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THE EFFECT OF AGENTS WHICH MODULATE LEVELS OF THE CYCLIC NUCLEOTIDES ON HUMAN LYMPHOTOXIN SECRETION AND ACTIVITY IN VITRO¹

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SUMMARY

The ability of concanavalin-A (Con-A) to activate lymphocytes to secrete human lymphotoxin (LT) was tested in the presence of agents known to modify the intracellular levels of cyclic nucleotides (cAMP and cGMP). Lymphocytes were treated with these agents at different stages of activation: (1) during the first encounter with mitogens, and (2) after they had been fully activated and were restimulated. Two agents, Dibutyryl cAMP and theophylline, dramatically inhibit the amount of LT secreted during both "primary" and "secondary" activation by Con-A. In contrast, DL-isoproterenol had a weak effect during primary activation, but greatly reduced the level of LT secreted during secondary activation. Agents which affect the intracellular level of cGMP were also tested. Imidazole had no effect on LT secretion by either primary or secondary Con-A activated cells. In contrast, carbamyl choline greatly reduced LT secretion to a level comparable to Dibutyryl cAMP and theophylline. All agents tested protected, to some degree, the sensitive α -L cell against LT-induced destruction in vitro. Agents which affect the levels of cyclic nucleotides affect both the effectiveness of the aggressor cell and the sensitivity of the target cells in vitro.

Cyclic nucleotides have been shown to have an important role in the regulation of cellular biosynthesis and metabolism (19) in inflammation and immunity (1). They appear to be important in both immediate and delayed hypersensitivity reactions. Production and/or secretion of antibodies by B lymphocytes is reduced when intracellular levels of cyclic adenosine 3',5'-monophosphate are raised (16, 23). Destruction of cells by immune lymphocytes activated by contact with target cell antigens, and in vitro measure of cell-mediated immunity, is suppressed when the effector lymphocytes are treated with pharmacologic drugs which increase the adenosine 3',5'-monophosphate (cAMP) level (14, 21, 22). On the other

hand, their effectiveness is enhanced when agents increasing guanosine 3',5'-monophosphate (cGMP) are employed (21, 22). These findings are consistent with the concept of a bidirectional control system involving these two key cyclic nucleotides (8).

Activated lymphocytes secrete in vitro a number of soluble molecules, collectively termed lymphokines (LK), which may be important effectors in CMI reactions (7). It has been shown that production of interferon by PHA-stimulated human lymphocytes is decreased by agents which increase the intracellular level of cAMP (1). The activity of migration inhibitory factor (MIF) on the indicator macrophage is reduced by drugs which increase cAMP (11, 18), however, the role of cyclic nucleotides in the release of MIF by the activated lymphocyte is still not clear (11).

Lymphotoxin (LT) is a cell toxin found in the supernatant of mitogen- or antigen-activated lymphocytes and may be an important mediator of lymphocyte-induced cell lysis or inhibi-

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tion of cell division in vitro (7). The relationship between the role of cyclic nucleotides, the release of LK by activated lymphocytes, and the activity of LK on the target cells is important and must be clarified. In the present report the effect of agents which influence the intracellular level of cyclic nucleotides on LT release by Con-A-activated human lymphocytes and on LT-induced target cell cytolysis have been studied.

MATERIALS AND METHODS

Agents which modulate intracellular levels of cyclic nucleotides. The agents employed were DL-isoproterenol (10^{-4} M), lot 71C-1740; dibutyl cyclic adenosine 3',5' monophosphate (10^{-4} M), lot 91C-7020; theophylline (10^{-3} M), lot 320-A; carbamyl choline chloride (10^{-12} M), lot 21C-141; imidazole (10^{-7} M), lot 53C-5430, and were purchased from Sigma Chemical Co., St. Louis, Missouri. Stock solutions of each agent were prepared fresh each day. The amount of the various agents employed has been shown to modify the intracellular levels of cyclic nucleotides (21). It was also verified that these levels were not toxic. The indicated molarity was chosen in order to avoid toxicity on lymphocytes.

Target L cells. Target cells employed were highly LT-sensitive α -L 929 mouse fibroblasts (α -L cells) isolated in our laboratory and maintained by biweekly passage as previously described (15, 20). The cells were maintained as monolayers in 8- or 160-ounce prescription bottles in Eagle's Minimum Essential Medium supplemented with 4 mM glutamine, 3% fetal calf serum (Microbiological Associates, Bethesda, Maryland) penicillin (100 U/ml) and streptomycin (100 μ g/ml) (MEMS).

Lymphocyte cultures. Lymphocytes were obtained from surgically removed adenoids of healthy children as described previously (24). Cells were cultured at a concentration of 3×10^6 viable lymphocytes/ml for primary stimulation experiments and 4×10^6 viable lymphocytes/ml for secondary stimulation experiments. The cultures were maintained in MEM supplemented with 4 mM glutamine, nonessential amino acids, 1 mM sodium pyruvate, 5×10^{-5} M mercaptoethanol (EtSH), 5% newborn calf serum, 30 μ g/ml garamycin, 2.0 μ g/ml amphotericin B, penicillin (100 U/ml), and streptomycin (100 μ g/ml). Aliquots of 2.0 ml were cultured in screw-capped tubes (16 \times 125 mm) under a 5%

CO₂-air atmosphere at 37 C. Lymphocyte activation was effected by addition of a predetermined optimal dose of concanavalin-A (Con-A, Sigma, lot 123C-5030). The cultures were established under two basic experimental protocols: (1) "primary" activation, effected by adding Con-A up to 20 μ g/ml to nonactivated cells, and (2) "secondary" stimulation of previously activated cells, which were temporarily deactivated. The latter procedure has been described in detail previously (3, 4). Briefly, after a 48-hr activation by incubation with Con-A at 20 μ g/ml, the cells were washed free of Con-A by the addition of phosphate buffered saline (PBS, 0.15 M NaCl, 0.01 M PO₄ buffer, pH 7.2) containing 5×10^{-2} M α -methyl-mannoside (MAM, Sigma), a competitive inhibitor of Con-A. The cells were then maintained with MEMS + MAM for 24 hr at 37 C. After this interval, the MAM was removed by washing, and the cells were subjected to a second stimulation with Con-A, 10 μ g/ml, with or without addition of one of the various test agents. To verify if the cells were still viable, and the effects reversible, treated cells were washed and recultured in fresh medium with 10 μ g/ml Con-A. Supernatants were collected at each step and tested for LT activity on target cells (Fig. 1). Lymphocyte viability at each step was determined by microscopic observation of the cells in an 0.1% eosin Y solution.

Assay for LT-induced cytotoxicity. A highly sensitive LT assay was performed as previously described (20). After trypsinization, target L cells were adjusted to 1×10^5 cells/ml and treated with 0.5 μ g/ml mitomycin C. The cell suspension (1 ml) was added to each screw-capped tube (16 \times 125 mm) and incubated 24 hr on a 5-degree slant under 5% CO₂ air at 37 C in darkness. The medium was discarded and 1 ml of a 5-fold serial dilution of LT-containing or control medium was added and the cultures incubated at 37 C for an additional 24 hr. Duplicate or triplicate tubes were tested for each dilution. The number of remaining viable target cells in each tube was counted in a model F Coulter Counter. The LT titer is defined as the reciprocal of the dilution necessary to effect a 50% reduction in the number of viable cells.

RESULTS

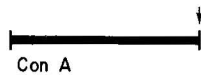
Modification of LT secretion during primary activation by Con-A. The various agents were added to the lymphocyte cultures simultane-

ously with an optimum activating dose of Con-A ($20 \mu\text{g/ml}$). After 48 hr of incubation at 37°C , the supernatant fluid was collected and 5-fold serial dilutions of the medium tested for LT activity. These experiments were repeated a total of 5 times and the results of a typical experiment are shown in Table 1. DibutylcAMP and theophylline significantly reduced the LT titer when compared to the Con-A stimulated control. In contrast, isoproterenol

had little or no effect on LT secretion. The two agents which affect intracellular levels of cGMP were also tested in the same conditions. No modification in the amount of LT secretion was observed with 10^{-7} M imidazole. In contrast, 10^{-12} M carbamyl choline strongly reduced LT secretion below stimulated control levels. While the data are not shown, no LT release was induced when the agents were added to the lymphocytes in the absence of Con-A. Experiments were performed to test the possibility that the reduction in titer could be due to the effect of the agents on: (1) the target cell, or (2) on LT itself. Each of the various agents were tested for their effects on mitomycin C-treated L fibroblasts. The data shown in Table 2 revealed that of all the materials tested, only theophylline had a weak, but not significant, toxic effect on the target L cells and only at the highest concentration. In addition, the drugs at the same concentrations used in Table 2 were added to a standard LT batch, and subsequently diluted. We found at these dilutions they did not modify the endpoint titer in a significant way, as is shown in Table 3.

Kinetics of LT secretion during in vitro secondary activation. Recent data indicate that LT secretion requires a continual membrane stimulation by Con-A, and is rapidly inhibited after removal of Con-A by washing and addition of the Con-A competitive inhibitor, α -methylmannoside (MAM) (3). A secondary accelerated responsiveness was observed when the blocked lymphocytes were restimulated with fresh mitogen (4). To illustrate this, one typical experiment resulted in a titer of 1333 ± 333 LT units after 48 hr of a primary stimulation with Con-A ($20 \mu\text{g/ml}$), which dropped to 89 ± 11 LT units after a 24-hr culture in 5×10^{-2} M MAM, then

A) Primary Activation



B) Secondary Activation

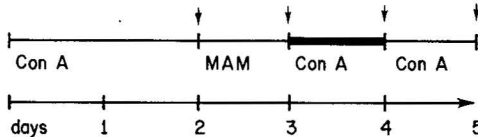


FIGURE 1. The experimental protocol of "primary" and "secondary" activation of human lymphocytes in vitro with Con-A. The agents employed in these studies were added at various times, as illustrated above (heavy lines). (A) During primary activation by Con-A, $20 \mu\text{g/ml}$ for 48 hr, or (B) during secondary activation for 24 hr. In the latter case, lymphocytes were first activated by Con-A ($20 \mu\text{g/ml}$) for 48 hr. Then the free Con-A washed away and bound Con-A was inhibited by adding 5×10^{-2} M α -methylmannoside (MAM) for 24 hr. Secondary activation was produced after removal of MAM and restimulated with Con-A ($10 \mu\text{g/ml}$) with or without addition of the various agents for 24 hr. To test for reversibility, the agents were washed out of the cultures, and the cells were restimulated. The supernatants were tested for the level of LT activity (titer) at each time indicated by an arrow.

TABLE 1. Primary activation of lymphocytes by Con-A with or without agents which modify intracellular level of cyclic nucleotides^a

Agents (molarity)	LT released in presence of various agents (units/ml \pm SD)	% Activated controls \pm SD	
Isoproterenol	1.10^{-4} M	1698 ± 365	$90\% \pm 21\%$
Dibutyl cAMP	1.10^{-4} M	615 ± 219	$32.5\% \pm 11.4\%$
Theophylline	1.10^{-3} M	738 ± 83	$39\% \pm 6\%$
Carbamyl choline	1.10^{-12} M	410 ± 20	$21.6\% \pm 5.3\%$
Imidazole	1.10^{-7} M	1945 ± 315	$103\% \pm 19\%$
None		1890 ± 196	

^a The LT titers were determined on 48-hr supernatants from lymphocyte cultures. The amount of LT in the supernatant was determined as described in Methods. The data are expressed as the mean of triplicate cultures.

TABLE 2. Activity of agents which modulate cyclic nucleotide levels of viability of mitomycin C-treated target L cells^a

Dilutions tested	Theophylline (1.10 ⁻³ M)	Dibutyryl cAMP (1.10 ⁻⁴ M)	Isoproterenol (1.10 ⁻⁴ M)	Imidazole (1.10 ⁻⁷ M)	Carbamyl (1.10 ⁻¹² M)
1/1	88 ± 5.7	107 ± 6.5	101 ± 5.5	105 ± 9	100 ± 7
1/5	91 ± 13.2	112 ± 8	91 ± 13	105 ± 4.3	103 ± 4.6
1/25	102 ± 5	113 ± 5.8	102 ± 5	105 ± 6	101 ± 4.8
1/125	105 ± 7	109 ± 8.2	95 ± 7	106 ± 4.7	92 ± 5.6
1/625	98 ± 9	112 ± 4.4	92 ± 6	105 ± 4.8	107 ± 4.9

^a The agents in the culture medium were tested for 24 hr at the initial concentration shown, and at 5-fold dilutions on previously mitomycin C-treated target cells. All data are expressed as percentages.

TABLE 3. Effect of low concentrations of agents which modulate levels of cyclic nucleotides on cytolysis of L cells in vitro by human LT^a

Agents	Supernatant LT activity (units/ml ± SD)	% of LT activity compared to control
Isoproterenol	753 ± 181	66.5 ± 24
Dibutyryl cAMP	1130 ± 341	100 ± 34
Theophylline	1012 ± 6	96.5 ± 1.8
Carbamyl choline	1305 ± 49	115 ± 22
Imidazole	913 ± 208	80.7 ± 35
None	1131 ± 200	

^a The agents were added to a batch of human LT at the initial high concentration. Then the agent and LT were diluted by serial 5-fold dilutions in MEM. Each dilution was tested for toxicity on L cells by the standard method outlined in the text. The final titer of each medium was compared to the control titer obtained from L cells treated with LT alone and is expressed as 100%.

raised to 6781 ± 115 LT units after 24 hr, upon restimulation with Con-A (10 µg/ml), indicating that once activated, the cells became hyperresponsive to a small secondary stimuli. We next designed experiments to study the action of the various drugs on LT secretion during Con-A restimulation. After 24 hr of exposure to the agents and Con-A, the supernatants were collected and the amount of LT secreted was determined. To examine if the effect of the agent on the lymphocytes was reversible, the cells were washed once to remove the drugs, then resuspended in fresh MEM containing Con-A (10 µg/ml), and the experiments were tested after 24 hr reincubation at 37 C. These experiments were repeated a total of 8 times. The results from one experiment are summarized in Figure 2, A and B. The amount of LT activity detected in the different supernatants

is expressed as a percentage of the untreated stimulated control. Isoproterenol, dibutyryl cAMP, and theophylline dramatically inhibited LT secretion by restimulated cells as shown in Figure 2A. However, the suppressive effect was not reversible within the following 24 hr for isoproterenol and dibutyryl cAMP (23 and 32% of the stimulated control), but was for theophylline (115% of the stimulated control) (Fig. 2B). Imidazole had no inhibitory effect on LT secretion of secondary stimulated cells (Fig. 2A). In contrast, carbamyl choline (1×10^{-12} M) strongly reduced the LT titer to about 20% of the control (Fig. 2, A and B). It was determined that lymphocyte viability at the end of the experiment was similar in both the treated and control cell suspensions.

Effect of agents which modulate cyclic nucleotide levels on LT-induced L cell destruction. Experiments were designed to test the possibility that the agents which modulate intracellular levels of cyclic nucleotides may also affect the action of LT on the target L cell. It was previously demonstrated that the majority of the agents had no significant cytotoxic effect on the mitomycin C-treated target cells. Tube cultures of mitomycin C-treated target cells were exposed to constant high concentrations of the different agents added to a serial 5-fold dilutions of a standard batch of LT. The results of these experiments are shown in Table 4. It is readily apparent that all the agents employed at the indicated molarity "protected" the target L cells to some degree from LT-induced cytolysis.

DISCUSSION

The cyclic nucleotides cAMP and cGMP have been identified as important intracellular effectors operative in the induction and control of

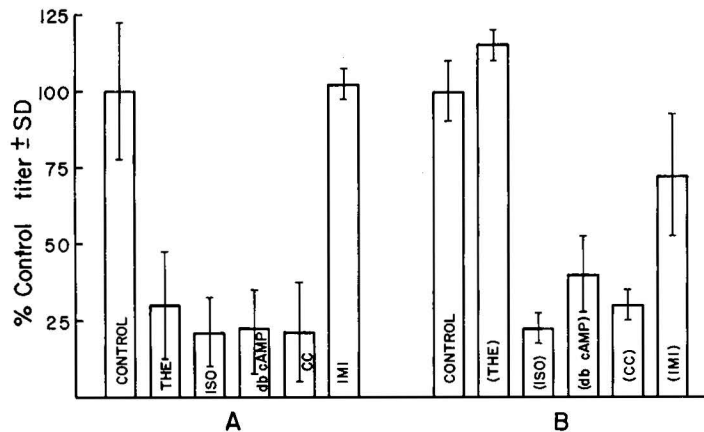


FIGURE 2. The effect of agents which modulate cyclic nucleotide levels on LT secretion by Con-A-restimulated human lymphocytes in vitro. Lymphocytes were stimulated with Con-A, 20 $\mu\text{g}/\text{ml}$, for 48 hr, and deactivated by washing out free Con-A and adding 5×10^{-2} M α -methyl-mannoside (MAM) for 24 hr. (A) The MAM was removed and the cells were resuspended in fresh medium containing one of the agents and Con-A, 10 $\mu\text{g}/\text{ml}$, at the following concentrations: theophylline, 1×10^{-3} M (THE); isoproterenol, 1×10^{-4} M (ISO); dibutyl cAMP, 1×10^{-4} M (db-cAMP); carbamyl choline, 1×10^{-12} M (CC); imidazole, 1×10^{-7} M (IMI). The supernatants were tested for LT activity after 24 hr culture. (B) The lymphoid cells from A were resuspended in fresh MEM and restimulated with Con-A. Supernatants were collected from each culture, which had been previously treated with one of the agents indicated in parentheses. The results are expressed as the percentage \pm SD of the titer of each test culture related to the titer of the mitogen-stimulated control. Representative data from two separate assays from supernatants obtained in Experiment 7 of eight experiments.

TABLE 4. Effect of agents which modulate cyclic nucleotides on LT-induced cytolysis of mitomycin C-treated L cells in vitro^a

Agents employed (molarity)	Theophylline (1.10^{-3} M)	Dibutyl cAMP (1.10^{-4} M)	Isoproterenol (1.10^{-5} M)	Carbamyl choline (1.10^{-12} M)	Imidazole (1.10^{-7} M)
% Reduction of LT titer	87.5	75	70	50	67

^aThe percentage reduction represent the amount of reduction of the LT titer compared to the controls without agents. The agents were added at a constant molarity to 5-fold dilutions of a standard LT batch and tested for toxicity on mitomycin-treated cells. Representative data from Experiment 3 of four experiments.

numerous cellular processes, indeed, they have been referred to as a "second messenger system". The actual mechanism(s) of how these substances cause the effects is complex and not well understood. In certain situations, raising the level of intracellular cyclic AMP results in increased synthesis and secretion of protein by certain cells, i.e., salivary gland and pancreatic islet cells (19), while the same treatment of murine leukocytes in vitro induces an inhibition of secretory and functional events (1). The concept has evolved that the regulating events may be effected not by one agent alone, but perhaps the relative levels or balance of one to another. There exists a host of substances which can be interacting with receptors on lymphoid

cells, affecting the levels of cyclic nucleotides and perhaps the immune response itself.

The present studies demonstrate that the pharmacological agents which affect the levels of intracellular cyclic nucleotides also have a decided effect on the secretion of LT Con-A-stimulated human lymphocytes in vitro. These studies confirm and extend the observations of Lies and Peter (15). It appears that agents which raise the level of intracellular cyclic AMP greatly reduce LT release. However, the situation is more complex, because in high concentrations these same agents block LT action on target cells. Thus, it is very important to show that this is truly a reduction of LT release or secretion and not protection of the target indi-

cator cells. We were able to separate these two events because they are very concentration-dependent, namely, protection of the target cell only occurs at high concentrations, whereas the agents were rapidly diluted out beyond their effective levels during the titrating procedure employed to determine the amount of LT present. This is clearly demonstrated in the experiments where the agents were diluted serially with a standard batch and, with the exception of isoproterenol, were found to have no effect on the endpoint titer. Once activated, the secretory mechanism is fully induced, and the restimulated cells were inhibited to a much greater degree by the same concentrations of the various agents than were the cells upon primary activation. This was particularly evident in studies employing isoproterenol, which did not modify the primary secretion, whereas it was inhibited by almost 80% during secondary stimulation. However, there is no obvious explanation for this observation; it was shown by Strom et al. (21) in a direct cytotoxic system employing rat lymphoid cells, that isoproterenol had an inhibitory effect, but only when the drug had been put on the lymphocytes 2 min before mixing with the target cells. A similar time-dependence was also shown by Henney et al. (9) studying cytotoxic mouse lymphoid cells. It was surprising to find that of all the agents we employed to affect cAMP levels, only theophylline was readily reversible.

The two agents we employed which are reported to affect the intracellular levels of cyclic GMP had opposite effects. Imidazole activates cyclic AMP phosphodiesterase and blocks cyclic GMP phosphodiesterase, leading to a decrease in the level of cyclic AMP and an increase in the level of cyclic GMP (6). Imidazole had little or no effect on LT levels when present in cultures of primary or restimulated human cells. This might be explained by the observation that in mitogen-stimulated cells the level of cyclic GMP may have already reached a high threshold, and the activated cell would no longer be responsive to further increases (8). In contrast, carbamyl choline, which is a cholinergic agonist, inhibited the amount of LT secreted by both primary and secondary activated cells. We have no explanation for this observation, since it was not toxic for the lymphocytes. However, it cannot be excluded that the blocking effect was based on a process altogether unrelated to its effect on cyclic GMP.

All the agents tested had a protective effect at high molarity on LT-induced cytotoxicity of L cells *in vitro*. The protection of target cells by these agents occurred if the cells were treated simultaneously with the various agents and LT; however, in other experiments, we found no modification of killing when the cells were pretreated with the agents for 4 hr. It has been demonstrated that changing the levels of cyclic AMP and GMP in fibroblasts has an effect on the growth rate (2, 10, 17). This is not a factor involved in the cytotoxic assay since: (1) LT activity on target cells is independent of DNA synthesis (12, 20); (2) target cell growth is inhibited in our assays by adding mitomycin C. The actual mechanism involved in the protection is not clear and needs further investigation. It could be because of affecting numbers of LT receptors on the cell surface or in some way modifying the cellular repair mechanism involved in resistance to cytolysis.

The events which serve to regulate cell-mediated immune reactions are just beginning to be understood yet they are central to our understanding of this complicated phenomenon. While the present *in vitro* system employing mitogen-activated nonimmune cells may not be a reflection of an immune reaction, it does provide important insight into some of the events which may be regulating lymphocyte effector molecule secretion and cell-mediated immune reactivity.

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