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ROLE OF CYCLIC NUCLEOTIDES IN THE REGULATION OF
LYMPHOCYTE TRANSFORMATION

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) Studies were conducted to define the roles of cyclic-AMP (cAMP) and cyclic-GMP (cGMP) in lymphocyte mitogenesis. The lymphocyte mitogens staphylococcal enterotoxin B (SEB), concanavalin-A (CON-A), phytohemagglutinin (PHA), and endo- toxin, did not stimulate the adenylate cyclase-cAMP system of human or rodent lymphocytes. Similarly, no effect of SEB, CON-A, and PHA on rodent lymphocyte cGMP content was observed during incubations up to 2 hours. Nevertheless, clear- cut and diametrically opposite effects of cAMP and cGMP on lymphocyte DNA syn- thesis were demonstrated, with cAMP inhibiting and cGMP potentiating this process.		

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These results suggest that the intracellular levels of cAMP and cGMP may be important determinants of lymphocyte growth. However, mitogens do not appear to mediate rapid changes in lymphocytes in cyclic nucleotide content.

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Foreword

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This work has been authorized under Project Order No. 4604, DA Project No. 3A762760A834, Task No. 34762760A834 02, and Work Unit No. 3A762760A834 02 920. These investigations are being conducted in collaboration with Captain Terry V. Zenser, Ph.D., U.S. Army Medical Research Institute of Infectious Diseases.

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences-National Research Council.

Summary

The purpose of these investigations is to define the roles of the cyclic nucleotides, cyclic-AMP (cAMP) and cyclic-GMP (cGMP), in lymphocyte mitogenesis. There is evidence to implicate these nucleotides in the regulation of growth and function of a number of cell lines including lymphocytes, but their precise roles are unresolved.

This report summarizes the first eight months of progress on the contract. During this period work was completed and two manuscripts published dealing with the effects of mitogens on lymphocyte cAMP levels and on local release of prostaglandin. In addition, considerable time was expended in procuring the equipment, supplies, methodology and technical assistance needed to begin work on cGMP.

Our results to date have shown that the mitogens, staphylococcal enterotoxin B (SEB), concanavalin-A (CON-A), phytohemagglutinin (PHA) and endotoxin do not activate the adenylate cyclase-cAMP system of rodent or human lymphocytes. Moreover, agents which do increase cAMP, such as prostaglandins of the E type, and exogenous cAMP itself inhibit basal and mitogen-stimulated DNA. Further, mitogens and antigens trigger the release of prostaglandins (PG) of the E type from lymphocytes. These results suggest that cAMP may act to suppress lymphocyte growth and that auto-regulation of growth and function following lymphocyte activation may be achieved through the local release of PGE.

Studies to date have failed to demonstrate an effect of SEB, CON-A or PHA on mouse splenic lymphocyte cGMP content during incubation up to 2 hours. However, they have clearly shown that the effects of exogenous cGMP on lymphocyte DNA synthesis are diametrically opposite those of cAMP at equimolar concentrations. Thus, while exogenous cAMP and agents which increase cAMP inhibit DNA synthesis, cGMP potentiates this process.

Accordingly, it is suggested that cAMP and cGMP may be key intracellular regulatory agents controlling lymphocyte growth and responses of these cells to mitogens and antigens. As such, these nucleotides may play a fundamental role in lymphocyte-mediated immune responses of the host. Since both cAMP and cGMP in lymphocytes are likely amenable to pharmacologic manipulation, these observations have potential therapeutic as well as theoretical implications. However, mitogens do not appear to mediate rapid alteration in lymphocyte cyclic nucleotide content and, therefore, our data do not support the notion that the actions of mitogens is expressed by their acute stimulation of either cAMP or cGMP synthesis.

Statement of Problems

The purpose of our investigations is to delineate the roles of cAMP and cGMP in the regulation of lymphocyte mitogenesis. To this end, we have been assessing the effects of mitogens on the adenylate cyclase-cAMP and guanylate cyclase-cGMP systems of rodent and human lymphocytes, while in concomitant studies we have been examining the actions of cAMP and cGMP on lymphocyte DNA synthesis.

Background

In addition to their well recognized roles as intracellular second messengers which mediate the actions of numerous hormones, the cyclic nucleotides, cyclic-AMP (cAMP) and cyclic-GMP (cGMP), have been implicated in the modulation lymphocyte function and transformation (1). Accordingly, they may be key factors in regulating the immune response. However, the precise role of cAMP and cGMP in lymphocyte transformation remains unresolved (2). Agents such as staphylococcal enterotoxin B (SEB), phytohemagglutinin (PHA) and concanavalin-A (CON-A) can transform lymphocytes in vitro from a resting to an actively dividing and secreting blast form (3). This in vitro phenomenon has been widely studied as a model of the processes involved in the activation of lymphocytes in the immunologically stimulated host in vivo, and appears to be a suitable system for defining the effects of cAMP and cGMP on lymphocyte growth and function.

Since there is evidence that both antigens and mitogens may program lymphocytes for transformation through events occurring at the cell surface (4) and since cAMP and cGMP have been implicated in the regulation of growth and activity of several cell lines (2), it is not surprising that these cyclic nucleotides have been investigated as potential intracellular second messengers which might mediate the actions of antigens and mitogens. Increases in both lymphocyte cAMP (5) and cGMP (6) levels have been reported in response to mitogenic agents in vitro and each nucleotide has accordingly been invoked as a mediator of mitogen and possibly of antigen action. Our own work has been directed at more clearly defining the role of cAMP and cGMP in the regulation of lymphocyte transformation, using the in vitro model system of mitogen stimulated cells.

Materials and Methods

SEB was kindly provided by Dr. Joseph D. Metzger, USAMRIID, Frederick, Md., PGE₁ by Dr. John Pike, the Upjohn Co., Kalamazoo, Mich. Concanavalin-A was purchased from Pharmacia Fine Chemicals, Piscataway, N.J. and a partially purified preparation of PHA (PHA-Mr69) was obtained from Burroughs-Wellcome, Research Triangle Park, N.C.

The techniques of cell preparation, cell culture, and assay of cellular cAMP content and adenylate cyclase activity have been described in detail in two recent publications (7,8). Any current modification of these techniques are specified in the appropriate sections of the Results. The cGMP content of mouse spleen cell suspensions were determined by the radioimmunoassay procedure of Steiner, et al (9). Anti-cGMP antibody and 125 I-labeled cGMP antigen (succinyl cyclic GMP tyrosine methyl ester) was obtained commercially (Schwarz/Mann, Orangeburg, N.Y.). The lower limit of assay sensitivity (0.1 pmol cGMP) was quite adequate to assess the content of incubated lymphocyte suspensions. Cell incubations were terminated by the addition of TCA. Both cells and media were extracted for their cGMP and/or cAMP content by the TCA-ether method (9). These extracts were then purified further by chromatography on AG1-X8 formate as described by Murad et al (10).

Results

A. cAMP

In our initial studies we extensively examined (1) the effect of several mitogens, including highly purified preparations of SEB, on the cAMP content of human peripheral and mouse splenic lymphocytes, and (2) the effect of agents which increase lymphocyte cAMP content on basal and mitogen-stimulated DNA synthesis and blast transformation. These studies demonstrated that purified mitogens, or crude preparation of PHA when employed in optimal mitogenic doses, do not activate the adenylate cyclase-cAMP system of either rodent or human lymphocytes. Further, agents which do enhance lymphocyte cAMP levels, such as prostaglandin (PG) E_1 and cholera toxin, uniformly inhibit lymphocyte DNA synthesis and blast transformation in a dose, time-dependent, and reversible fashion. Since PG of E types can modulate lymphocyte function and proliferation and since PGE is often released locally at its site of action, we examined the possibility that mitogen-activated lymphocytes might release PGE as part of an auto-regulatory mechanism to quench cell proliferation and a given immune response. Release of PGE from rodent and human lymphocytes was demonstrated following activation of these cells in vitro with mitogen and antigen. During the current fiscal year, we have two publications summarizing the above described work (7,8).

B. cGMP

Mitogens have previously been reported to increase lymphocyte cGMP content rapidly (6) but this finding has been neither confirmed or definitely refuted in the literature. Since our own studies (7,8) and those of others indicate that cAMP inhibits cell transformation and since the effects of cGMP in some cells is physiologically antagonistic to those of cAMP, we examined the possibility that the action of

mitogens to stimulate lymphocytes might be mediated by cGMP. To date these studies have been conducted almost exclusively with relatively heterogeneous preparations of mouse spleen cells.

(1) Verification of the validity of the cGMP assay procedure.

Considerable initial effort was directed at assessing the validity of the cGMP assay procedure in our hands. Some of these results are shown in Table I. As indicated, extracted spleen cell cGMP content was proportional to cell number and tissue dilution. Extracted tissue cGMP was additive with cold exogenous cGMP, but values were not effected by added exogenous cAMP or PGE_1 stimulated increases in endogenous cAMP (Table II). Measurable extracted tissue cGMP was completely destroyed by treatment with cyclic-phosphodiesterase. Consistent with previously reported results of changes in dog thyroid cGMP content measured by a protein binding method for cGMP (11), 4 to 6 fold increases in cGMP were observed when this tissue was incubated with either carbamylcholine or acetylcholine and eserine. Extracted cGMP from this tissue was also destroyed by phosphodiesterase. Carbamyl or acetyl choline were not found to increase the cGMP content of mouse spleen cells, a finding contrary to the results of Illiano, et al. (12). In addition, we have measured the basal cGMP content of both rat liver (20 pmol/g wet wt) and kidney cortex (35 pmol/g wet wt) and have obtained values consistent with those reported in the literature (13).

(2) Effects of mitogens on mouse spleen cell cGMP content.

Table II summarizes the results of experiments assessing the effects of SEB, CON-A, PHA and PGE_1 on spleen cell cGMP and cAMP. As shown none of these agents increased cGMP over control when monitored sequentially during a 120 min incubation. Incubations were conducted in both the presence or absence of 10% heat-inactivated human serum or 10 mM theophylline in Krebs Ringers Bicarbonate buffer. Mean cellular cGMP did increase upon prolonged incubation but this change was not influenced by the mitogens. Consistent with our previous studies (7) PGE_1 , but not mitogen, increased lymphocyte cAMP content. The viability of each cell preparation was routinely monitored by assessment of their cAMP response to PGE_1 (3-6-fold increase) and their ability to exclude trypan blue (> 92%). Additional experiments were also conducted in the complete lymphocyte culture medium (RPMI-1640, 25 mM HEPES, and 10% serum). No increases in cGMP were observed in response to SEB, PHA, and CON-A under these conditions.

Since cGMP levels have been reported to show a rapid peak-decline pattern in some tissues, we examined changes at 1, 2, 3, 5, 10 and 20 min after exposure of cells to either CON-A, SEB, PHA, PGE_1 or carbamyl choline. None of these agents increased cGMP at any of these time intervals, although PGE_1 increased cAMP content of the same cells at 1 minute. Results for only CON-A are shown in Table III.

Table IV shows the effects of a wide dose range of CON-A (1, 10 and 100 $\mu\text{g/ml}$) on spleen cells cGMP levels. This last dose far exceeds that necessary to stimulate cell transformation *in vitro*. In similar studies SEB was tested at 5, 50 and 200 $\mu\text{g/ml}$ and PHA at 3, 30, and 100 $\mu\text{g/ml}$. No increase in cGMP were noted at any of the doses tested.

(3) Effects of cGMP on ^3H -Tdr incorporation by cultured mouse spleen cells.

As noted above our previous studies had demonstrated that exogenous cAMP and agents which increase lymphocyte cAMP content reversibly inhibit lymphocyte DNA synthesis and blast transformation. Table V compares the effects of equimolar doses (4×10^{-3} M) of cAMP, cGMP and 8-bromo-cGMP on ^3H -Tdr incorporation by cultured mouse spleen cells incubated in the presence and absence of CON-A. In contrast to the inhibitory effects of cAMP, both cGMP and its 8-bromo derivative significantly potentiated basal ^3H -Tdr incorporation. This effect was greater with the 8-bromo derivative and maximal at 4×10^{-3} M; 2×10^{-3} M cGMP gave stimulation equivalent or somewhat less than 4×10^{-3} M. These effects of cGMP could not be ascribed to enhanced cell viability (trypan blue exclusion), which averaged $75 \pm 5\%$ under each condition shown. With serum included in the culture media, 8-bromo-cGMP did not significantly change the response of cells to an optimal dose of CON-A. However, when the same cell preparation was cultured in the absence of serum (Table VI) several differences were noted. The effect of 8-bromo-cGMP to enhance DNA synthesis was increased while that of CON-A was blunted. The dose of CON-A employed in the absence of serum was less than that used in presence of serum. Nevertheless, the doses employed were those that gave maximal responses under each condition, with lesser responses noted when either higher or lower doses of CON-A were employed.

In contrast to the results observed in culture media containing serum, the combination of maximal doses of CON-A and 8-bromo-cGMP resulted in a significantly greater increase in ^3H -Tdr incorporation than did either of these agents alone. In additional studies (not shown), the effects of maximal doses of CON-A and 8-bromo-cGMP were roughly additive. This is consistent with, but not proof of, a possible action of these agents on two different subpopulations of lymphocytes. In the absence of serum, cell viability at the end of a 48 h culture was markedly reduced ($45 \pm 5\%$) compared to viability in the presence of serum (75%).

As shown in Table VII, cholera toxin, an agent known to increase lymphocyte cAMP levels, significantly inhibited the actions of both CON-A and 8-bromo-cGMP to potentiate ^3H -Tdr incorporation when cells were cultured with serum. In the absence of serum, cholera toxin clearly depressed CON-A stimulated increase in ^3H -Tdr incorporation but its effect on 8-bromo-cGMP stimulated DNA synthesis was insignificant.

Discussion

The results of our current studies indicate that the lymphocyte mitogens, SEB, CON-A and PHA, tested over wide dose and time ranges and under several incubation conditions, do not increase the cGMP content of mouse spleen cells. These observations are inconsistent with the notion that mitogens initiate transformation of such cells by rapidly increasing their cGMP levels (6). However, our data do support the hypothesis that both cGMP and cAMP influence lymphocyte transformation (2,14) and have opposite effects on DNA synthesis. Exogenous cAMP and agents which increase the endogenous cAMP content of lymphocytes inhibit, while cGMP and its 8-bromo derivative, significantly stimulate DNA synthesis. The differences in the effects of 8-bromo-cGMP observed when cells were cultured in the presence and absence of serum imply that cGMP and cAMP probably represent only two of several interdependent factors which regulate lymphocyte growth and function. Clearly, the precise action of these two nucleotides remains to be defined. However, an understanding of their role as lymphocyte regulatory agents may be of potential practical as well as theoretical importance. Since it is likely that lymphocyte cGMP content, like cAMP content, will prove amenable to pharmacologic manipulation, such manipulation could have therapeutic applications.

Conclusions and Recommendations

Our data indicate that cGMP stimulates and cAMP inhibits DNA synthesis and support the hypothesis that these nucleotides may be involved in the regulation of lymphocyte proliferation and function. However, we have as yet found no evidence that mitogens increase rodent lymphocyte cGMP content. Nevertheless, it seems very likely that the intracellular levels of cAMP and cGMP are important determinants of lymphocyte growth and function and, by implication, of lymphocyte-mediated immune responses of the host. Accordingly, an understanding of the precise roles of these nucleotides may make it possible to influence the immune response of the infected host through pharmacologic agents directed at influencing lymphocyte cAMP or cGMP levels. Clearly, numerous additional questions are raised by these initial findings and await further investigation. The additional studies planned to assess some of these questions are outlined in our proposal for renewal of research support for fiscal year 7/75 - 7/76, which accompanies this report.

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Appendages to Progress Report

Tables I through VII.

Table I

Verification of cGMP Assay Procedure

Mouse Spleen Cells

Value	Basal	Carbamylcholine 5 x 10 ⁻⁴ M
(A) pmol/10 ⁷ cells	1.9 ± 0.2 (n = 6)	1.7 ± 0.2 (n = 6)
(B) pmol extracted from incubates containing 6 x 10 ⁷ /cells	11.0, 9.7	—
(C) pmol from 3 x 10 ⁷ cells	6.0, 5.4	—
(D) 1:1 dilution of (B)	5.1, 4.8	—
(E) 10 pmol exogenous cGMP + (B)	23.5, 20.6	—
(F) PDE treated (B)	< 0.1, < 0.1	—
(G) 50 nmol exogenous cAMP + (B)	10.3, 9.3	—

Dog Thyroid Slices

(A) pmol/g wet weight	34 ± 2 (n = 6)	215 ± 39 (n = 6)*
(B) PDE treated extract of thyroid tissue	< 0.1 pmole	< 0.1 pmole

Mouse spleen cells and dog thyroid slices were incubated for 20 min at 37°C in Krebs-Ringers bicarbonate glucose buffer (pH 7.4) containing 10 mM theophylline. Spleen cells were incubated at a density of 6 x 10⁷ cells/ml with both cells and media extracted for cGMP; PDE, phosphodiesterase. * indicates p < 0.001 versus basal.

Table II

Effect of Mitogens on Mouse Spleen Cell cGMP Content

Agent	Incubation Time (Min)			cAMP
	5	30	60	120
<u>Exp. I - KRBG</u>				
None	0.69 ± 0.08	0.75 ± 0.10	0.78 ± 0.08	0.98 ± 0.10 †
SEB	0.74 ± 0.10	0.62 ± 0.09	0.81 ± 0.05	1.13 ± 0.10
CON-A	0.61 ± 0.07	0.75 ± 0.07	0.69 ± 0.10	1.06 ± 0.09
PHA	0.77 ± 0.09	0.80 ± 0.10	0.76 ± 0.07	0.91 ± 0.08
PGE ₁	0.64 ± 0.05	---	---	---
<u>Exp. II - KRBG + Serum</u>				
None	1.04 ± 0.06	1.21 ± 0.10	1.27 ± 0.13	1.65 ± 0.14 †
SEB	0.95 ± 0.09	1.25 ± 0.14	1.14 ± 0.10	1.49 ± 0.15
CON-A	1.15 ± 0.08	1.19 ± 0.11	1.36 ± 0.17	1.40 ± 0.14
PHA	1.11 ± 0.10	1.28 ± 0.15	1.36 ± 0.10	1.50 ± 0.10
PGE ₁	0.92 ± 0.08	---	---	---
<u>Exp. III - KRBG + Theophylline</u>				
None	2.02 ± 0.16	1.73 ± 0.12	2.23 ± 0.16	2.65 ± 0.19 †
SEB	1.86 ± 0.10	1.75 ± 0.14	2.19 ± 0.15	2.44 ± 0.20
CON-A	1.93 ± 0.12	1.85 ± 0.14	2.07 ± 0.10	2.62 ± 0.25
PHA	2.07 ± 0.15	1.89 ± 0.10	2.33 ± 0.15	2.51 ± 0.20
PGE ₁	1.66 ± 0.11	---	---	---
				5
				77 ± 6 *
				59 ± 5 *
				25 ± 2
				24 ± 2
				20 ± 2
				29 ± 3
				121 ± 9 *

Table II (Continued)

Spleen cells were incubated at a density of approximately 5×10^7 cells/ml at 37°C for the times shown in either Krebs Ringers Bicarbonate Glucose Buffer (KRBG) alone, KRBG with 10% heat-activated human serum, or KRBG with 10 mM theophylline. In the experiments shown staphylococcal enterotoxin B (SEB) was employed at $5 \mu\text{g}/\text{ml}$, concanavalin-A (CON-A) at $1 \mu\text{g}/\text{ml}$, phytohemagglutinin at $3 \mu\text{g}/\text{ml}$ and prostaglandin E_1 (PGE_1) at 10^{-5} M. Incubations were terminated by addition of TCA to the incubates and both cells and media were extracted for cyclic nucleotide content.

* Indicates $p < 0.01$ vs control at same time. † indicates $p < 0.05$ comparing 5 min control to 120 min control. cAMP and cAMP values shown each represent mean $\pm$ SE of 4 determinations expressed as pmol/ 10^7 cells.

Table III

Evaluation of Mouse Spleen Cell cGMP Content After Brief Periods of Exposure To Concanavalin - A (CON-A)

Agent	Incubation Time (min)					cAMP pmol/10 ⁷ cells
	1	2	3	5	10	20
None	1.55 ±0.06	1.59 ±0.08	1.50 ±0.05	1.64 ±0.05	1.78 ±0.09	1.73 ±0.07
CON-A (1 ug/mL)	1.61 ±0.05	1.42 ±0.06	1.50 ±0.05	1.71 ±0.09	1.63 ±0.07	1.69 ±0.07
PGE ₁ (10 ⁻⁵ M)	1.66 ±0.09	—	—	—	—	—
						41* ±3

Mouse spleen cells (4×10^7 /ml) were incubated at 37 C in Krebs-Ringers bicarbonate glucose buffer containing 10 mM theophylline. Incubations were terminated by addition of TCA to the incubates, and both cells and media were extracted for cyclic nucleotide content. * indicates $p < 0.01$ versus control.

Values shown represent mean $\pm$ SE of 4 determinations.

Table IV

Effects of Incubation Time and Dose on cGMP Response of Mouse Spleen Cell to Concanavalin - A (CON-A)

Agent	Incubation Time (min)		
	10	20	60
None	1.76 ± 0.11	1.76 ± 0.11	2.24 ± 0.14
CON-A (ug/mL)			2.36 ± 0.14*
1	1.88 ± 0.06	1.96 ± 0.03	2.50 ± 0.22
10	1.87 ± 0.0	1.73 ± 0.15	2.32 ± 0.09
100	1.95 ± 0.21	1.91 ± 0.03	2.34 ± 0.09

Mouse spleen cells were incubated at a density of 4×10^7 cells/mL in Krebs-Ringers bicarbonate glucose buffer containing 10 mM theophylline. Values represent mean $\pm$ SE of 4 determinations.

* indicates $p < 0.05$ vs 10 min value of control.

Table V

Effects of Cyclic Nucleotides on ^3H -Thymidine (^3H -Tdr) Incorporation by Non-Stimulated and Concanavalin-A (CON-A) Stimulated Mouse Spleen Cells Cultured in the Presence of Serum

Test Agent	Dose	^3H -Tdr Incorporation cpm/ 10^6 cells	
		Control	CON-A 4 $\mu\text{g}/\text{ml}$
None		1578 $\pm$ 251	184,582 $\pm$ 19,866 *
cAMP	4 $\times 10^{-3}$ M	5830 $\pm$ 670 *	---
8-bromo-cGMP	4 $\times 10^{-3}$ M	12,257 $\pm$ 750 *	166,563 $\pm$ 7998 *
	4 $\times 10^{-4}$ M	2,534 $\pm$ 100 *	---
	4 $\times 10^{-5}$ M	1,959 $\pm$ 145	---
cAMP	4 $\times 10^{-3}$ M	523 $\pm$ 45 *	37,893 $\pm$ 4861 * $\mp$

Cells were cultured at a density of $4 \times 10^6/\text{ml}$ at 37°C for 48 hours with 10% heat-inactivated human serum present in the culture medium. ^3H -Tdr (4 $\mu\text{Ci}/\text{ml}$) was added to the cultures 4 hours before termination of the incubation. To determine ^3H -Tdr incorporation a 250 μl culture aliquot was placed on a Millipore EAMP filter which was washed with saline, 5% TCA, and 10% ethanol. The filter was then dried and solubilized before counting. Cyclic nucleotides and CON-A were added at hour 0. Values represent mean $\pm$ SE, with an n of 6 for each group. * indicates $p < 0.001$ vs control cultures (no cyclic nucleotide or CON-A); $\mp$ indicates $p < 0.01$ vs. CON-A alone. The dose of CON-A used gave maximal ^3H -Tdr incorporation under these experimental conditions.

Table VI

Effects of Cyclic Nucleotides on ^3H -Thymidine (^3H -Tdr) Incorporation by Non-Stimulated and Concanavalin-A (CON-A) Stimulated Mouse Spleen Cells Cultured Without Serum

Test Agent	Dose	^3H -Tdr Incorporation cpm/ 10^6 cells	
		Control	CON-A 0.4 $\mu\text{g}/\text{ml}$
None	---	1508 $\pm$ 40	30,416 $\pm$ 637 *
cGMP	4 x 10^{-3} M	6204 $\pm$ 298 *	---
8-bromo-cGMP	4 x 10^{-3} M	25,696 $\pm$ 939 *	46,424 $\pm$ 2019 * †
	4 x 10^{-4} M	6153 $\pm$ 300 *	---
	5 x 10^{-5} M	1938 $\pm$ 57	---
cAMP	4 x 10^{-3} M	492 $\pm$ 38 *	4941 $\pm$ 782 * †

Cells were cultured at a density of $4 \times 10^6/\text{ml}$ at 37°C for 48 hours in culture media without serum.

^3H -Tdr (4 $\mu\text{Ci}/\text{ml}$) was added 4 hour prior to the termination of the culture and its incorporation assessed as outlined in Table V. Cyclic nucleotides and CON-A were added at hour 0. Values represent mean $\pm$ SE, with an n of 6 for each group. * indicates $p < 0.01$ vs. Control cultures (no cyclic nucleotides or CON-A). † indicates $p < 0.01$ vs. CON-A alone. The dose of CON-A used gave maximal ^3H -Tdr incorporation under these experimental conditions.

Table VII

Effects of Cholera Toxin on the Stimulation of Mouse Spleen Cell ^3H -Thymidine (^3H -TdR) Incorporation

Mediated By Concanavalin-A (CON-A) and 8-bromo-cGMP

Culture Condition	^3H -TdR Incorporation cpm/ 10^6 cells	Cholera Toxin 0.5 $\mu\text{g}/\text{ml}$
		+
Serum Present Control	1074 $\pm$ 96	547 $\pm$ 48 *
8-Bromo-cGMP (4×10^3 M)	7130 $\pm$ 297*	4611 $\pm$ 314 * $\mp$
CON-A (4 $\mu\text{g}/\text{ml}$)	166,101 $\pm$ 18,951 *	79,510 $\pm$ 6898 * $\mp$
Serum Absent Control	348 $\pm$ 43	258 $\pm$ 47
8-Bromo-cGMP (4×10^3 M)	16,416 $\pm$ 1315 *	13,926 $\pm$ 590 *
CON-A (0.4 $\mu\text{g}/\text{ml}$)	25,775 $\pm$ 1632 *	8,831 $\pm$ 589 * $\mp$

Culture conditions with and without serum were as outlined in Tables V and VI. Cyclic nucleotide, CON-A, and cholera toxin were added at hour 0. Values shown are mean $\pm$ SE with an n of 6 for each group.

* indicates $p < 0.01$ vs. appropriate corresponding control culture; $\mp$ indicates $p < 0.01$ vs. corresponding culture without cholera toxin added.