THE INFLUENCE OF INTRACELLULAR LEVELS OF CYCLIC NUCLEOTIDES ON CELL PROLIFERATION AND THE INDUCTION OF ANTIBODY SYNTHESIS*, ‡

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Precursor antibody-forming cells (AFC)[1] are those bone marrow-derived (B) lymphocytes that are committed to follow several terminal pathways. Precursor cells can respond to antigen by proliferation and maturation to AFC (induction), or by inactivation in that cells are rendered noninducible (paralysis). Haptens coupled to immunogenic carriers elicit hapten-specific immune responses in animals, but haptens on nonimmunogenic carriers do not (1–7). Haptens on nonimmunogenic carriers are not inert because they induce a hapten-specific unresponsiveness which is due to a direct inactivation of the precursors of AFC, and not to an indirect suppressive event (1, 4–8). Since haptens on nonimmunogenic carriers inactivate precursor cells in the absence of thymus-derived (T) cells, the interaction between haptens and immunoglobulin receptors on the surface of precursor cells is sufficient to initiate the intracellular events that constitute the paralytic pathway. A number of haptens on nonimmunogenic carriers have been shown to elicit hapten-specific immune responses when a T-cell signal acting on precursor cells is provided by allogeneic lymphoid cells (4, 6, 8) or by bacterial lipopolysaccharides (LPS) (9, 10), implying that the T-cell signal diverts those cells binding hapten from a paralytic to an inductive pathway. These experiments also suggest that the inductive stimulus is delivered to precursor cells via two membrane-mediated events: the first by the binding of haptenic determinants to surface immunoglobulin receptors and the second by a T-cell signal acting on precursor cells. However, it is not known whether the intracellular changes initiated by hapten binding are required for both inductive and paralytic pathways (11), or whether the changes initiated in precursor cells by the T-cell signal alone are sufficient to induce maturation to AFC (12, 13).

The experiments described here question the possible involvement of the cyclic nucleotides, adenosine and guanosine 3′,5′-cyclic monophosphates as the intra-

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[1] *Abbreviations used in this paper:* AFC, antibody-forming cells; 5′ AMP, adenosine 5′-monophosphate; BSS, balanced salt solution; carbachol, carbamylcholine chloride; cyclic AMP, adenosine 3′5′-cyclic monophosphate; cyclic GMP, guanosine 3′,5′-cyclic monophosphate; dibutyryl cyclic AMP, $N^6,O^{2'}$ dibutyryl cyclic AMP; dibutyryl cyclic GMP, $N^6,O^{2'}$ dibutyryl cyclic GMP; 5′ GMP, guanosine 5′-monophosphate; LPS, lipopolysaccharides; monobutyryl cyclic AMP, N^6, monobutyryl cyclic AMP; PFC, plaque-forming cells; TNP, trinitrophenyl.

cellular mediators of the membrane-mediated signals that regulate the inductive and paralytic pathways in precursor cells. These two cyclic nucleotides have ubiquitous effects on the proliferation (14–23) and differentiation (24–26) of mammalian cells. The addition of mitogenic concentrations of LPS to mouse spleen cultures is shown here to transiently increase intracellular levels of guanosine 3',5'-cyclic monophosphate (cyclic GMP) but not adenosine 3',5'-cyclic monophosphate (cyclic AMP). In addition, cyclic GMP itself is shown to have mitogenic properties. An analysis of a number of mammalian cell populations indicates that the intracellular ratios of cyclic AMP to cyclic GMP are consistently lower in dividing cells compared to nondividing cells. Agents that interact with lymphoid cells to increase intracellular levels of cyclic AMP inhibit the proliferative response of B lymphocytes to LPS. The effects of raising intracellular levels of cyclic nucleotides on the induction of in vitro immune responses to erythrocyte antigens have been described elsewhere (14, 15). LPS and cyclic GMP enhance immune responses in T-cell-depleted cultures (14). These results lead to the suggestion that antigen raises intracellular levels of cyclic AMP and T-helper cells (and LPS) function by raising intracellular cyclic GMP levels, and that it is the intracellular ratio of cyclic AMP to cyclic GMP which determines the pathway a precursor AFC follows.

Materials and Methods

Mice. C57BL/6 mice were purchased from Jackson Laboratories, Bar Harbor, Maine. Congenitally athymic (nude) mice ($H\text{-}2^d$) were bred at the Salk Institute, San Diego, Calif. (27).

Chemicals. The chemical agents utilized in this study are indicated as follows: carbamylcholine chloride (carbachol) and D-L isoproterenol hydrochloride from Sigma Chemical Co., St. Louis, MO.; N^6,O^2 dibutyryl adenosine 3',5'-cyclic monophosphate (dibutyryl cyclic AMP), cyclic AMP, adenosine 5'-monophosphate (5' AMP), $N^6,O^{2'}$-dibutyryl guanosine 3',5'-cyclic monophosphate (dibutyryl cyclic GMP), cyclic GMP, and guanosine 5'-monophosphate (5' GMP), all sodium salts, from Plenum Scientific Company, New York. *Escherichia coli* 0127:B8 LPS was obtained from Difco Laboratories, Detroit, Mich.

Spleen Cell Cultures. Immune responses to sheep erythrocytes (SRBC) were studied in spleen cultures prepared from C57BL/6 or nude mice exactly as detailed elsewhere (27, 28). Cells were cultured at a starting density of 10^7 cells/ml. The number of hemolytic plaque-forming cells (PFC) was determined on day 4 and has been calculated as the number of PFC per 10^6 recovered cells (27, 28).

To study polyclonal responses, spleen cultures were incubated for 3 days with various concentrations of LPS. The increase in AFC specific for trinitrophenyl (TNP) was used as the assay for a polyclonal response. Each spleen culture was assayed in a hemolytic plaque assay using SRBC or TNP coupled to SRBC as target cells (29). The difference in PFC detected using SRBC and TNP-SRBC in the plaque assay has been reported as the TNP-specific AFC.

Mitogenic responses were also assayed in nude spleen cultures. The chemical agents designated in the test were added in 0.1-ml vol dissolved in a balanced salt solution (BSS) to 1-ml cultures. The cultures were incubated for 24 h and then incubated for an additional 6 h with 10^{-7} M fluorodeoxyuridine, 10^{-6} M thymidine, and 0.5 μCi[^{3}H]thymidine (52 Ci/mmol; New England Nuclear, Boston, Mass.). Cells were then collected by membrane filtration (Whatman GF/c), and were washed successively with 10-vol of BSS, 10% TCA, and 95% alcohol. Filters were dried and then radioactive measurements were made.

Autoradiography was performed as described elsewhere (30). Only those cells with more than 20 grains were counted as labeled. Approximately 1,000 cells were counted in each assay to determine the percentage of labeled cells.

Cyclic Nucleotide Measurements. Nude spleen cells were seeded at a density of 2×10^7 cells/ml

in Eagle's medium supplemented with 5% fetal bovine serum, left 16 h after preparation, and then various agents added for the periods described in the text. Four to eight cultures were pooled, mixed with 0.1 vol of 50% TCA, and boiled. Samples were centrifuged at 10,000 *g* for 20 min., the supernates removed and extracted with ether, then lyophilized to dryness. The dry residues were then dissolved in 1.0 ml of 0.1 M Tris-HCl buffer (pH 8.5) containing 0.5 mM $MgCl_2$ and separated into two 0.5-ml samples. One sample was treated with 5 μg 3',5'-cyclic nucleotide phosphodiesterase (Sigma Chemical Co.), to check the authenticity of the products assayed (31) and then both treated and untreated samples were separately mixed with 1-2 ml Dowex resin (Bio-Rad AG 1-X8, 200-400 mesh, formate form, Bio-Rad Laboratories, Richmond, Calif.), and the cyclic AMP and cyclic GMP fractions eluted with 2 N and 4 N formic acid, respectively. Solutions were lyophilized to dryness and redissolved in 0.05 M sodium acetate (pH 6.2). Both cyclic AMP and cyclic GMP were measured using the radioimmune assay of Steiner et al. (32) purchased in assay kits from Collaborative Research, Boston, Mass. The cyclic nucleotide measurements for the enzyme-treated samples have been subtracted from the data presented. Radioactive cyclic nucleotide markers were added to control cultures at the start of extractions to check the recovery of each cyclic nucleotide. In general, recoveries of radioactive cyclic AMP and cyclic GMP were between 63% and 70%. These radioactive markers were chromatographed as described below to check that the recovered material was undegraded. Dowex resins were washed and recycled before use.

The results obtained with the radioimmune assay were then checked using a radioactive labeling procedure to measure intracellular changes in levels of cyclic AMP and cyclic GMP. Nude spleen cells were radioactively labeled with 20 μCi/ml $[^{32}P]H_3PO_4$ for 10-20 h. The agents indicated in the text were then added and cyclic AMP and cyclic GMP were isolated 15 min later as described above. Approximately 2×10^7 cpm/10^7 of ^{32}P-labeled acid soluble material was recovered after the initial TCA extraction. After chromatography over Dowex AG 1-X8 columns, cyclic AMP- and cyclic GMP-containing fractions were lyophilized and dissolved in 0.1 M ammonium acetate (pH 7.0). Samples were applied to Whatman 3MM paper and the chromatogram developed in a solvent of butanol:acetone:0.5 M ammonium acetate pH 7.0 (7:5:8) for 30-40 h. The chromatogram was dried and cut into strips of ^{32}P-labeled cyclic AMP and cyclic GMP. Radioactivity in the cyclic nucleotide regions were recorded for duplicate experiments which agreed to within 20%.

Results

Effect of LPS on Intracellular Cyclic Nucleotide Levels. The effect of mitogenic concentrations of LPS on intracellular cyclic nucleotide levels has been examined in mouse spleen cultures. The addition of 25 μg *E. coli* 0127:B8 LPS to nude mouse spleen cultures results in a rapid, transient elevation of intracellular levels of cyclic GMP (Fig. 1). Maximum stimulation generally occurs between 5 and 20 min after the addition of LPS to cultures. In general, considerable variation in the magnitude of the elevation of cyclic GMP levels is observed in different experiments. These increases are consistently in the range of threefold to fivefold above background values obtained in nonstimulated cultures. The intracellular levels of cyclic GMP then decrease to a level which is generally slightly higher than the initial background value. In contrast to these rapid changes in the intracellular levels of cyclic GMP little change in cyclic AMP levels is observed. In some experiments a transient decrease in cyclic AMP levels has been observed within 30 min after LPS addition to cultures.

To verify the results obtained with the radioimmune assay procedure, experiments were performed to measure cyclic nucleotide levels in spleen cells utilizing a radioactive-labeling procedure. Spleen cells were labeled with $[^{32}P]H_3PO_4$ for 12 h and then 25 μg LPS was added to each culture. After 15 min, acid soluble material was isolated and cyclic AMP and cyclic GMP recovered from samples. Each cyclic nucleotide preparation was cochromatographed with

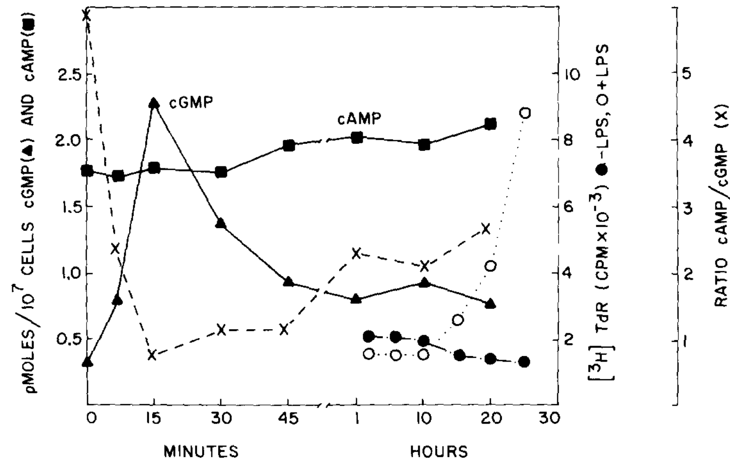

FIG. 1. Effect of LPS on intracellular levels of cyclic AMP (cAMP) and cyclic GMP (cGMP). Nude spleen cells were cultured at a density of 10^7 cells/ml and left 16–20 h. Then 25 μg *E. coli* 0127:B8 LPS was added to each culture for the times indicated, cyclic AMP and cyclic GMP extracted from the cells, and measured using a radioimmune assay procedure (32). For DNA synthetic responses, [^{3}H]thymidine was added to cultures at the time shown, and after 4-h incubation incorporation was measured as described in the Materials and Methods. While in the above experiment the ratio of cyclic AMP to cyclic GMP at the start was 6.0, in most experiments this ratio in resting cells has varied between 9.0 and 20.0.

radioactive markers to separate cyclic AMP and cyclic GMP from their respective precursor nucleotides and the total radioactivity associated with specific cyclic nucleotide regions determined (Table I). The total radioactivity associated with cyclic GMP consistently increased by 15–20 min after LPS stimulation to fourfold above the values obtained in nonstimulated cultures (Table I), while smaller changes in the radioactivity associated with cyclic AMP were observed. The comparison of ^{32}P-labeled counts per minute of cyclic AMP and cyclic GMP do not reflect their relative concentrations in the cell as the intracellular specific activities of their precursor molecules (ATP, and GTP) are different for a 12 h labeling period.

The initiation of DNA synthesis is not detected until 12–14 h after the addition of LPS to cultures. The question arises then as to how a transient early change in the intracellular level of cyclic GMP can function as a mitogenic signal. The data shown in Table II compare intracellular levels of cyclic AMP and cyclic GMP in spleen cells to those in other cells. Nude spleen cells cultured in the absence of LPS show a basal level of 0.4 ± 0.2 pmol/10^6 cells for cyclic AMP and 0.05 ± 0.03 pmol/10^6 cells for cyclic GMP (Table II) and less than 1% of these cells show significant incorporation of radioactive thymidine into nuclei (Table II). The ratio of cyclic AMP to cyclic GMP varies in resting cells in the range of 9.0–20.0. 24 h after the addition of LPS to cultures 16–20% of cells show increased DNA synthetic activity (Table II), and while the levels of both cyclic nucleotides have increased, the ratio of cyclic AMP to cyclic GMP has generally fallen below 5.0. This change towards a lower ratio is continued in cultures examined at 48 h where the number of dividing cells are in the range of 30–42%. For comparative purposes the intracellular concentrations of cyclic AMP and cyclic GMP have been examined in nondividing and dividing 3T3 cultures. While the absolute

TABLE I

The Incorporation of [^{32}P]H_3PO_4 into Cyclic GMP in Spleen Cultures

Treatment	^{32}P-labeled counts per minute	
	Cyclic AMP	Cyclic GMP
None	19,800	46,500
25 μg LPS (15 min)	24,600	93,200
25 μg LPS (20 min)	26,700	124,200

Nude spleen cells were seeded at a density of 10^7 cells/ml and radioactively labeled with 20 μCi/ml [^{32}P]H_3PO_4 for 10–12 h. At that time 25 μg *E. coli* 0127:B8 LPS was added and cyclic AMP and cyclic GMP were isolated 15 min and 20 min later. Approximately 2×10^7 cpm 10^7 cells of ^{32}P-labeled acid soluble material was recovered after the initial TCA extraction. Samples were chromatographed over Dowex AG 1-X8 columns to separate cyclic AMP from cyclic GMP and the counts per minute associated with each cyclic nucleotide determined by paper chromatography (see Materials and Methods). The counts per minute in the cyclic nucleotide regions were recorded for duplicate experiments and agree to within 20%.

TABLE II

Intracellular Levels of Cyclic AMP and Cyclic GMP in Various Nondividing and Dividing Cell Cultures

Cells	Labeled nuclei	pmol/10^6 cells		cAMP/cGMP
		cAMP	cGMP	
	%			
Nude spleen control	1.0	0.46	0.05	9.2
Nude spleen + 25 μg LPS				
At 24 h	16	0.96	0.28	3.4
At 48 h	36	0.70	0.32	2.1
3T3 (minus serum)	2.1	8.6	0.54	15.9
3T3 plus 10% horse serum	54	3.2	0.94	3.4
P3 plus 10% horse serum	62	5.2	3.6	1.4
S49 plus 10% horse serum	64	1.09	0.96	1.1
RAW-8 plus 10% horse serum	71	2.5	2.8	0.89

Nude spleen cells were cultured at a density of 10^7 cells/ml and after 16 h treated with LPS for the times indicated. RAW-8 and P3 tumor cells were used at a density of 10^6 cells/ml. 3T3 cells were grown in Eagle's medium supplemented with 10% horse serum and used during growth or after cultures had incubated in medium lacking serum for 16 h. Parallel cultures were incubated with radioactive thymidine for 10 h as described in the Materials and Methods and then examined by autoradiographic techniques to determine how many cells were active in DNA synthesis. The tumor cells were provided by Dr. P. Ralph, Salk Institute, San Diego, Calif., and the conditions for maintaining cells in culture have been described elsewhere (33).

levels of cyclic AMP and cyclic GMP in 3T3 cells is higher than spleen cells, the ratio of these two cyclic nucleotides is always high (>10) in nondividing cells and much lower (<4) in dividing cultures. Also, the cyclic AMP to cyclic GMP ratio in rapidly growing lymphoid (P3 and S49) and myeloid (RAW-8) tumor cells is always low (0.8–2.0) (Table II). The data show that the intracellular ratio of cyclic AMP and cyclic GMP is very different in nondividing and dividing mammalian cells and raise the question of whether a mitogenic signal delivered by LPS is read by B cells as a change in the absolute level of cyclic GMP or a change in ratio of cyclic AMP to cyclic GMP.

Effects of Cyclic Nucleotides on LPS Activity. LPS exerts several major effects in mouse spleen cultures: LPS induces DNA synthesis in most B lymphocytes and causes their maturation to AFC cells which can be measured by an increase background immune response to a wide variety of determinants termed the polyclonal response (12, 13, 27, 28). Lower LPS concentrations also stimulate immune responses to a variety of antigenic determinants presented in nonimmunogenic forms. This can be done by using heterologous erythrocyte antigens in T-cell-depleted cultures. The data presented in Table III compare the effects of exogenously added cyclic nucleotides or drugs which increase intracellular levels of cyclic nucleotides, on each of these three responses to LPS in nude spleen cultures. Dibutyryl cyclic AMP and isoproterenol, a beta-adrenergic agonist which increases cyclic AMP levels (14, 34, 35) but not 5′ AMP inhibited the mitogenic, polyclonal, and inductive effects of LPS. Cyclic GMP and carbachol, a cholinergic agonist which increases cyclic GMP levels (14, 34, 35) showed little effect (Table III). Raising intracellular levels of cyclic AMP therefore appears to inhibit the events which are normally a consequence of LPS-induced cell proliferation.

The effect of isoproterenol and carbachol on intracellular cyclic AMP and cyclic GMP levels in nude spleen cultures is described elsewhere (14). Isoproterenol stimulates increases in intracellular levels of cyclic AMP but not cyclic GMP. Conversely, carbachol enhances intracellular levels of cyclic GMP without changing markedly cyclic AMP levels (14). An attempt was also made to measure intracellular cyclic AMP and cyclic GMP levels after the addition of exogenous dibutyryl cyclic AMP and cyclic GMP to spleen cultures. This procedure was unsuitable for measuring intracellular changes after the addition of cyclic nucleotides to cells in culture because of extracellular binding to cell surfaces (data not presented).

Cyclic Nucleotides and the Initiation of DNA Synthesis. The effect of cyclic AMP and cyclic GMP on the initiation of DNA synthesis in nude spleen cultures is shown in Table IV. Cyclic GMP and its butyrated derivatives all stimulate DNA synthesis in nude spleen cultures in concentrations of between 10^{-4} M and 10^{-3} M. The magnitude of the stimulation after 24 h varies in different experiments in the range of threefold to fivefold. After 48 h and 72 h, cyclic GMP shows little enhancement of DNA synthesis in mouse spleen cultures (Fig. 2). The effects of other guanosine nucleotides. 5′ GMP (Table IV) and 2′,3′ GMP (not shown), have shown quite variable effects upon DNA synthetic responses. Both nucleotides have shown stimulatory effects as shown for 5′ GMP in Table IV in many experiments. Carbachol has no detectable mitogenic effects. Also,

TABLE III

Effect of Cyclic AMP and Cyclic GMP Elevating Agents on LPS Activity

Nude spleen cells + 10 μg LPS	Counts per minute × 10^{-3} (day 3)	Polyclonal response TNP-PFC/10^6 (day 3)	Induced response to SRBC (day 4)	
			No SRBC	SRBC
No LPS	1.92	<10	<10	<10
LPS alone	75.17	544	98	440
1×10^{-3} M DBcAMP	<1.0	<10	<10	<10
5×10^{-4} M DBcAMP	46.40	320	50	210
1×10^{-4} M DBcAMP	81.20	440	90	490
5×10^{-4} M Isoproterenol	<1.0	<10	<10	<10
1×10^{-4} M Isoproterenol	30.71	454	47	220
1×10^{-5} M Isoproterenol	76.17	468	68	520
1×10^{-3} M 5'AMP	68.20	510	90	400
5×10^{-4} M 5'AMP	71.40	490	70	480
1×10^{-4} M 5'AMP	72.90	560	84	416
1×10^{-3} M cGMP	74.10	540	102	390
5×10^{-4} M cGMP	78.20	590	90	460
1×10^{-4} M cGMP	79.50	460	80	510
1×10^{-3} M Carbachol	93.73	647	90	560
5×10^{-4} M Carbachol	98.88	670	84	620
1×10^{-4} M Carbachol	79.67	614	104	490

E. coli O127:B8 LPS was used at a concentration of 10 μg/ml in nude spleen cultures seeded at an initial density of 10^7 cells/ml. On day 3, the incorporation of radioactive thymidine was measured as described in the Materials and Methods. The polyclonal response was measured in parallel cultures by determining the number of PFC directed against TNP. To measure the induced or antigen-dependent response to SRBC, nude spleen cultures were seeded with LPS as indicated ± 3×10^6 SRBC. On day 4 the number of PFC directed against SRBC was determined. Each figure represents the mean of triplicate cultures.

cyclic AMP, its butyrated derivatives, and isoproterenol have no stimulatory effect on DNA synthesis in nude spleen cultures in concentrations between 10^{-6} M and 10^{-3} M. At high concentrations all of these cyclic AMP-elevating agents have marked inhibitory effects on the incorporation of radioactive thymidine in spleen cultures.

The stimulation of DNA synthesis by cyclic GMP in spleen cultures was also examined by autoradiographic techniques. When spleen cultures were incubated for 24 h with various concentrations of cyclic GMP (1×10^{-4} M–1×10^{-3} M) and then labeled with radioactive thymidine for 6 h, between 5% and 10% of the cells showed labeled nuclei (Table V). This shows that cyclic GMP itself stimulates significant numbers of lymphocytes into DNA synthesis.

Effect of Cyclic Nucleotides on Immune Responses. It has been shown elsewhere that the addition of cyclic AMP or agents known to stimulate increases

TABLE IV

Effect of Cyclic Nucleotides on DNA Synthesis in Nude Spleen Cultures

Concentration	Dibutyryl cyclic GMP	Monobutyryl cyclic GMP	Cyclic GMP	5'GMP	Carbachol	Dibutyryl cyclic AMP	Monobutyryl cyclic AMP	Cyclic AMP	Isoproterenol
M									
None	3,020	3,140	3,360	3,090	3,246	2,706	2,800	2,970	2,780
1×10^{-3}	7,580	13,952	12,714	7,640	3,410	<200	<200	340	<200
5×10^{-4}	9,633	15,704	14,974	9,120	3,350	<200	<200	649	<200
2×10^{-4}	10,026	15,000	11,476	8,969	3,560	460	656	1,948	757
1×10^{-4}	9,452	8,456	9,660	8,576	3,080	1,894	892	2,850	1,082
5×10^{-5}	3,503	3,110	4,137	4,077	2,970	1,850	2,408	2,787	1,975
1×10^{-5}	3,200	3,261	3,442	3,352	2,990	2,950	3,030	3,250	2,543

Nude spleen cells were cultured at a density of 5×10^6 cells/ml with the agents described above. After 24 h cells were incubated for a 6-h period with radioactive thymidine and the incorporation measured as described in the Materials and Methods. Each figure represents the counts per minute incorporated per culture and is the mean of triplicate cultures.

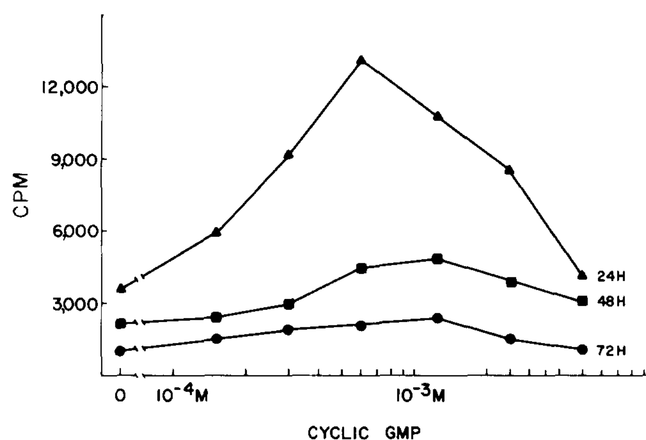

FIG. 2. Mitogenic effects of cyclic GMP. Nude spleen cells were cultured at a density of 5×10^6 cells/ml with the concentrations of cyclic GMP indicated. At 24, 48, and 72 h DNA synthetic responses were measured as described in the Materials and Methods. Each point represents the mean of triplicate cultures.

in the intracellular levels of cyclic AMP inhibit the induction of primary immune responses to SRBC in C57BL/6 spleen cultures (14, 15). Dibutyryl cyclic AMP, monobutyryl cyclic AMP, cyclic AMP, but not 5' AMP, all inhibit the induction of immune responses in the range of 10^{-4} M–10^{-3} M, indicating that it is cyclic AMP and not one of its various metabolites that is responsible for the inhibition. At concentrations between 10^{-5} M and 10^{-6} M cyclic AMP and its butyrated derivatives have an enhancing effect on the induction of immune responses (14, 15). Cyclic GMP or agents that stimulate increases in intracellular cyclic GMP in lymphoid cells have little effect in the same concentration ranges on the induction of immune responses to SRBC in C57BL/6 spleen cultures (14, 15).

TABLE V
Autoradiography of Nude Spleen Cells Stimulated by Cyclic GMP

Compound	Labeled nuclei
	%
None	1.0
1×10^{-3} M cGMP	5.5
5×10^{-4} M cGMP	10.8
2×10^{-4} M cGMP	6.1
1×10^{-4} M cGMP	3.0
25 μg LPS	19.6

Nude spleen cells were cultured at a density of 5×10^6 cells/ml with the agents described above. After 24-h cultures were incubated for 10 h with 0.5 μCi [^{3}H]thymidine and examined autoradiographically as described in the Materials and Methods. The figures presented are the percent of nuclei with more than 20 grains averaged from a total of 600–1,000 counted cells.

The addition of cyclic GMP to nude (T-cell depleted) spleen cultures has been reported to have two effects on the induction of primary immune responses to SRBC (14, 15). In the absence of SRBC small background immune responses to SRBC are observed. In the presence of SRBC there is a definite synergistic effect resulting in stimulations of immune responses above the background response. In many experiments these stimulations consistently fall in the range of threefold to fivefold above the responses detected in cultures lacking SRBC. 5′ GMP also shows some stimulation of immune responses to SRBC in nude spleen cultures (14, 15). Cyclic AMP shows no stimulation of immune responses to SRBC in nude spleen cultures in similar concentration ranges (14).

Discussion

The addition of mitogenic concentrations of LPS to mouse spleen cultures stimulates rapid but transient increases in the intracellular levels of cyclic GMP with little concomitant change in cyclic AMP levels (Fig. 1 and Table I). The increases in cyclic GMP levels are generally in the range of threefold to fivefold and reach a maximum at 5–20 min and then decrease to a level that is generally slightly higher than levels observed in nonstimulated cultures. Cyclic GMP itself stimulates DNA synthesis in mouse spleen cultures, which reaches a maximum in our culture conditions 24 h after addition to cultures (Fig. 2 and Tables IV and V). Spleen cells contain a mixture of cell types, but as it is known that LPS stimulates DNA synthesis and cell division in B lymphocytes (12–14), it is likely that LPS is stimulating cyclic GMP synthesis in B lymphocytes. A survey of a variety of nondividing and dividing cell cultures has shown that while the intracellular levels of cyclic AMP and cyclic GMP vary from one cell type to another, there is a striking difference in the ratio of cyclic AMP to cyclic GMP between nondividing and dividing cells (Table II). This ratio is always high in nondividing cells, and much lower in dividing cells (Table II).

This was consistent in spleen cells, lymphoid and myeloid tumor cells, and 3T3 cells. These observations suggest that the rapid, transient elevation of intracellular cyclic GMP levels may not be as important as a mitogenic signal, particularly as the initiation of DNA synthesis is not detected some 12–14 h later (Fig. 1), as the prolonged decrease in the ratio of cyclic AMP to cyclic GMP.

There is considerable evidence from other mammalian systems that cyclic GMP is involved as an intracellular mediator in proliferative processes (14–19), and that it is the ratio of intracellular cyclic AMP to cyclic GMP that actually modulates proliferative processes (17). The lymphocyte mitogens concanavalin A and phytohemagglutinin stimulate rapid increases in cyclic GMP levels in peripheral blood lymphocytes, although the reported increases are significantly higher than observed for LPS (16). Cyclic GMP stimulates DNA synthesis and cell division in nondividing 3T3 cultures (17), and in rat lymphocyte cultures (18). The addition of serum to 3T3 cells which have been maintained in a nondividing state by serum deprivation results in rapid, transient changes in cyclic GMP levels, followed by DNA synthesis some 18 h later (17). These reports indicate that a change in cyclic GMP levels is an early event after the delivery of a mitogenic stimulus to cells.

The mitogenic, polyclonal, and synergistic effects of LPS in mouse spleen cultures were blocked by simultaneously adding to cultures agents that raise intracellular levels of cyclic AMP (Table III). It has been shown previously that the addition of high concentrations of cyclic AMP to mouse spleen cultures for short times (<12 h) stimulates the induction of primary immune responses to erythrocyte antigens, but that treatment for longer periods inhibits immune responses (14, 15). The effect of cyclic AMP specifically on the activity of AFC precursors, studied by incubating nude spleen cells for various times with cyclic AMP-elevating agents, washing cultures, and challenging with erythrocyte antigens and nontreated T cells, has yielded similar results (14, 15). While there are also conditions where cyclic AMP enhances immune responses (14, 15), prolonged treatment of precursor cells with cyclic AMP leads to their inactivation. In contrast to these cyclic AMP effects, agents which enhance intracellular levels of cyclic GMP in mouse spleen cultures fall into three categories: (*a*) cyclic GMP which, at concentrations where cyclic AMP is inhibitory, stimulates DNA synthesis (Tables IV and V) and immune responses to SRBC in T-cell-depleted cultures (14, 15), but has no effect on immune responses in normal spleen cultures (14); (*b*) cholinergic agonists such as carbachol that are nonmitogenic (Table IV), but which may exert slight stimulatory effects on immune responses to SRBC in T-cell-depleted cultures (14); and (*c*) LPS which has mitogenic, polyclonal, and synergistic effects (Table III). A number of the cyclic GMP effects require consideration. 5′ GMP also has stimulatory effects on DNA synthesis (Table IV) and immune responses (14). While 5′ GMP has been reported to have no effect on DNA synthesis in quiescent 3T3 cells (17), it has been shown to stimulate DNA synthesis in human lymphocyte cultures (18). The 5′ GMP effects may be due to a general enhancing effect that purines appear to exert on mammalian cells (36). Although LPS enhances intracellular cyclic GMP levels, the addition of cyclic GMP to cultures does not have the same effect as LPS. High concentrations of cyclic GMP stimulate DNA synthesis reaching a

maximum earlier than does LPS (Fig. 2). Cyclic GMP does not stimulate polyclonal responses as does LPS, and has smaller mitogenic and synergistic effects in T-cell-depleted cultures (14). There are a number of reasons why cyclic GMP may have limited activity: it is unstable in culture having a half-life in culture of approximately 60 min, and it gives rise to metabolites, particularly guanosine, which exert inhibitory effects on growth. It is not known how efficiently cyclic GMP is taken up by cells. It has not been possible to measure accurately changes in the intracellular levels of cyclic GMP in the presence of high concentrations of extracellular cyclic GMP, thus it is difficult to directly compare the changes that occur intracellularly after treatment with LPS or cyclic GMP. While these data show that an increase in cyclic GMP levels is required to initiate proliferative events in nondividing cells, they also show that agents that raise intracellular levels of cyclic GMP are not necessarily mitogenic. For example, carbachol is not mitogenic (Table IV). As discussed above, the transient increase in cyclic GMP levels in spleen cells after LPS stimulation may not be as important to cells as the prolonged elevation which remains slightly above the background (Fig. 1). For a cell to continue on a proliferative pathway, the ratio of cyclic AMP to cyclic GMP may have to be lowered and maintained within definite limits. LPS and cyclic GMP may be mitogenic because of their long-term effects on this intracellular ratio, whereas agents such as carbachol may not have the prolonged effect on intracellular levels of cyclic GMP required for an agent to be mitogenic.

Both cyclic GMP and carbachol, however, have an effect that is not shared by LPS. It has been previously shown (14, 15), that the inactivating effect exerted by agents that raise intracellular levels of cyclic AMP on the induction of in vitro immune responses, is partially or completely prevented by the simultaneous presence of cyclic GMP or carbachol. Attempts to reverse these cyclic AMP effects with LPS have failed, but this may be due to changes in the immune responsiveness of lymphoid cells after prolonged LPS treatment (28).

An AFC precursor is a cell that is committed to respond to antigen in several very different ways: by maturation to AFC (proliferation), or by inactivation (see introduction). The data reported here suggest that the intracellular ratio of cyclic AMP to cyclic GMP is important in regulating the proliferative response of a lymphoid cell, and the question follows then as to whether this ratio also regulates the inactivation pathway in precursor cells.

Agents that elevate cyclic AMP levels in cells inactivate precursor cells in a period of some 20–30 h (14, 15). Since the maturation of precursor cells to AFC requires cell proliferation, and cyclic AMP is known to inhibit proliferation in a variety of normal and malignant cell cultures (20–23), it is not surprising that these various agents inhibit the induction of antibody synthesis. Thus, the problem is whether to regard the inactivation of precursor cells by cyclic AMP as merely a nonspecific toxic effect, or to regard this inactivation as a consequence of a specific cyclic AMP-induced biochemical pathway. There are a number of indirect reasons that favor inactivation via a specialized cell pathway. While high concentrations of cyclic AMP inhibit the growth of other mammalian cell types, the inhibitory effects tend to be reversible for considerable periods (21–23). These high concentrations of cyclic AMP also cause the expression of differentiated

functions characteristic of specialized cells (24–26). For example, the addition of high concentrations of cyclic AMP, or agents that raise intracellular levels of cyclic AMP, induce the expression of thymus cell-specific antigens on immature lymphocytes (24). Also tumors derived from T lymphocytes have been found which are rapidly killed by raising intracellular levels of cyclic AMP, not merely growth arrested as are other mammalian cells (26). Since such lymphoid tumors are rapidly killed, rather than growth inhibited by cyclic AMP, it is believed that cyclic AMP induces an inactivation process that is a specialized pathway in lymphoid cells (26). The inactivation of precursor cells by cyclic AMP is partially or completely prevented by the simultaneous presence of cyclic GMP (14, 15). This may not be expected if the inactivation process is due to a general toxic effect. It is unlikely that cyclic GMP is merely competing with cyclic AMP for uptake into cells to reduce the effective intracellular concentration of cyclic AMP in these experiments as carbachol, the cholinergic agonist, reverses the inactivating effects of both cyclic AMP and isoproterenol, the beta-adrenergic agonist (14). The intracellular levels of cyclic AMP are not affected by carbachol and possibly not by cyclic GMP in those reversal experiments (14), thus the reversal effects indicate it is not the absolute level of intracellular cyclic AMP that leads to inactivation, rather it is the intracellular ratio of cyclic AMP to cyclic GMP.

The interaction of haptenic determinants with surface immunoglobulin receptors on precursor cells initiates events that lead to their inactivation (see introduction). I suggest that this interaction may lead to the activation of adenylate cyclase and a subsequent increase in cyclic AMP levels. This increase, detected by the cell by the increase in the ratio of cyclic AMP to cyclic GMP initiates the biochemical events that constitute the inactivation pathway. This process may take some 20–30 h for completion to give time for the T cells to deliver a signal to precursor cells allowing cells to then proceed to an inductive pathway (see introduction). I suggest that the T-cell signal activates guanylate cyclase. The subsequent increase in the intracellular cyclic GMP level decreases the ratio of cyclic AMP to cyclic GMP and leads to a proliferative pathway.

The external signals that constitute inductive or paralytic stimuli may be mediated at the cell surface via the activation of the enzymes that synthesize cyclic AMP and cyclic GMP in cells. The changing ratio of these cyclic nucleotides may determine which pathway a cell will follow. Little is known of the steps involved in the activation of adenylate or guanylate cyclases after the binding of ligands to cell surface receptors, or of the metabolic pathways that may be regulated by changes in the intracellular ratio of cyclic AMP to cyclic GMP. Since this ratio can be altered in a number of ways, by increasing cyclic GMP, by decreasing cyclic AMP, or by differentially increasing or decreasing both, it is important to determine whether the ratio can be altered in only one or a variety of ways to initiate proliferation or inactivation pathways. For example, does initiation of the inductive pathway require the delivery of two membrane-mediated signals? Is an increase in the absolute level of both cyclic AMP and cyclic GMP, as well as a decrease in their ratio required?

These data may also reflect the mode of action of polyclonal B-cell mitogens (12, 13). The evidence using LPS and other B-cell mitogens has been interpreted that a single mitogenic signal is sufficient to induce both the maturation of AFC, and also their inactivation (12, 13). As LPS elevates cyclic GMP levels (Fig. 1), a

prolonged increase in cyclic GMP levels may be all that is required to stimulate maturation to AFC. Other polyclonal mitogens may function in this manner (12, 13) to initiate cell proliferation. This raises the question of whether the T-cell signal alone is sufficient to induce antibody synthesis. There is a fundamental difference between the action of polyclonal mitogens on one hand, and antigen and specific T cells, on the other, on individual precursor cells. Polyclonal mitogens appear to restrict most responding cells to only several division cycles (12, 13), however, antigen and specific T-helper cells stimulate cells to undergo many more division cycles (28). Perhaps this difference reflects a requirement for an elevation of cyclic AMP to enable lymphoid cells to maintain their proliferative capacity via a more mitogenic cyclic AMP to cyclic GMP ratio.

Summary

The intracellular ratio of adenosine 3′,5′-cyclic monophosphate (cyclic AMP) to guanosine 3′,5′-cyclic monophosphate (cyclic GMP) may control the developmental pathway followed by antibody-forming cell (AFC) precursors. The evidence for this is derived from several different types of experiments. First lipopolysaccharide (LPS) which is mitogenic for B lymphocytes, stimulates rapid, transient changes in intracellular levels of cyclic GMP but not cyclic AMP when added to mouse spleen cultures. Cyclic GMP itself stimulates DNA synthesis in these cultures, suggesting that the intracellular changes in cyclic GMP levels are involved in the mitogenic signal delivered by LPS to cells. The absolute amounts of cyclic nucleotides may vary widely in different cells under various conditions, however, the intracellular ratio of cyclic AMP to cyclic GMP is always high in nondividing cells and low in dividing cells. AFC precursors appear to respond to antigen in the absence of T-cell activity by inactivation (1–7). In the response to antigen in the presence of specific T cells, precursor cells proliferate and mature to AFC. Raising intracellular levels of cyclic AMP inhibits cell proliferation and leads to precursor cell inactivation (14, 15). It is suggested that the interaction of antigen with immunoglobulin receptors on the surface of precursors cells leads to the stimulation of adenylate cyclase activity and initiates the inactivation pathway. Since cyclic GMP stimulates immune responses in T-cell-depleted cultures (14, 15) and increasing cyclic GMP levels appear to be involved in the delivery of a mitogenic signal to cells, it is suggested that T-helper cells deliver a signal to precursor cells via the stimulation of guanylate cyclase to initiate the inductive pathway. It is suggested that it is the intracellular ratio of cyclic AMP to cyclic GMP that regulates the fate of precursor cells, not the absolute level of one cyclic nucleotide.

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