

## THE MODULATING INFLUENCE OF CYCLIC NUCLEOTIDES UPON LYMPHOCYTE-MEDIATED CYTOTOXICITY\*

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After sensitization with an allograft, the mouse develops thymus-derived (T) lymphocytes that are selectively cytotoxic for cells bearing donor alloantigens (1, 2). Intimate contact (3) between viable sensitized lymphocytes and target cells is a prerequisite for cytotoxicity (lymphocyte-mediated cytotoxicity or LMC)<sup>1</sup> (1-3) although the exact nature of the cytotoxic process is unknown. A variety of agents that stimulate adenylate cyclase, the enzyme that catalyzes the formation of adenosine 3',5'-cyclic monophosphate (cyclic AMP) from adenosine 5'-triphosphate (ATP), and methylxanthines, which inhibit the hydrolysis of cyclic AMP to 5'-adenosine monophosphate (5'-AMP), suppress LMC mediated by mouse (4-6) and rat (7) cells. Cholinergic agents, which have been shown to elevate intracellular guanosine 3',5'-cyclic monophosphate (cyclic GMP) in a variety of tissues (8-13), including the human lymphocyte (13) through activation of guanylate cyclase, enhance LMC (7).

The present studies employing a purified rat spleen lymphocyte population establish that the effects of activators of adenylate cyclase and cholinergic agents are mediated by an action upon the attacking and not target cells. They further reveal that cholinergic enhancement is not associated with a measurable change in cyclic AMP levels, is duplicated by 8-bromo-cyclic guanosine 3',5'-monophosphate (8-bromo-cyclic GMP), and is independent of the presence of immunoglobulin-bearing lymphocytes. Finally, the results indicate that the modulating effects of the intracellular levels of cyclic AMP and cyclic GMP upon the attacking cell population are dependent upon the concentration of cyclic nucleotides (cyclic AMP and cyclic GMP) at the moment of initial interaction with the target cells.

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<sup>1</sup> *Abbreviations used in this paper:* 8-bromo-cyclic GMP, 8-bromo-cyclic guanosine monophosphate; C, unabsorbed guinea pig complement; cyclic AMP, adenosine 3',5'-cyclic monophosphate [cAMP]; cyclic GMP, guanosine 3',5'-cyclic monophosphate; FITC, fluorescein isothiocyanate; LMC, lymphocyte-mediated cytotoxicity; PGE<sub>1</sub>, prostaglandin E<sub>1</sub>; pM, picomoles.

### Materials and Methods

The agents listed were obtained from manufacturers: carbamylcholine chloride (carbachol), imidazole, aminophylline, and DL-isoproterenol (Sigma Chemical Co., St. Louis, Mo.); RPMI-1640 medium and fetal calf sera (Grand Island Biological Co., Grand Island, N. Y.); fluorescein isothiocyanate (FITC) and unabsorbed guinea pig complement (C) (Nutritional Biochemical Corp., Cleveland, Ohio); desoxyribonuclease (DNase) (Pentex Biochemical, Kankakee, Ill.); and  $\text{Na}_2^{51}\text{CrO}_4$  (specific activity = 1 mCi/ml) (Nuclear-Chicago, Corp., Chicago, Ill.). Prostaglandin  $\text{E}_1$  ( $\text{PGE}_1$ ), cholera toxin, and 8-bromo-cyclic GMP were gifts received from Dr. John Pike, The Upjohn Co., Kalamazoo, Mich.; SEATO Cholera Research Program, National Institute of Allergy and Infectious Diseases; and Dr. Lionel Simon of ICN Nuclear Acid Research Institute (ICN Corp., Irvine, Calif.), respectively. Male Lewis, Brown Norway (BN), and (Lewis  $\times$  Brown Norway  $\text{F}_1$ ) (LBN) rats were obtained from Microbiological Associates, Inc., Bethesda, Md.

**Grafting and Cell Preparation.**—Lewis rats were given full-thickness skin grafts, 2.5 cm in diameter, from LBN rats. After exsanguination of anesthetized rats by aortic puncture, sensitized Lewis rat spleen tissue and normal BN thymus tissue were excised and placed in wash medium consisting of RPMI-1640 medium containing 0.5 vol percent of 1 M buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and 4% (vol/vol) heat-inactivated (56°C for 2 h) fetal calf serum. The organs were expressed manually through a 60 gauge steel mesh, and the resulting suspensions passed through a tube containing cotton to remove tissue fragments. The spleen and thymus cell preparations were purified by Ficoll-Hypaque gradient separation (14) such that the final suspensions consisted of lymphoid cells that appeared 98% viable by phase microscopy. After washing twice with sedimentation at 200 *g* at room temperature, 1.0 ml of the thymic cell suspension containing  $10^6$ – $10^7$  cells was incubated at 37°C with 400  $\mu\text{Ci}$  of  $^{51}\text{Cr}$  for 1 h. The suspension was then washed twice to remove free  $^{51}\text{Cr}$ . The spleen and thymus cells were suspended in culture medium consisting of RPMI-1640 medium to which 10% (vol/vol) heat-inactivated fetal calf serum and 0.5 vol percent of 1 M buffer were added. The number of thymus and spleen cells in suspension was determined on a Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.). 1 aliquot of spleen cells was used for quantitation of LMC and the other was placed in Tyrode's buffer for determination of intracellular cyclic AMP levels.

**Quantitation of Lymphocyte-Mediated Cytotoxicity (LMC).**—LMC was determined by a modification of the technique of Brunner et al. (15). The attacking cell population consisted of Lewis spleen cells harvested 7 days post-LBN to Lewis grafting, and the target cells were  $^{51}\text{Cr}$ -labeled BN thymocytes.  $5 \times 10^4$  BN thymocytes were added to  $5 \times 10^6$  sensitized Lewis spleen cells to achieve a final medium vol of 2 ml in triplicate samples placed in disposable  $10 \times 75$ -mm glass tubes. After incubation at 37°C for 30 min, the mixtures were centrifuged at 50 *g* for 10 min and further incubated for 3.5 h at 37°C. The cells were sedimented at 400 *g* for 10 min and the supernatants were decanted into disposable  $13 \times 100$ -mm glass test tubes and counted for  $^{51}\text{Cr}$  release.  $5 \times 10^4$  target cells suspended in 2 ml of culture medium were used to determine spontaneous  $^{51}\text{Cr}$  release. Pharmacologic agents diluted in saline just before use were interacted with the sensitized lymphocytes for a varying time interval at room temperature before introduction of the target cells. Parallel cultures of target or attacking cells alone were interacted with pharmacologic agents under identical conditions. The pharmacologic agents, in the concentrations reported, did not injure either target or attacking cells as determined in  $^{51}\text{Cr}$  release studies. 2-ml suspensions of  $5 \times 10^4$  target cells were frozen and thawed in a dry ice-ethanol bath three times to determine maximum releasable cell-bound chromium. The percent specific cell lysis was determined as follows:

$$\text{Specific cell lysis (\%)} = \left[ \frac{\text{Experimental cpm} - \text{control cpm}}{\text{Freeze-thaw cpm} - \text{control cpm}} \right] \times 100.$$

Spleen cells from Lewis rats sensitized with LBN skin grafts do not injure Lewis thymocytes and cause only barely detectable injury to Buffalo rat thymocytes (7), whereas the sensitized Lewis spleen cell populations used in this study produced 38–46% specific lysis of BN target cells. Unsensitized Lewis spleen cells do not injure BN thymocytes (7).

The percent augmentation or suppression of specific lysis noted with pharmacologic treatment was determined as follows:

% change (all data expressed as positive values)

$$= \left[ \frac{\% \text{ specific lysis in treated mixture}}{\% \text{ specific lysis in untreated mixture}} \times 100 \right] - 100.$$

*Depletion of Immunoglobulin-Bearing Lymphocytes.*—Two pooled sensitized Lewis rat spleens were incubated with heat-inactivated rabbit antirat immunoglobulin (Ig), prepared as previously described (16, 17), at a final dilution of 1:10 for 30 min at 37°C in the presence of a final 1:10 dilution of complement. DNase, 0.2 mg/ml, was then added for 10 min and the surviving cells were washed twice. A 43% reduction of the total cell population and total elimination of immunoglobulin-bearing splenocytes, as determined by inspection of suspensions incubated with FITC-conjugated rabbit antirat Ig (18), were achieved by this treatment.

*Quantitation of Lymphocyte Cyclic AMP Levels.*—Triplicate or quadruplicate samples of  $10^7$  sensitized Lewis spleen cells were maintained in Tyrode's buffer (2 cc/sample), exposed to the pharmacologic agent, and the intracellular levels of cyclic AMP quantitated at the same time that replicate cells were interacted with BN thymocytes. Cyclic AMP levels ranged from 0.55 to 10 pmol (pM)/ $10^7$  lymphocytes with an average of 3.55 pM as determined by the cyclic AMP protein binding assay (19) as previously described (20).

## RESULTS

### *Pharmacologic Augmentation of LMC.*—

*Cholinergic stimulation:* The previously observed effect of cholinergic agonists on LMC (7) did not distinguish whether the action was upon the attacking or target cells. As shown in Fig. 1, the augmentation of LMC produced by carbachol  $10^{-12}$  M is completely dependent upon a short period of preincubation of the attacking cells with this cholinergic agonist. Augmentation was not observed if the attacking cells were preincubated with carbachol for more than 6 min before introduction of the target cells, nor if the cholinomimetic was added after both attacking and target cells were mixed. Even at the higher concentration of  $10^{-10}$  M, the enhancement was limited to a particular preincubation interval (Table I), and the magnitude was not greater than that with the lesser dose. In the same experiment,  $10^{-10}$  M or  $10^{-12}$  M carbachol had no effect on sensitized spleen cell cyclic AMP levels (Table I, Fig. 1).

*Cyclic GMP effects:* Cholinergic stimulation of several tissues has been associated with tissue accumulation of cyclic GMP (8–13); therefore, the effect of exogenous 8-bromo-cyclic GMP upon LMC was studied. 8-bromo-cyclic GMP produced a dose-dependent augmentation of LMC in the dose range of  $5 \times 10^{-6}$  to  $1 \times 10^{-8}$  M with a maximum of 73% augmentation of LMC (Fig. 2). The enhanced cytotoxicity induced by 8-bromo-cyclic GMP could not be produced unless the agent was preincubated with the attacking cells, although the duration of preincubation was not as critical as in the carbachol experiments.

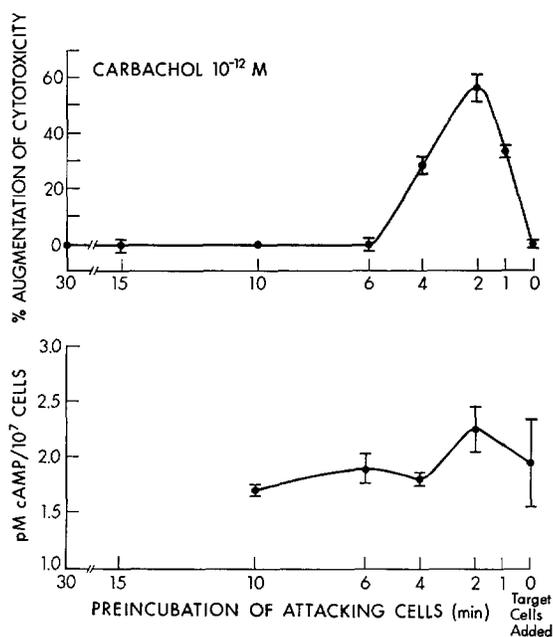


FIG. 1. The effect of carbachol  $10^{-12}$  M upon LMC (range indicated by brackets) and concomitant levels of cyclic AMP (brackets indicate SEM).

TABLE I  
*The Effect of Carbachol  $10^{-10}$  M upon LMC and Lymphocyte Cyclic AMP Levels*

Preincubation time	Augmentation LMC*	Cyclic AMP levels†
<i>min</i>	%	
0	0	$1.94 \pm 0.38$
2	3 (1-5)	$2.02 \pm 0.36$
4	27 (24-30)	$1.93 \pm 0.05$
6	5 (3-7)	$1.85 \pm 0.16$
10	1 (0-2)	$1.97 \pm 0.28$

\* The average augmentation of LMC is noted with the range of triplicate samples enclosed in parentheses.

† The average cyclic AMP concentration in pM/10<sup>7</sup> lymphocytes is given  $\pm$  the standard error of the mean for triplicate samples.

These findings again indicate that 8-bromo-cyclic GMP was acting on the attacking rather than the target cell. Attention was directed to determining whether the augmentation involved Ig-coated (B) lymphocytes as well as T lymphocytes. The deletion of Ig-coated cells had no effect on the capacity of 8-bromo-cyclic GMP to augment LMC (Fig. 3).

*Imidazole effects:* Imidazole, an agent known to stimulate cyclic AMP-phosphodiesterase activity (21) in broken cell preparations, is also capable of aug-

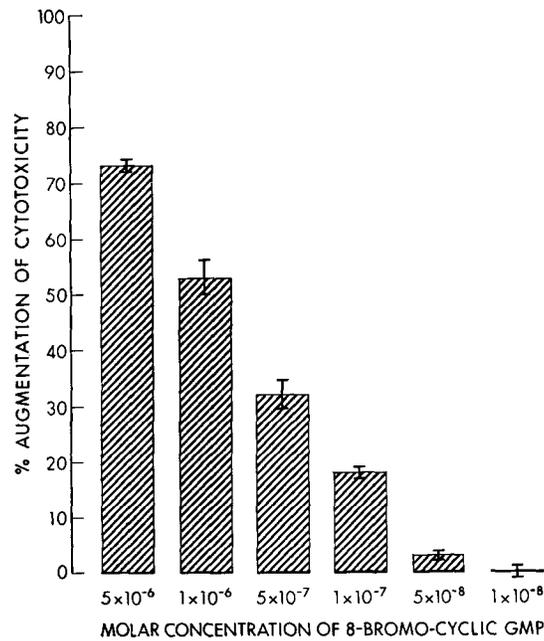


FIG. 2. The effect of 8-bromo-cyclic GMP upon LMC (range indicated by brackets).

menting allograft-sensitized LMC (22). The addition of  $10^{-7}$  M imidazole to suspensions of attacking cells 2 min before introduction of the target cells increased LMC by 50% in association with depletion of cyclic AMP levels from 6.93 pM/ $10^7$  lymphocytes to 3.97 pM/ $10^7$  (Fig. 4). Thus, imidazole-mediated enhancement is associated with changes not seen with cholinergic stimulation.

*Pharmacologic Attenuation of LMC.—*

*PGE<sub>1</sub> effects:* PGE<sub>1</sub> ( $10^{-4}$  M), an activator of lymphocyte adenylate cyclase (5), produced an increase in the level of cyclic AMP during the first 5 min after introduction with a maximal stimulation occurring after 1 h of incubation at 37°C and persisting for at least 2 h (Fig. 5). The percent suppression of cytotoxicity occurring after 1 h was not further increased after an additional hour. As the lymphocyte cyclic AMP level peaked 1 h after interaction with PGE<sub>1</sub>, a dose-response experiment was carried out utilizing a 60 min preincubation of attacking cells and PGE<sub>1</sub>. Under these circumstances the dose-related inhibition of LMC included one concentration ( $10^{-6}$  M) without an associated elevation of cyclic AMP (Fig. 6). That this dose of PGE<sub>1</sub> was stimulating the adenylate cyclase in attacking cells is revealed in Fig. 7 in which the introduction of the phosphodiesterase-inhibiting agent, aminophylline, resulted in a synergistic accumulation of cyclic AMP and an additive suppression of LMC.

*Beta adrenergic stimulation:* The beta adrenergic agonist, isoproterenol  $10^{-5}$  M,

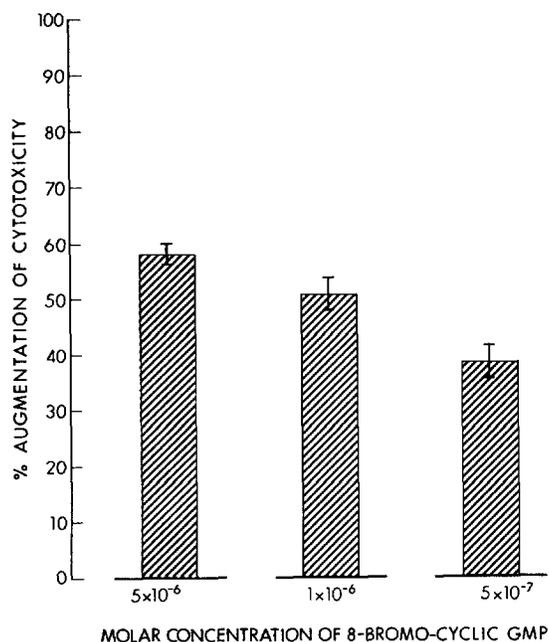


FIG. 3. The effect of 8-bromo-cyclic GMP upon LMC (range indicated by brackets) after depletion of immunoglobulin-bearing lymphocytes by anti-Ig and C. The percent specific lysis of BN thymocytes before and after depletion of immunoglobulin-bearing lymphocytes was 38%.

produced a modest inhibition of LMC without appreciably altering cyclic AMP levels when added to attacking cells 2 min before mixing with target cells (Fig. 8). The effect was not observed if interaction exceeded 2 min and could not be studied at higher concentrations of isoproterenol because of a direct toxic effect upon the target cells. The combination of isoproterenol and aminophylline  $10^{-3}$  M, which independently suppressed LMC and elevated cyclic AMP, caused an additive inhibition of LMC and a synergistic increase in intracellular cyclic AMP levels (Fig. 8).

*Cholera toxin effect:* In order to establish conclusively that modulation of LMC may result from an effect exclusively upon the attacking cell, experiments were carried out with cholera toxin, a prolonged activator of adenylate cyclase that can be removed after its interaction with the cell without loss of adenylate cyclase stimulation (23). Cholera toxin ( $1 \mu\text{g}/\text{ml}$ ) was preincubated with sensitized Lewis spleen cells for 10, 30, 60, 120, and 180 min; and the cells were washed three times before introduction of target cells. The cyclic AMP levels were determined at the time of introduction of the target cells. Cholera toxin had little effect on either LMC or cyclic AMP levels until 180 min of preincu-

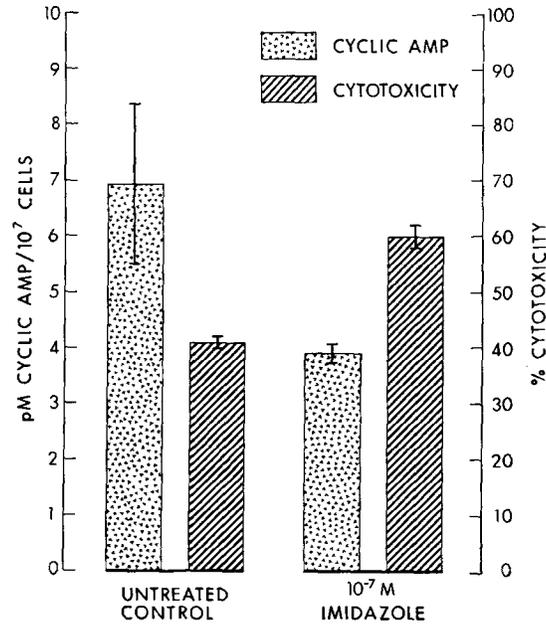


FIG. 4. The effect of imidazole  $10^{-7}$  M upon LMC (range indicated by brackets) and cyclic AMP levels (brackets indicate SEM).

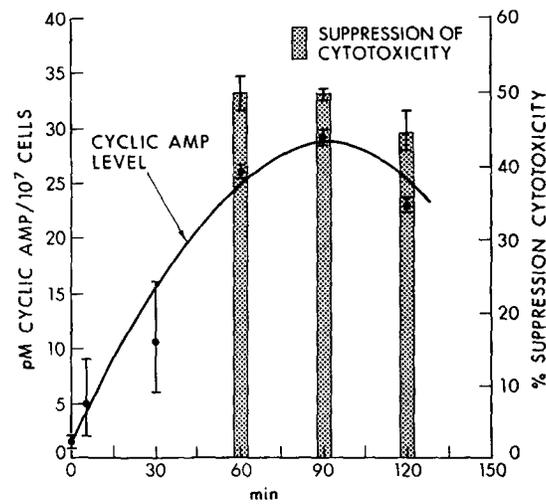


FIG. 5. The time-course effect of  $PGE_1$   $10^{-4}$  M upon LMC (range indicated by brackets) and cyclic AMP (SEM indicated by brackets).

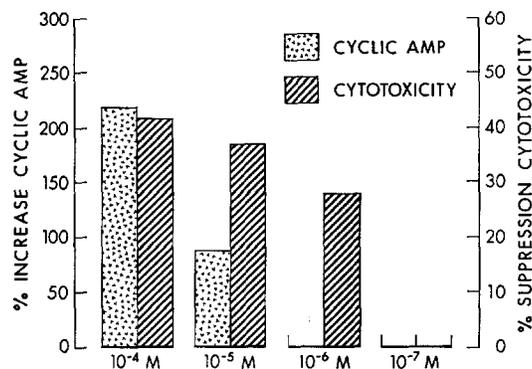


FIG. 6. The effect of PGE<sub>1</sub> upon LMC and cyclic AMP.

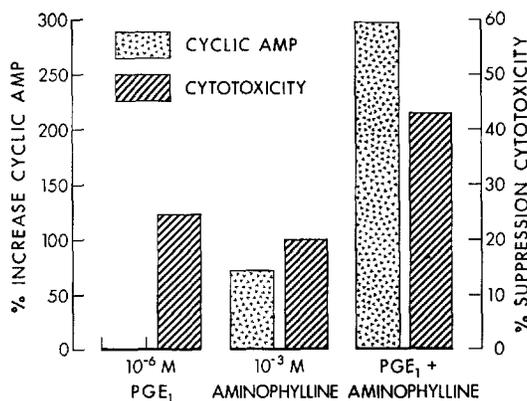


FIG. 7. The effect of PGE<sub>1</sub> 10<sup>-6</sup> M, aminophylline 10<sup>-3</sup> M, and the combination of PGE<sub>1</sub> and aminophylline upon LMC and cyclic AMP.

bation when both a marked inhibition of LMC and increase in cyclic AMP occurred (Fig. 9).

#### DISCUSSION

The capacity of allosensitized T lymphocytes to destroy target cells bearing donor alloantigens is modulated by the cellular levels of cyclic AMP and cyclic GMP. Increases in attacking lymphocyte cyclic AMP levels, as achieved after stimulation with PGE<sub>1</sub>, isoproterenol, and cholera toxin, inhibit cytotoxicity; whereas, depletion of cyclic AMP with imidazole, cholinergic stimulation with carbachol, or addition of 8-bromo-cyclic GMP enhance LMC. Chemically and pharmacologically distinct agents such as isoproterenol (Fig. 8), PGE<sub>1</sub> (Figs. 5-7), and cholera toxin (Fig. 9) having in common the ability to activate adenylate cyclase and thereby increase intracellular cyclic AMP, suppress LMC. Methylxanthines, aminophylline (Figs. 7 and 8) and theophylline (7), inhibit

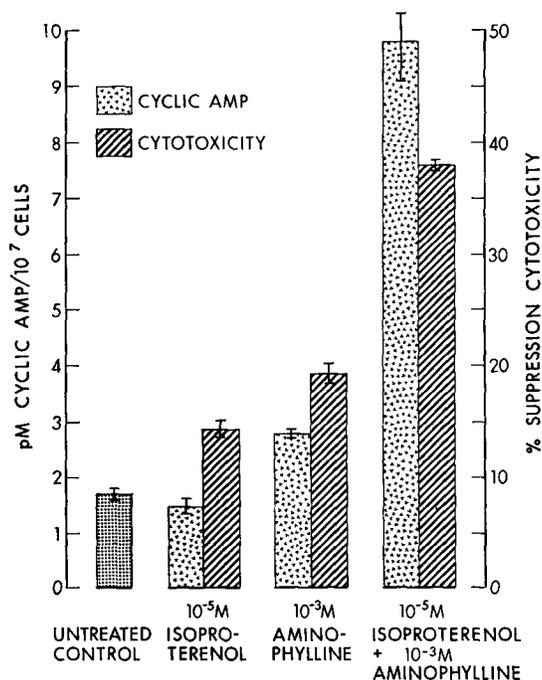


FIG. 8. The effect of isoproterenol  $10^{-5}$  M, aminophylline  $10^{-3}$  M, and the combination of isoproterenol and aminophylline upon LMC (range indicated by brackets) and cyclic AMP levels (SEM indicated by brackets).

phosphodiesterase protecting cyclic AMP from breakdown, and also inhibit LMC. The combination of adenylate cyclase activators and methylxanthines produces an additive attenuation of LMC associated with a synergistic increase in cyclic AMP (Figs. 7 and 8). Suppression of LMC is also produced by dibutyl cyclic AMP (7). Finally, the kinetic, inverse correlation between cyclic AMP levels and cytotoxicity in a system in which the attacking cells exclusively have been treated with cholera toxin is the strongest evidence obtainable that elevations of attacking lymphocyte cyclic AMP suppress their ability to kill target cells (Fig. 9). The consistent inverse association between cyclic AMP levels in the total attacking cell population and modulation of target cell destruction, as assessed both kinetically and by dose-response stimulation employing chemically and pharmacologically distinct agents, suggests that the cyclic AMP levels in the total attacking lymphocyte population reflects that of the subpopulation of T lymphocytes actually involved in the cytotoxic process.

In contrast, imidazole, an agent known to be capable of activating cyclic AMP phosphodiesterase (21) and herein demonstrated to deplete cyclic AMP concentrations, was found to enhance LMC (Fig. 4), suggesting that bidirectional alterations in cyclic AMP levels may influence LMC. Imidazole has been

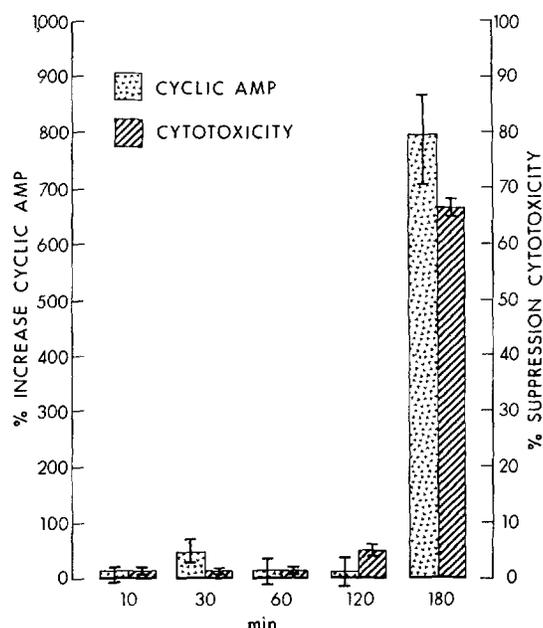


FIG. 9. The time-course effect of cholera toxin ( $1 \mu\text{g/ml}$ ) upon LMC (range indicated by brackets) and cyclic AMP (SEM indicated by brackets).

shown in broken cell preparations to inhibit cyclic GMP phosphodiesterase (24), an action capable of elevating cyclic GMP. Although the enhancement of LMC by imidazole may reflect both a depletion of cyclic AMP and an increase in cyclic GMP, it seems unlikely that the depletion of cyclic AMP is secondary to the rise of cyclic GMP (25) as cholinergic augmentation is not associated with a reduction of cyclic AMP concentration (Fig. 1, Table I).

That cholinergic enhancement of LMC may be mediated through changes in the intracellular concentrations of cyclic GMP is suggested by the findings of others that cholinergic stimulation of various tissues (8–13), including the human lymphocyte (13), transiently increases the levels of cyclic GMP and by the demonstration herein that exogenous 8-bromo-cyclic GMP enhances cytotoxicity (Figs. 2 and 3). Furthermore, there is no change in cyclic AMP levels after cholinergic stimulation at various concentrations of carbachol over an incubation period of 10 min during which time a significant enhancement of LMC was appreciated (Fig. 1, Table I). These findings are comparable with studies of the immunologic release of chemical mediators from passively sensitized human lung fragments in which depletions of cyclic AMP after stimulation with imidazole or alpha adrenergic agonists augment release, while similar enhancement after cholinergic stimulation occurs without a measurable change in cyclic AMP levels (20, 26).

In vitro LMC is dependent upon intimate contact (3) between viable lymphocytes (1, 2) and target cells and is independent of B lymphocytes (27), macrophages (27, 28), antibody formation (29), and complement (3). Although direct measurement of cyclic AMP in the mouse lymphocyte-attacking cell population in previous investigations has also revealed inhibition of LMC in association with elevation of cyclic AMP, the attacking cell population was not purified to isolate a relatively homogeneous lymphoid population, the agents were present throughout the period of attacking and target cell interaction and the system did not reveal the enhancement operative in the present system (4-6). Selective destruction of immunoglobulin-bearing (B) lymphocytes from sensitized spleen cell populations by pretreatment with anti-Ig and C did not alter cytotoxicity nor did it prevent the enhancement of LMC by exogenous 8-bromo-cyclic GMP (Fig. 3).

A brief preincubation of the attacking cell population with carbachol (Fig. 1) as well as 8-bromo-cyclic GMP (Fig. 2) before introduction of the target cells is a prerequisite for cholinergic enhancement of LMC, an effect consistent with the known brief increases in cyclic GMP after cholinergic stimulation (8-13). Furthermore, these data strongly suggest that cholinergic agents augment LMC via an effect upon the attacking cells alone, since the addition of carbachol to attacking and target cell mixtures fails to enhance LMC. Similarly, isoproterenol attenuates LMC only when the attacking cells are briefly preincubated before entry of target cells into the mixture (Fig. 8). These data and the demonstration that cholera toxin-treated (Fig. 9) attacking cells are inhibited in their ability to injure target cells only when cyclic AMP levels are elevated at the time of initial attacking-target cell interaction suggest that the level of cyclic nucleotide (cyclic AMP and cyclic GMP) within the attacking cells at the time of initial contact between the protagonist cell populations determines largely the extent of cytotoxicity.

#### SUMMARY

The capacity of allosensitized thymus-derived lymphocytes to destroy target cells bearing donor alloantigens is modulated by the cellular levels of cyclic AMP and cyclic GMP. Increases in the cyclic AMP levels of attacking lymphocytes by stimulation with prostaglandin E<sub>1</sub>, isoproterenol, and cholera toxin inhibit lymphocyte-mediated cytotoxicity; whereas, depletion of cyclic AMP with imidazole enhances cytotoxicity. The augmentation of cytotoxicity produced by cholinergic stimulation with carbamylcholine is not associated with alterations in cyclic AMP levels and is duplicated by 8-bromo-cyclic GMP. The effects of activators of adenylate cyclase, cholinomimetic agents, and 8-bromo-cyclic GMP are upon the attacking and not the target cells and occur at the time of initial interaction of attacking and target cells. Indeed, the level of cyclic nucleotide (cyclic AMP and cyclic GMP) at the time of initial cell-to-cell interaction determines the extent of cytotoxicity.

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