMP accumulation parallels the development of inhibitory activity. Both activities, inhibition of the in vitro hypersensitivity reactions and cyclic AMP accumulation, are blocked by cholera antitoxin and by a toxoid prepared from the toxin (choleragenoid). These are specific antagonists in that they do not block the inhibiting activity or rise in cyclic AMP levels caused by other adenyl cyclase stimulators. Because cholera enterotoxin has no known activity other than the stimulation of adenyl cyclase and because of its unusual time course and the availability of specific antagonists, this data considerably strengthens the hypothesis that the cyclic AMP system influences the expression of these two forms of hypersensitivity phenomena.

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Effects of Cholera Toxin on In Vitro Models of Immediate and Delayed Hypersensitivity

FURTHER EVIDENCE FOR THE ROLE OF CYCLIC ADENOSINE 3',5'-MONOPHOSPHATE

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ABSTRACT Cholera enterotoxin inhibits the antigen-induced, IgE-mediated release of histamine from human leukocytes and the lysis of allogeneic mastocytoma cells by splenic lymphocytes from specifically immunized mice. This effect requires a prolonged preincubation time of the toxin with the lymphocyte/leukocyte preparations: a demonstrable inhibition requires about 30 min of preincubation and the toxin activity is still increasing at 90–180 min. Cholera enterotoxin also stimulates adenylyl cyclase and leads to increased levels of cyclic AMP in the lymphocyte/leukocyte preparations. The concentration of toxin required for both cyclic AMP accumulation and inhibition of the biologic responses is about the same (ca. 1 ng/ml), and the time course of cyclic AMP accumulation parallels the development of inhibitory activity. Both activities, inhibition of the in vitro hypersensitivity reactions and cyclic AMP accumulation, are blocked by cholera antitoxin and by a toxoid prepared from the toxin (choleragenoid). These are specific antagonists in that they do not block the inhibiting activity or rise in cyclic AMP levels caused by other adenylyl cyclase stimulators. Because cholera enterotoxin has no known activity other than the stimulation of adenylyl cyclase and because of its unusual time course and the availability of specific antagonists, this data considerably

strengthens the hypothesis that the cyclic AMP system influences the expression of these two forms of hypersensitivity phenomena.

INTRODUCTION

Cyclic AMP acts as an intracellular "second messenger" mediating the hormonal control of enzymatic or secretory processes in many tissues (1). Several years ago we implicated cyclic AMP as a regulator of the IgE-mediated allergic response, in a model involving the antigen-induced release of histamine from basophils of allergic patients (2–4). This work has since been confirmed in several other models of immediate hypersensitivity (5–7). The release of histamine and other mediators is prevented by agents which stimulate production of cyclic AMP (including prostaglandins, catecholamines, and histamine), block its intracellular degradation (methylxanthines), or mimic the effect of the nucleotide (dibutyryl cyclic AMP) (2–4, 8). More recently, we have shown that the same agents inhibit the activity of lymphocytes in an in vitro model of cellular immunity which involves the lysis of allogeneic mastocytoma cells by splenic lymphocytes obtained from specifically immunized mice (9, 10).

In both model systems the measured changes in cyclic AMP correlated well with the relative inhibitory effects of the amines and prostaglandins studied. The evidence was circumstantial rather than conclusive, however, because in both cases the nucleotide was measured in a heterogeneous cell population, whereas histamine release or cytolytic activity involved a subpopulation of cells. In

This is publication 40 from the O'Neill Research Laboratories of The Good Samaritan Hospital, Baltimore, Md.

Dr. Lichtenstein is the recipient of Research Career Development Award AI42373 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

Received for publication 4 May 1972 and in revised form 29 August 1972.

the case of human leukocytes, the histamine-containing basophils comprised less than 1% of the cells in which cyclic AMP was measured. The percentage of lymphocytes in mouse splenic-cell preparations was larger (>80% of the leukocytes present), but the actual number of these lymphocytes involved in specific cytolytic activity was only of the order of 2-4% (11).

Consequently, we have sought to design critical experiments which could disprove the hypothesis that cyclic AMP inhibits both immediate and cellular hypersensitivity reactions *in vitro*. For this purpose we used the enterotoxin of *Vibrio cholerae*, which stimulates adenylylase activity and the production of cyclic AMP in many tissues (12-15), including gut mucosa, where this action is probably the pathogenic basis of cholera (16).

We found that cholera enterotoxin stimulated cyclic AMP production and inhibited both histamine release

and lymphocyte-mediated cytotoxicity. The effects on cyclic AMP and cell function required low molar concentrations of the toxin, were prevented by specific antagonists, and exhibited a distinctive time course, different from that of any other compound so far tested. These results add considerable weight to the postulated causal relation between intracellular cyclic AMP and the inhibition of *in vitro* models of immediate and delayed hypersensitivity.

In an accompanying paper we report further observations related to the unique mechanism of action of cholera enterotoxin, and results of experiments in which the toxin was used to test two other hypotheses that implicate cyclic AMP as a regulator of leukocyte function (17).

METHODS

Histamine release. Leukocytes from donors sensitive to ragweed and grass were separated from the other formed elements of the blood by dextran sedimentation, washed several times, and resuspended in a serum-free Tris buffer containing calcium, magnesium, and crystallized human albumin. The cells were then challenged with either antigen E of ragweed or grass group I antigen and the histamine released from these cells into the supernatant fluid measured by the fluorometric assay described by Shore et al. (18-20). These techniques have previously been described in detail. We have currently used May's modification of our earlier method (21, 22). All experimental points are carried out in duplicate; the values agree within $\pm 5-10\%$.

Lymphocytic-mediated cytotoxicity. The system employed was that originally described by Brunner, Mauel, Cerottini, and Chapuis (23). Briefly, C_{57} B1 mice were injected intraperitoneally with 10^7 DBA/2 mastocytoma cells. 11 days later the immune mice were sacrificed, and splenic lymphoid cell suspensions prepared. 10,000,000 viable lymphocytes were incubated at 37°C for varying periods of time with 10^5 ^{51}Cr -labeled mastocytoma cells. Total cytotoxicity was evaluated by measurement of ^{51}Cr release and specific cytotoxicity by correction for the ^{51}Cr released in the presence of 10^7 normal (i.e., nonimmune) C_{57} B1 lymphocytes. These methods have previously been described in detail (11, 23).

Cyclic AMP measurements. The method of Gilman was used (24). Its application to the present systems has been described previously (4, 10), and a modification of the extraction procedure is described in detail in the accompanying paper (17).

Cholera enterotoxin, toxoid, and antitoxin. Cholera enterotoxin (choleragen) and the naturally occurring toxoid (choleragenoid) were generously supplied by Dr. Richard A. Finkelstein (25). The antiserum employed was raised by immunization of a dog with choleragen.¹ This serum had 1354 antitoxin U by comparison to a standard cholera antitoxin made by The Swiss Serum and Vaccine Institute and distributed by Dr. John Seale (National Institutes of Health Standard).

RESULTS

Immediate hypersensitivity. Cholera enterotoxin inhibits the IgE-mediated release of histamine from the human leukocytes of allergic individuals. Two variables

¹ Pierce, N. F. Personal communication.

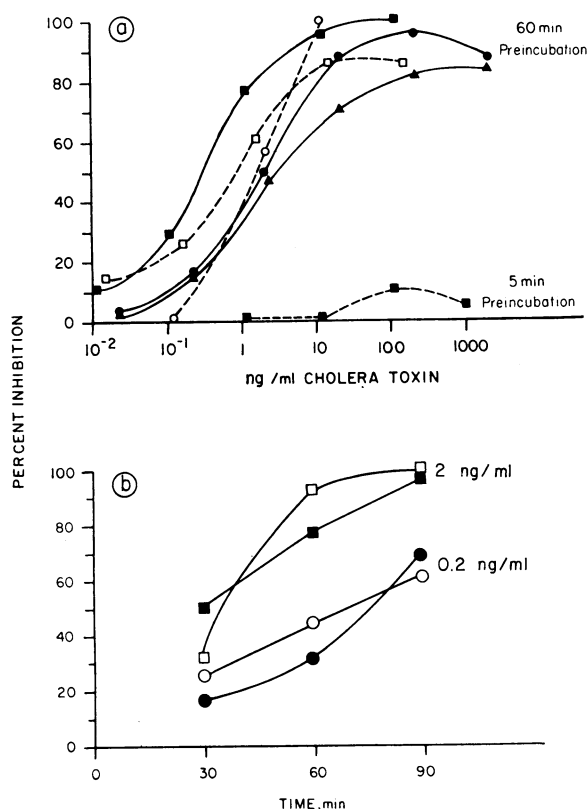


FIGURE 1 (a) Inhibition of IgE-mediated histamine release from human leukocytes incubated with cholera enterotoxin for 5 and 60 min before antigen addition. Each curve is a separate experiment except those depicted by the closed squares at 5 and 60 min preincubation, which were carried out simultaneously. (b) Time course of cholera enterotoxins' inhibition of histamine release. Cells were preincubated for 30, 60, or 90 min with the stated concentrations of cholera enterotoxin before antigen addition. Two separate experiments are shown: (○) and (■) and (□) and (●) were carried out together.

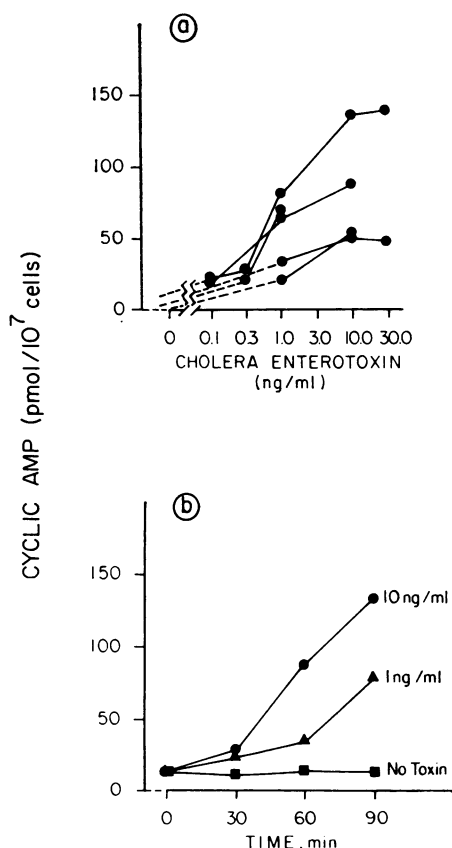


FIGURE 2 Effect of cholera enterotoxin on accumulation of cyclic AMP in leukocytes. (a) Effect of varying concentrations of cholera enterotoxin on cyclic AMP content of leukocytes from five subjects, incubated for 90 min. The origin of each dotted line represents cyclic AMP content of leukocytes incubated for 90 min in the absence of toxin. (b) Time course of accumulation of cyclic AMP in leukocytes of one subject exposed to 10 ng/ml cholera enterotoxin (●), 1.0 ng/ml (▲), or in the absence of the toxin (■). All values are the mean of duplicate determinations, differing by not more than 10%.

are important: time and concentration. This is illustrated in the dose-response curves shown in Fig. 1a, in which varying concentrations of cholera enterotoxin were incubated with the leukocytes for either 5 or 60 min at 37°C before antigen was added to initiate histamine release. At 60 min there was a profound effect, with 50% inhibition occurring usually at about 0.5–3.0 ng/ml, while little inhibition was noted after the shorter preincubation, even at 1000 ng/ml. Similar dose-response curves of inhibition have been noted in more than 10 different experiments with the cells of different donors. The time dependency of cholera enterotoxin activity is demonstrated more clearly in Fig. 1b which shows two experiments in which cells were preincubated with 2 and 0.2 ng/ml of cholera enterotoxin for 30, 60, and 90 min before the start of the reaction. Inhibition increased di-

rectly with time; the cells cannot be studied for longer than 90 min without a decrease in their viability, so it is not clear when this effect levels off.

The effect of cholera enterotoxin on cyclic AMP levels in human leukocytes after 90 min incubation is shown in Fig. 2a. The results are entirely concordant with the inhibition data: a definite effect is seen with concentrations as low as 0.1–0.3 ng/ml; 1 ng/ml provides a strong stimulus and the effect levels off at about 10 ng/ml as in the inhibition dose-response curves. Fig. 2b shows that the time course of cyclic AMP accumulation in the leukocytes after treatment with cholera enterotoxin is similar to the inhibition time course: little activity at 30 min and an increase which is still continuing at 90 min. The activity of cholera enterotoxin on cyclic AMP is potentiated by 10⁻³ M theophylline, an agent which at this concentration inhibits cyclic AMP destruction by phosphodiesterase (data not shown).

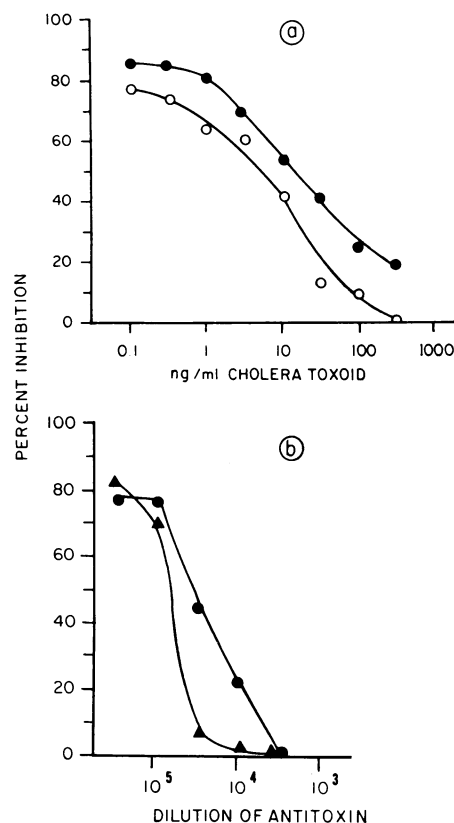


FIGURE 3 Inhibition by (a) cholera enterotoxin and (b) antitoxin of the inhibiting effect of cholera enterotoxin on histamine release. The abscissa records the dilution of antiserum (a) or the actual concentration of cholera enterotoxin (toxoid). Two separate experiments are shown in (a) and (b). In each case the cells were treated with toxoid or antitoxin first, mixed rapidly, and the enterotoxin added immediately thereafter.

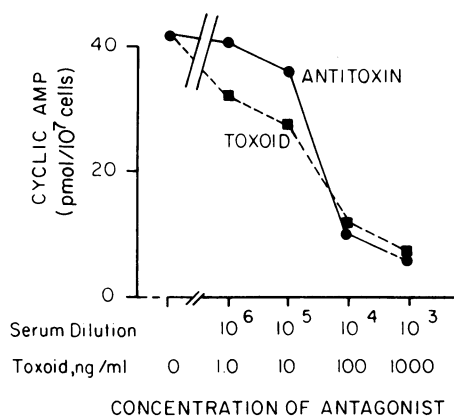


FIGURE 4 Inhibition of cholera enterotoxin-induced accumulation of cyclic AMP in leukocytes by specific antagonists. Leukocytes were incubated for 90 min in the presence of cholera enterotoxin, 10 ng/ml, and increasing concentrations of canine antiserum (●) or cholera toxin (■) before assay of cyclic AMP. The cells were exposed to the antagonists immediately before the addition of toxin. In this experiment leukocytes incubated in the absence of cholera enterotoxin contained 4.5 pmol cyclic AMP per 10⁷ cells. Each value shown is the mean of duplicate determinations, which differed by no more than 10%.

In order to demonstrate the specificity of cholera enterotoxin its effects were blocked by first treating the cells with cholera toxin and with an antitoxin raised in dogs. In the experiments shown in Fig. 3a, cells were treated with 2.5 ng/ml of cholera enterotoxin together with varying levels of the toxoid. After 60 min preincubation, antigen was added. The toxin without toxoid caused, in these two cases shown, 80 and 84% inhibition; we have plotted the percent inhibition of toxin activity by the toxoid against the concentration of cholera toxin: 50% neutralization of toxin activity was noted at about 7 and 15 ng/ml of toxoid, a three- and sixfold multiple (by weight) of the toxin level used. A similar experiment carried out with the antitoxin is shown in Fig. 3b: 50% inhibition was noted at a 1.5 and 3.0 × 10⁵ dilution of the antiserum. The effects of cholera toxin and antitoxin on cyclic AMP accumulation in human leukocytes stimulated by cholera enterotoxin are shown in Fig. 4: 50% inhibition of the cyclic AMP increase is noted at about 10 ng/ml of toxoid and at a 2–3 × 10⁵ dilution of the antitoxin. Further studies with the cholera toxin (not shown) indicate that it is specific for cholera toxin in the sense that it does not block the action of prostaglandin E1 or of isoproterenol. Similarly, the toxin is not affected by the beta blocker propranolol which inhibits the activity of isoproterenol.

Lymphocyte-mediated cytotoxicity. Lymphocyte-mediated cytotoxicity requires the specific interaction of two cell types: an effector lymphoid cell and an antigen-bearing target cell. The destruction of target cells (as

measured by ⁵¹Cr release) is a specific lytic event characterized kinetically by a linear destruction of target cells resulting from interactions of a single target cell with a single "sensitized" lymphocyte (a "one-hit destruction") (11). Compared with the in vitro release of histamine, the destruction of target cells is a relatively slow process, somewhere between 1 and 2 h being required before specific cytotoxicity is demonstrable even at a ratio of 100 lymphocytes to each target cell. In spite of these vast kinetic differences, all of the cyclic AMP active agents studied thus far are similar in the two systems (10). Isoproterenol, for example, has an effect which is rapidly dissipated in in vitro cytotoxicity inasmuch as the cyclic AMP levels rise and fall rapidly (17). Cholera

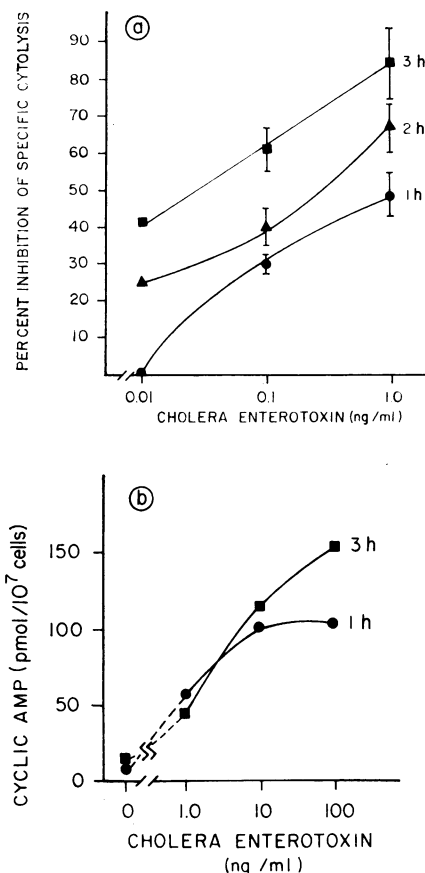


FIGURE 5 (a) Inhibition of specific lymphocyte-mediated cytotoxicity of mastocytoma target cells by cholera enterotoxin. Target cell lysis is reflected by ⁵¹Cr release (see methods). The lymphocytes were exposed to the noted concentrations of toxin for 1, 2, or 3 h before the addition of target cells; inhibition of lysis was measured four hours later and was calculated relative to uninhibited controls. (b) Effect of cholera enterotoxin on the accumulation of cyclic AMP in spleen-cell suspensions. The cells were exposed to the noted concentrations of toxin for 1 or 3 h before the cyclic AMP level was measured. All values are the mean of duplicate determinations, differing by not more than 10%.

TABLE I
Effect of Cholera Toxin on Lymphocyte-Mediated Cytolysis

Target cells	Lymphocytes	Inhibitor	Cytolysis*	Mean percent inhibition of specific cytolysis†	cAMP
			% \pm SD		pmol/10 ⁷
10 ⁵ DBA/2 mastocytoma cells	Normal C ₅₇ B1	None	8.8 \pm 1.5		
	Immune§ C ₅₇ B1	None	63.2 \pm 7.1	—	6.9
		Toxin	16.8 \pm 2.2	85.3	80.0
		Toxoid¶	64.1 \pm 4.2	0	9.2
		Toxin \pm toxoid	49.6 \pm 5.9	25.0	16.0
		Antitoxin**	62.5 \pm 2.0	<5	5.0
		Toxin \pm antitoxin	62.0 \pm 3.1	<5	17.2

* 10⁷ lymphocytes incubated for 6 h with 10⁵ ⁵¹Cr target cells. Total cytolysis is mean of at least three experiments performed in triplicate.

† Calculated relative to uninhibited controls. Lymphocytes and inhibitor were incubated together at 37°C for 3 h before washing, and the addition of target cells. Cytolysis was then evaluated 4 h later.

§ Lymphocytes prepared from animals 11 days after intraperitoneal immunization with 10⁷ DBA/2 mastocytoma cells.

|| Toxin 1 ng/ml.

¶ Toxoid 10 ng/ml.

** Antitoxin 1/100 final dilution.

enterotoxin, however, proved to be a potent agent with a long lasting action. Preincubation of lymphocytes for 2 h with 0.1 ng/ml cholera enterotoxin inhibited, by approximately 50%, the subsequent specific cytolytic activity of the lymphoid cells (Fig. 5a): This represents an inhibitory activity comparable with that shown by the toxin in the histamine-release system. The inhibition observed was not due to nonspecific toxicity of the cholera enterotoxin preparation. Trypan blue exclusion tests showed no decrease in viability of the lymphoid cell preparations after incubation with toxin.

The time course of inhibition of cytolysis by cholera enterotoxin showed a delayed pattern which was similar to that observed in the immediate hypersensitivity reaction (Fig. 5a). Measurement of the cyclic AMP accumulation in mouse splenic lymphocytes stimulated by cholera enterotoxin is shown in Fig. 5b; again the data are concordant with those derived from inhibition studies, though the concentration of toxin required for a *measurable* cyclic AMP increase is slightly higher than that required for a discernible inhibition of target cell lysis.

Experiments devised to measure the effects of the choleragenoid preparation and the dog antitoxin on the inhibition of cytolysis shown by cholera enterotoxin are shown in Table I. As in the histamine-release assay, both agents, toxoid and antitoxin antibody, interfered with toxin-stimulated cyclic AMP accumulation, and prevented the inhibition of cytolysis shown by cholera toxin.

DISCUSSION

As far as is known, cholera enterotoxin has no activity other than that of stimulating adenyl cyclase and such toxicity as it engenders is presumably due to this action, which has been noted in every cell system thus far studied (12). In addition to this broad range of activity, the protracted time necessary for its effect to become demonstrable is unique. Both characteristics make it an agent of considerable utility in attempting to analyze whether the cyclic AMP system plays a role in a particular process under study. Here we have demonstrated that the toxin inhibits both IgE-mediated histamine release and cell-mediated cytolysis and stimulates cyclic AMP accumulation in both preparations of cells used in these model systems, human leukocytes, and mouse lymphocytes. The concentration of toxin required for a measurable increase in the intracellular level of cyclic AMP is usually slightly higher than that needed to inhibit the *in vitro* model hypersensitivity reactions. This phenomenon has been noted with other agents in the two systems herein described, (4, 10) as well as in a variety of other situations (reference 1, p. 296). The explanation offered is that there is a discrepancy between the concentration of cyclic AMP required to lead a cell into a particular response (or lack thereof) and that which can be measured, with the former requiring a considerably lower concentration.

We have previously shown that there is complete agreement between the effects of the catecholamine series (isoproterenol, epinephrine, norepinephrine, and phenylephrine) as to their known beta agonist activity, their inhibition of histamine release, and their stimulation of adenylyl cyclase. The beta-blocker propranolol blocks both the inhibition of the allergic response and the cyclase stimulation. Similarly, in both hypersensitivity systems, the prostaglandin series, PGE1 and PGE2 and PGF1 α , shows complete concordance between the decreased immunologic response and increased cyclase activity (4, 10). Kinetic studies with these agents, presented in the accompanying paper, are similarly in agreement (17). With cholera enterotoxin acting on human leukocytes no effect on cyclic AMP is seen until about 30 min and the effect rises linearly at 60 and 90 min. Inhibition of histamine release after incubation of these cells with the toxin follows entirely similar kinetics. In cell-mediated specific immune cytotoxicity there is a linear rise with time in the cyclic AMP level and the inhibition of target cell lysis. These time course correlations add another dimension to the postulated causal association between increased cyclic AMP levels and *in vitro* manifestations of both immediate and delayed hypersensitivity.

The specificity of the response to cholera enterotoxin is amply demonstrated by the ability of the antitoxin and cholera toxin preparations to block the response. The action of the antitoxin is clear; it would appear from this work (seen also the following paper) that the toxoid competes with the toxin for cell receptor sites and thereby blocks its ability to stimulate cyclase.

It is perhaps curious that these two reactions should have such identical inhibition profiles. We feel this similarity is merely a reflection of the fact that both reactions are basically secretory in nature. This argument has been well-aided with respect to the IgE-mediated release of histamine (3): a similar conclusion with respect to the cell-mediated response appears to be tenable, based on the similarity in inhibition profiles. Further study is required to support this concept. Also to be established are the substance or substances "secreted" when sensitized lymphocytes are triggered by contact with homologous target cells, and how these act to kill the target cell. We do not doubt, however, that substantial differences in the mechanisms of the two reactions will come to light as they are further studied.

Finally, there is the question of how the cholera enterotoxin stimulates adenylyl cyclase and why so much time is required. Experimental approaches to this problem are considered in the following report (17).

ACKNOWLEDGMENTS

This work was supported by grants AI7290, AI8290, HL09964, and AI10280 from the National Institutes of Health.

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