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Elevated Cyclic AMP Levels in Mouse Lymphoid Tissue after Stimulation by Cholera Enterotoxin *in Vitro*[1] (38468)

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Clinical symptoms associated with cholera infections result from the action of a toxin elaborated by *Vibrio cholerae* organisms in the small bowel (1, 2). Substantial evidence indicates that cholera enterotoxin (CT) mediates the outpouring of fluid and electrolytes from the gut by activating the adenylate cyclase system, the enzyme which catalyzes the conversion of adenosine triphosphate (ATP) to cyclic 3′,5′-adenosine monophosphate (cAMP) and inorganic pyrophosphate (PP). Elevation of cAMP by CT is not restricted to intestinal cells, but has been noted in every cell system thus far studied (1–4).

Initial studies of cAMP revealed that this nucleotide acts as an intracellular "second messenger" which stimulates the hormonal control of enzymatic or secretory processes in many tissues (5). Recently, it was established that cAMP can also inhibit the "effector" component of immunocompetent spleen cells. Henney and colleagues have demonstrated that agents which stimulate the accumulation of cAMP inhibit the thymus (T-cell)-derived, lymphocyte-mediated cytolysis of allogeneic target cells (6). These same agents also inhibit the production or secretion of antibody by bone marrow (B-cell)-derived spleen cells (7). No data, however, have appeared to date in respect to changes in the level of cAMP in T- or B-cell populations contained in the spleen.

Studies in this laboratory recently demonstrated that CT also influences the inductive phase of antibody formation (8). The response to sheep erythrocytes was enhanced or depressed, depending upon the dose of CT and the time of injection relative to erythrocyte challenge. Modulation of the humoral immune response appeared to be related to changes in cAMP content in the spleen. Since the response to sheep erythrocytes requires the cooperation of T and B lymphocytes, it seemed relevant to determine whether CT could elevate cAMP levels in both thymus and bone marrow cell populations *per se*, as well as compare the content of this cyclic nucleotide in cells from different lymphoid organs. This report demonstrates that CT elevates the cAMP content in all major lymphoid tissues, with the greatest increases occurring in thymus or T-derived lymphocytes.

Materials and Methods. Animals. Young adult Balb/c mice, 6–8 wk of age, were obtained from Cumberland View Farms, Clinton, TN. The animals were fed Purina mouse pellets and acidified water (pH 2.5) *ad libitum*. For some studies, athymic (nu/nu) Balb/c mice were used; they were a gift from Dr. D. Forbes, Skin and Cancer Institute, Temple University, Philadelphia, PA.

Preparation of lymphoid cells. The spleen, thymus, peripheral and mesenteric lymph nodes and femurs were removed from individual mice after exsanguination. Bone marrow cells were obtained by forcing medium through the femur cavity. Single cell suspensions were prepared from the spleen, thymus and lymph nodes by squeezing the organs individually through a nylon mesh screen. RPMI 1640 medium was used for the isolation of all cells. All procedures were carried out at 4°. Each cell suspension was adjusted to 10^7 viable cells/ml medium, using the standard trypan blue dye exclusion test.

Cell transfer. Fifty million isogeneic bone marrow, thymus or spleen cells were given intravenously to Balb/c mice 6–8 hr after exposure to 750 R whole body X-irradiation. Seven days after cell transfer, mice were killed and spleen cells prepared as described above.

Cholera toxin. A preparation of cholera enterotoxin (lot 0572) was prepared by Dr. R. A. Finkelstein, Southwestern University Medical School, Dallas, TX, and was gener-

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ously supplied by Dr. Finkelstein and Dr. C. E. Miller, NIH, Bethesda, MD. The lyophilized toxin was reconstituted with sterile water to a stock concentration of 1.0 mg/ml and stored at 4°. Appropriate dilutions of the stock solution were prepared in RPMI 1640 on each day of assay.

Antitoxin. Antiserum to CT was prepared in New Zealand white rabbits. Three injections of 20 μg CT were given intraperitoneally at 2-wk intervals; the bleeding was taken 1 wk after the last injection. For some experiments, fluorescein-labeled anti-CT was used. The globulin fraction was separated from the serum by $(NH_4)_2SO_4$ precipitation and conjugated with fluorescein isothiocyanate (FITC), as described by Cherry (9). The fluorescein/protein ratio (A_{495}/A_{280}) of this conjugated antiserum was 2.8.

Binding of enterotoxin to lymphoid cells. One-milliliter aliquots of the various lymphoid cell suspensions (10^7 cells/ml) were incubated with varying concentrations of CT (1–1000 ng/ml) for 30 min at room temperature. After two washings with phosphate-buffered saline (PBS), pH 7.2, the cell pellets were resuspended in 1.0 ml of FITC-labeled rabbit anti-CT serum, diluted 1:25. The cells were incubated for 30 min at 37° and washed three additional times with PBS. The number and percentage of fluorescent-positive cells were determined per 500 nucleated cells, using a Zeiss fluorescent microscope. Cells not mixed with enterotoxin were always negative when stained with the same dilution of fluoresceinated serum.

Cyclic AMP assay. Cyclic AMP was measured, using Gilman's method (10). Two-milliliter aliquots of lymphoid cells (10^7 cells/ml) were incubated at 37° with or without CT for up to 24 hr in a 5% CO_2 atmosphere. At varying times, cell cultures were centrifuged and washed in RPMI 1640 medium. The pellets were resuspended in 2.0 ml ice-cold 5% trichloroacetic acid (TCA) and homogenized. To remove the TCA precipitate, homogenates were centrifuged for 10 min at 2500 rpm and the supernatants extracted five times with 2 vol acidified water-saturated ether. Following extraction, supernatants were lyophilized to dryness and resolubilized in 0.1 ml 50 m*M* acetate buffer (pH 4.0). A 0.02-ml sample was used for each cAMP assay and a standard curve was prepared and used with each assay to insure comparable results. All reagents necessary for determination of intracellular cAMP were purchased from Nuclear Dynamics, Inc., El Monte, CA. Cyclic AMP concentrations are reported as picomoles (pmol) cAMP per 10^7 viable cells.

Results. Preliminary experiments demonstrated that CT produced a delayed, dose-dependent increase in the endogenous cAMP content of murine spleen cells (Fig. 1). Cyclic AMP levels began to increase within 1–2 hr and were maximal at 4–5 hr for each dose of CT studied. At 24 hr, however, intracellular cAMP concentrations returned to baseline values.

Ten ng/ml concentrations of CT stimulated marked increases in cAMP levels in cells of both central and peripheral lymphoid organs (Table I). Thymus cells responded the most (40x) and bone marrow cells responded the least (1.5x). The same dose of CT increased intracellular levels of cAMP in spleen and lymph node cells approximately 10- to 12-fold. One ng/ml concentrations of CT also produced an increase in the level of cAMP in thymocytes (19x), splenocytes (3x) and lymph node cells (3x), but not in bone marrow cells (Table I). No increase in intracellular cAMP levels was observed when anti-CT serum (diluted 1:1000) was mixed with 10 ng CT at the time of cell culture initiation. Furthermore, in other studies it was found that addition of antitoxin (1:1000) 10 min after CT failed to prevent accumulation of cAMP (unpublished data).

Cell transfer studies with X-irradiated recipient mice were performed to determine further whether CT stimulated accumulation of cAMP preferentially in T-derived lymphocytes, as compared to B-derived cells. As seen in Table II, CT (10 ng/ml) elevated the cAMP content of T-derived spleen cells eight- to ninefold, while only a twofold increase was noted in the cAMP content for spleens of recipient mice given bone marrow cells (B-derived splenocytes). For mice repopulated with isogeneic spleen cells (mixture of T- and B-cells) a four- to fivefold increase in the cAMP content occurred follow-

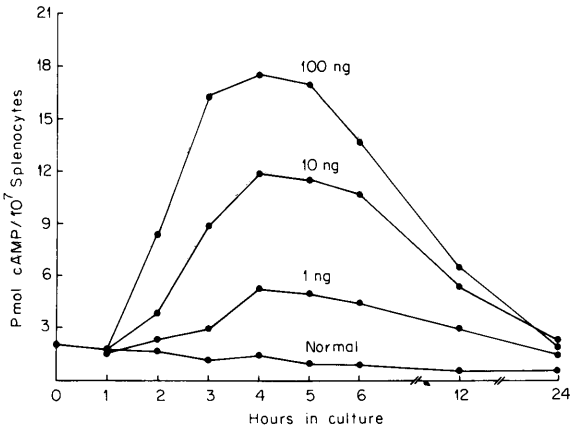

FIG. 1. The kinetics of accumulation of cyclic AMP in splenocytes from normal mice after exposure in tissue culture for varying periods of time with graded concentrations of cholera enterotoxin. Each point represents the average cAMP level for spleens from four mice at the time indicated after incubation with the indicated dose of toxin.

TABLE I. CHANGE IN cAMP LEVELS IN LYMPHOID CELLS FROM NORMAL MICE AFTER INCUBATION WITH CT.

Lymphoid cells[a]	Antitoxin[b]	Pmol cAMP/10^7 cells[c]		
		None	10.0 ng/ml	1.0 ng/ml
Thymus	−	2.1 ± 0.5	78.2 ± 7.9 (40×)[d]	40.4 ± 5.1 (19×)
	+	2.5 ± 0.7	3.1 ± 0.6 (1.2×)	−
Bone marrow	−	1.7 ± 0.2	2.4 ± 0.4 (1.5×)	1.6 ± 0.3 (0×)
Spleen	−	1.8 ± 0.4	19.8 ± 3.6 (10.5×)	5.5 ± 1.1 (3.0×)
	+	1.9 ± 0.3	2.0 ± 0.2 (1.0×)	−
Lymph node	−	1.7 ± 0.4	21.5 ± 5.2 (12×)	5.7 ± 0.8 (3.3×)

[a] Lymphoid cells from normal Balb/c mice incubated *in vitro* for 4 hr with or without indicated dose of CT.

[b] Antitoxin (1:1000) mixed with 10 ng/ml CT immediately before adding to thymus and spleen cell cultures.

[c] Average response of five mice.

[d] Number in parenthesis represents increase in cAMP content over normal.

ing incubation with CT, a value intermediate between those obtained for spleen cells from T- and B-cell repopulated mice.

Experiments with normal and athymic (nu/nu) mice also showed that thymus-derived spleen cells provided the largest increase in cAMP levels following incubation with CT *in vitro*. For these experiments, CT (10 ng/ml) stimulated a 15-fold increase in the cAMP content of spleen cells from normal mice while only a three- to fourfold increase in the cAMP content of spleen cells from athymic mice was observed (Table III).

Fluorescent antibody assay studies showed that cells from mouse lymphoid tissues readily bound CT when the concentration was 100 ng/ml or more (Table IV). The fluorescence was distributed either as a continuous ring around the cell or as discrete areas on the cell surface. The largest percentage of fluorescent-positive cells occurred with spleen and lymph node suspensions; approximately 80% of these cells were positive for CT-binding activity. The percentage appeared approximately equal to the summation of the percentages obtained for thymocyte (60%) and bone marrow cell (21%) suspensions. At a 10 ng/ml concentration of

TABLE II. CHANGES IN cAMP LEVELS IN SPLEEN CELLS FROM X-IRRADIATED MICE AFTER TRANSFER OF BONE MARROW, THYMUS OR SPLEEN CELLS.

Cells transferred (5×10^7)[a]	CT treatment *in vitro* (10 ng/ml)[b]	Pmol cAMP/10^7 spleen cells	Increase
None	−	1.1 ± 0.3	−
	+	0.9 ± 0.2	0
Bone marrow	−	0.9 ± 0.3	−
	+	2.0 ± 0.5	2.2×
Thymus	−	0.9 ± 0.3	−
	+	7.5 ± 1.7	8.3×
Spleen	−	1.0 ± 0.2	−
	+	4.8 ± 1.0	4.8×

[a] Indicated lymphoid cells given iv to recipient mice 6–8 hr after whole body X-irradiation (750 R); spleen cells tested 7 days later.

[b] CT added to spleen cell cultures from indicated mouse group and tested for cAMP levels 4 hr later.

TABLE III. CHANGE IN cAMP LEVELS IN SPLEEN CELLS FROM NORMAL AND ATHYMIC (nu/nu) MICE AFTER *in Vitro* INCUBATION WITH CT.

Mouse group[a]	CT treatment (10 ng/ml)	Pmol cAMP/10^7 spleen cells[b]	Increase
Normal Balb/c	−	1.5 ± 0.3	−
	+	22.0 ± 3.9	15.0×
nu/nu Balb/c	−	2.0 ± 0.4	
	+	6.7 ± 2.1	3.3×

[a] Spleen cells from indicated mice incubated *in vitro* for 4 hr with or without CT.

[b] Average response of five mice.

CT, bone marrow cell suspensions contained less than 1% fluorescent cells, while 7–10% of the cells in thymus, spleen and lymph node suspensions were fluorescent-positive. Few, if any, fluorescent cells were observed, however, when similar cell suspensions were incubated with 1.0 ng/ml CT (Table IV).

Discussion. The results of the experiments described in this report indicate that cholera enterotoxin elevates the cAMP content in cells from all lymphoid tissues, with the greatest increases occurring in thymocytes or T-derived lymphocytes. Immunofluorescent

TABLE IV. PERCENTAGE OF CT-BINDING LYMPHOID CELL AMONG DIFFERENT LYMPHOID TISSUES AFTER EXPOSURE TO DIFFERENT CONCENTRATIONS OF CT.

Cell suspension[a]	Cholera enterotoxin concentration for binding (ng/ml)[b] 1000	100	10
Thymus	60	19	7
Bone marrow	21	6	<1
Spleen	80	27	10
Lymph node	90	21	5

[a] Indicated type of cells from Balb/c mice incubated with indicated dose of CT *in vitro* before immunofluorescent staining for CT-binding cells.

[b] Average percent of fluorescent-positive cells for 500 lymphoid cells from five mice incubated for 30 min with indicated concentration of enterotoxin.

studies showed that cells from the various lymphoid tissues also bind CT directly, although not equally. The greatest number of CT-binding cells were observed in spleen and lymph node suspensions incubated with 1000 or 100 ng CT/ml. Bone marrow suspensions contained the fewest CT-binding cells.

Substantial evidence is available which demonstrates that spleen and lymph node cell suspensions contain both thymus-derived and bone marrow-derived lymphoid cells (11, 12). Whether CT-binding cells in the spleen and lymph nodes also contain theta or Ig surface markers which differentiate T- and B-cells is currently under investigation. It seems important to note, however, that at lower concentrations of CT (10 ng/ml), few or no CT-binding cells were found in bone marrow cell suspensions and less than 10% of the cells in thymus, lymph node, or spleen cell suspensions were binding CT. At this concentration, however, thymocytes accumulated three to four times more cAMP than spleen or lymph node cells and 25–30 times more cAMP than bone marrow cells. Such results suggest that thymocytes contain more adenylate cyclase than other lymphoid cells and/or they have more CT receptors. Undoubtedly, the immunofluorescence assay does not detect all cells binding CT and it is possible that several explanations may be possible as to why low doses of CT can stim-

ulate cAMP increases without demonstrable binding of CT by such cells.

Several investigators have proposed that the G_{M1} ganglioside is the receptor structure for CT, since such gangliosides block the biological effects of CT on isolated fat cells and cells from the small intestine (13, 14). No information, however, is available concerning the number of such possible receptors on B- and T-lymphocytes and whether or not such receptors are actually the same as those present on other cells which respond to CT. Holmgren *et al.* (15) have demonstrated recently, however, that thymocytes bind up to 40,000 molecules of toxin within minutes. Cuatrecasas has demonstrated that a single fat cell can bind up to 20,000 molecules of CT within the same time (16). With such a rapid binding characteristic it is not surprising that addition of anti-CT antibody 10 min after addition of CT to lymphoid cell suspensions does not influence the level of cAMP stimulation. Others have observed that antitoxin does not inhibit the biological activity of CT when added shortly after addition of CT to lipocytes or intestinal mucosa cells (1–4).

It is now generally accepted that cyclic AMP acts as a second messenger for cells involved in many biological functions, including antibody production and other immunological reactions such as hypersensitivity (17, 18). It is noteworthy, however, that stimulation of antibody formation to antigens such as sheep erythrocytes by CT occurs only under certain conditions. When CT is administered 12–24 hr before antigenic challenge, there is a depression of the expected immune response (8). This has been interpreted as being due to a reduction in the endogenous cAMP content after an initial increase. Several recent reports demonstrate that immune activity can be depressed by elevated cAMP levels. Such studies are, however, concerned mainly with effector cells and not with the induction of the immune response (6, 7, 18).

It seems noteworthy that recent studies in this laboratory concerning the effects of CT on antibody formation *in vitro* after addition of antigen to lymphoid cells in culture have also revealed a dichotomous effect, i.e., stimulation or enhancement of antibody activity under certain conditions and depression under others (19). A major factor involved in these contrasting responses appears to be the dose of CT and the time when CT is added to the spleen cells relative to the time of antigenic stimulation. Since T-lymphocytes may serve as a helper for B-cells in the immune response to sheep erythrocytes, it seems likely that the effect of CT on cAMP levels in thymocytes may play an important role in the immune response.

Whether the T-cells which respond to CT with increased cAMP levels are also the same cells which serve as "helpers" for antibody formation to sheep erythrocytes or are the effector cells which mediate T-cell-dependent cytolysis is conjectural at present. However, continued use of cholera enterotoxin and other cAMP stimulators provide pharmacological probes to study the mechanism and role of this nucleotide in the induction of immune responsiveness. Studies of this type are in progress.

Summary. Addition of CT to suspensions of thymus, lymph node, spleen, or bone marrow cells *in vitro* resulted in a marked accumulation of cAMP with peak levels occurring 4–5 hr after incubation of cells with CT. Thymus cells showed the largest increase in cAMP, approximately 40-fold at 10 ng/ml CT. Bone marrow cells accumulated the least cAMP (1.5x), while intermediate levels were observed for spleen and lymph node cells (10–12x). Antiserum to CT prevented stimulation of increased cAMP levels.

Repopulation studies using X-irradiated mice also showed that thymus-derived spleen cells accumulated more cAMP/10^7 cells than spleen cells from recipients given spleen or marrow cells. Spleen cells from athymic (nu/nu) mice also responded much less than did spleen cells from normal mice.

Thymocytes appeared to bind CT to a greater degree than bone marrow cells. Spleen and lymph node cell suspensions also contained CT-binding cells and the number of CT-binding cells in these peripheral lymphoid tissues appeared approximately equal to the summation of the numbers observed in thymocyte and bone marrow cell suspensions.

Stimulation of cAMP in lymphoid cells, especially thymocytes, by CT provides a pharmacological tool to investigate the mechanism and role of this nucleotide in the early events of antibody formation.

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