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Research Article

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The addition of various nucleotides to the culture medium also inhibited incorporation of labeled precursors. The best inhibitor, dibutyryl cyclic AMP (DU cyclic AMP), produced maximal inhibition only if present during the 1st hr after initial exposure to PHA. Among the various cyclic nucleotides derivatives of guanosine and adenine were the most effective inhibitors (substantial inhibition at 0.1 mM concentrations). However, the inhibition was not specific for nucleotides containing the cyclic phosphodiester moiety since the tri-, di-, and monophosphates of adenosine and guanosine were equally effective in diminishing thymidine uptake. The above inhibitions were not due to secondary effects of the inhibitors on the interaction of PHA with lymphocytes as judged by ^{125}I-labeled PHA binding studies.

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Human Lymphocyte Metabolism. Effects of Cyclic and Noncyclic Nucleotides on Stimulation by Phytohemagglutinin

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ABSTRACT The effects of extracellular nucleotides and agents which elevate intracellular cyclic adenosine 3′,5′-monophosphate (cyclic AMP) concentrations on human lymphocyte metabolism have been studied. Aminophylline, isoproterenol, and prostaglandins, all of which elevate lymphocyte cyclic AMP levels, inhibited incorporation of ^{3}H-labeled thymidine, uridine, and leucine into the DNA, RNA, and protein of phytohemagglutinin (PHA)-stimulated lymphocytes. Aminophylline inhibition was maximal only when the inhibitor was added within 1 hr after exposure of cells to PHA, suggesting that a relatively early step in the lymphocyte transformation process may be affected.

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Low concentrations (1–10 μmoles/liter) of cyclic AMP produced slight stimulation of thymidine-^{3}H uptake in resting lymphocytes (lymphocytes not stimulated with PHA). However, the effects were quite small as compared with those produced by PHA itself. Attempts to demonstrate increased thymidine uptake 48 hr after pulsing lymphocytes with aminophylline or isoproterenol were unsuccessful. The relationship of these observations to a possible regulatory role for cyclic AMP in PHA-stimulated lymphocytes is discussed.

INTRODUCTION

In the preceding paper we have presented evidence that phytohemagglutinin (PHA) produces a defined sequence of changes in the adenyl cyclase activity and cyclic adenosine 3′,5′-monophosphate (cyclic AMP) concentration of human peripheral blood lymphocytes. Changes in cyclic AMP concentration occur very early raising the possibility that cyclic AMP fulfills the role of a secondary messenger for PHA in lymphocytes, acting as an intracellular regulator in the complex changes in intracellular metabolism which accompany lymphocyte transformation. In examining this possibility further we have evaluated a number of pharmacologic agents which raise intracellular cyclic AMP levels (β-adrenergic agents, prostaglandins, and methyl xanthines) for inhibitory and stimulatory effects on protein, RNA, and DNA synthesis in control and PHA-stimulated cells. We have made a similar evaluation of the effects of exogenous cyclic and noncyclic nucleotides on lymphocyte metabolism. In this paper we will demonstrate that exogenous cyclic and noncyclic nucleotides (1×10^{-4} to 1×10^{-3} mole/liter) inhibit the response of human lymphocytes to PHA whereas low concentrations of cyclic AMP ($1–10 \times 10^{-6}$ mole/liter) are weakly stimulatory to cells unexposed to PHA. Evidence will also be presented showing that pharmacological agents which raise the endogenous level of cyclic AMP markedly inhibit transformation in PHA-stimulated lymphocytes. The relationship of these findings to a possible

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messenger role of cyclic AMP in lymphocytes will be discussed. Two brief resumes of this work have appeared previously (1, 2).

METHODS

Preparation of lymphocytes. Purified lymphocytes were prepared as described in the previous paper. In most experiments nylon column fractionation was not carried out and the preparation contained 40–70% lymphocytes.

Culture medium. The culture medium was a fortified Eagle's minimum essential medium as described in the previous paper.

Reagents. Phytohemagglutinin P (PHA-P), lot Nos. 52650, 52936, and 545526 from Difco Labs (Detroit, Mich.) was reconstituted in 5.0 cc of sterile H_2O and diluted to 1:5 in Gey's balanced salt solution. In most experiments 0.05 cc of the reconstituted and diluted PHA-P was added to 1.5 cc of culture medium. This concentration of PHA-P gave maximal uptake of thymidine-^{3}H into DNA as measured with a 4 hr thymidine pulse beginning 48 hr after the addition of PHA.

Cyclic AMP, N_6, O'_2 dibutyryl cyclic AMP (DU-cyclic AMP), adenosine 5′-mono-, di-, and triphosphates (AMP, ADP, ATP), cyclic 3′,5′-guanosine monophosphate (cyclic GMP, guanosine-5′-mono- and triphosphates (GMP, GTP), cyclic uridine 5′-monophosphate (cyclic UMP), cyclic cytidine 5′-monophosphate (cyclic CMP), cyclic inosine 5′-monophosphate (cyclic IMP), aminophylline, norepinephrine, and isoproterenol were purchased from the Sigma Chemical Company, St. Louis, Mo. Guanosine 5′-diphosphate (GDP) was purchased from the Schwarz BioResearch, Orangeburg, N. Y. These reagents were dissolved in Gey's balanced salt solution and filtered through Swinnex 0.45 μ filters (Millipore Corp., Bedford, Mass.).

Prostaglandins were dissolved in a 10% ethanol solution as described in the previous paper. Final ethanol concentrations were 1% or below and control solutions containing comparable levels of ethanol were included in each experiment.

Radioisotopes included thymidine-methyl-^{3}H (New England Nuclear, specific activity 6.7 Ci/mmole), leucine-4,5-^{3}H (New England Nuclear, 59.1 Ci/mmole), and uridine-5-^{3}H (Amersham/Searle, 22.8 Ci/mmole).

Culture conditions. Peripheral blood leukocytes (or purified lymphocytes where indicated) were suspended in fortified Eagle's minimum essential medium at a concentration of 10^6 lymphocytes per ml; 1.5 ml of the cell suspension was added to each culture tube (No. 2005, Falcon Plastics, Div. of Bioquest, Los Angeles, Calif.). The pH of the incubation medium remained within 0.1 pH U of 7.3 after the addition of reagents. The cells were incubated at 37°C in a 5% CO_2 atmosphere.

Measurement of incorporation of thymidine-^{3}H into DNA. At 48 hr 1 μCi thymidine-^{3}H was added, and 4 hr later the cells were harvested. In some experiments thymidine-^{3}H was added at 48 hr and the cells were harvested at 72 hr. Using 4-hr pulses the uptake of radioactivity into DNA was linear over the time period from 48 to 60 hr. After incubation cells were washed three times with cold phosphate-saline. DNA was precipitated by incubating cells in 2.0 ml of cold 10% trichloroacetic acid (TCA) for 20 min. The precipitates were washed once with 2.0 cc of cold 5% TCA and allowed to air dry at room temperature before dissolving in 0.5 cc of hydroxide of Hyamine 10-X (Packard Instrument Co., Downers Grove, Ill.). Hyamine solutions were quantitatively transferred to counting vials with 10 cc of Bray's solution and measured for radioactivity in a Packard liquid scintillation counter. 95% of the radioactivity has been shown to be in DNA using this procedure (3). All experimental conditions were performed in triplicate.

Measurement of incorporation of leucine-^{3}H into cellular protein. At 24 hr 1 μCi of leucine-^{3}H was added to each culture tube and 12 hr later the cells were harvested using the procedure for measuring thymidine-^{3}H incorporation into DNA. Under these conditions leucine uptake into TCA-precipitable protein was stimulated 3-fold by PHA as compared with control cells.

Measurement of incorporation of uridine-^{3}H into nucleic acid. At 24 hr 1 μCi of uridine-^{3}H was added to each culture tube and 12 hr later the cells were harvested using the procedure for measuring DNA synthesis as described above. Under these conditions the amount of TCA-precipitable radioactivity was stimulated 12-fold by PHA as compared with control cells.

Blast cell changes in PHA-stimulated lymphocytes. Lymphocyte morphology was evaluated in some experiments by making thin smears of cultured cells and staining with Wright's stain. In the presence of PHA typical blast cell transformation was demonstrated in up to 70% of cells. Under conditions in which there was a decrease in thymidine-^{3}H uptake decreased blast cell transformation was observed.

Binding of ^{125}I-labeled PHA to purified lymphocytes. Chromatographically purified erythroagglutinating PHA (4) labeled with ^{125}I was generously provided by Dr. Stuart Kornfeld, Washington University School of Medicine. Purified (99%) lymphocytes in which the erythrocytes had been lysed by hypotonic shock (5) were incubated with labeled PHA (specific activity 6×10^4 cpm/μg) under the conditions described in the legend to Table IV. The hypotonic lysis has been shown not to alter PHA binding to lymphocytes (5) whereas it essentially eliminates PHA binding to erythrocytes. Lymphocytes treated in this way responded to PHA (thymidine-^{3}H uptake) and exhibited the expected inhibition of thymidine uptake by the substances listed in Table IV. Binding of radioiodinated PHA to lymphocytes takes place largely at the external cell membrane as judged by the results of elution studies (S. Kornfeld, personal communication). Within the time period of the experiment presented in Table IV approximately 90% of the cell-bound radioactivity can be removed by fetuin or fetal calf serum. Moreover, a purified glycopeptide isolated from human erythrocytes markedly inhibits the PHA-^{125}I lymphocyte interaction providing strong evidence that a specific binding process is involved (6).

RESULTS

Effect of reagents which elevate endogenous cyclic AMP levels on PHA-stimulated thymidine-^{3}H uptake. Aminophylline, isoproterenol, norepinephrine, and prostaglandins inhibited thymidine-^{3}H uptake into PHA-P–stimulated lymphocytes (Fig. 1). These agents raise the level of intracellular cyclic AMP in lymphocytes as demonstrated in the previous paper. Interestingly, the prostaglandin which was the least potent stimulator of cyclic AMP, $PGF_{1\alpha}$ (See Table IV, preceding paper) was also the least effective inhibitor of lymphocyte transformation (Fig. 2). The degree of inhibition at specified

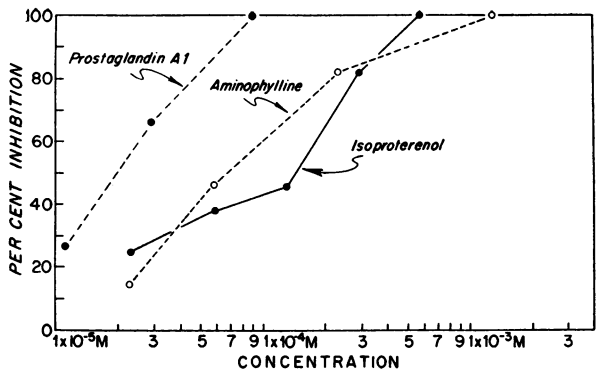

FIGURE 1 Peripheral blood leukocytes were incubated for 72 hr with PHA with thymidine-^{3}H present during the final 24 hr of the culture. The inhibitors (final concentrations indicated on the abscissa) were added at 0 time just before the addition of PHA.

levels of isoproterenol, aminophylline, and prostaglandin varied as much as 15–20% with different lymphocyte donors, but marked inhibition was consistently demonstrated.

The inhibition by isoproterenol and prostaglandins was not readily reversible, whereas cells which had been exposed to aminophylline for 72 hr and carefully washed were fully responsive to PHA. At the concentrations used none of the inhibitors appeared to have adverse effects on cell viability (as defined by uptake of vital dyes).

Inhibition by aminophylline was most marked when the reagent was present in the culture medium during the 1st hour of incubation; isoproterenol and prostaglandin were effective inhibitors when added at 6 and 24 hr after PHA-P stimulation (Fig. 3). The inhibition by aminophylline, isoproterenol, and prostaglandins was equally striking when purified lymphocytes (> 99% pure) were used instead of mixed cell populations.

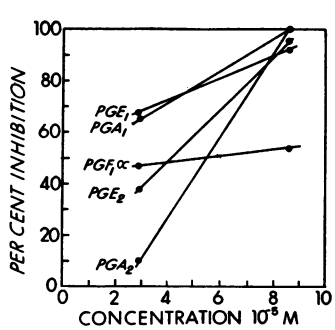

FIGURE 2 Inhibition of DNA synthesis by prostaglandins. Peripheral blood leukocytes were incubated for 52 hr with PHA with thymidine-^{3}H present during the final 4 hr of the culture. The prostaglandins were added at 0 time, just before PHA.

The possibility that lymphocyte transformation might be stimulated by isoproterenol, aminophylline, and the prostaglandins under altered experimental conditions was evaluated. In one set of experiments cells were exposed to various concentrations (3×10^{-5} to 8×10^{-7} mole/liter) of these agents for periods of 1–6 hr, washed, and reincubated under standard conditions in the absence of PHA and inhibitor. In other experiments low concentrations of the above agents (1×10^{-4}, 2.5×10^{-5}, 1×10^{-5}, 2.5×10^{-6}, and 1×10^{-6} mole/liter) were maintained for the entire 72 hr of the culture. Unequivocal stimulation of thymidine uptake was not observed in either set of experiments. In one experiment norepinephrine present throughout the culture period (at 1.6 and 5.0×10^{-5} mole/liter) produced low grade stimulation. In two other experiments the same concentrations of norepinephrine failed to produce stimulation.

Effects of exogenous nucleotides on PHA-stimulated DNA synthesis. PHA-stimulated uptake of thymidine-^{3}H into the DNA of lymphocytes was inhibited by cyclic GMP, cyclic AMP, cyclic UMP, cyclic CMP, and cyclic IMP (Fig. 4). The dibutyryl derivative of cyclic AMP was the most effective inhibitor followed by cyclic GMP and cyclic AMP. Noncyclic mono-, di-, and trinucleotides of guanosine and adenine inhibited thymidine-^{3}H uptake just as effectively as the respective cyclic nucleotides (Fig. 4). Adenosine inhibited

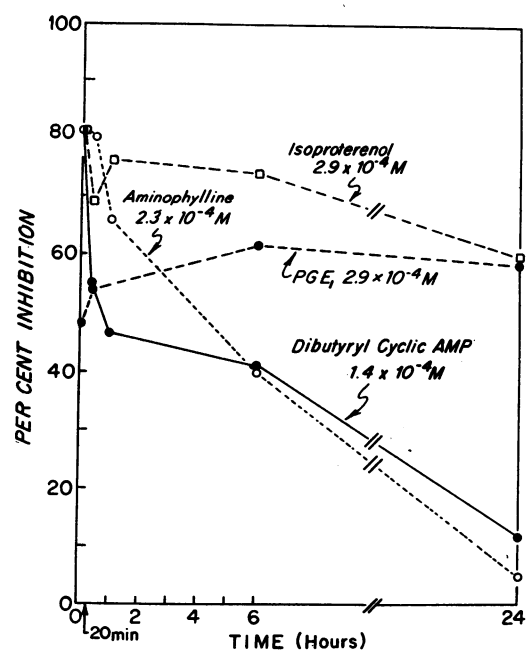

FIGURE 3 Peripheral blood leukocytes were incubated for 72 hr with PHA with thymidine-^{3}H present during the final 24 hr. Inhibitors were added at various times after PHA (time indicated on the abscissa).

thymidine-^{3}H uptake just as effectively as the adenine nucleotides.

The possibility that inhibition of lymphocyte transformation by cyclic AMP might be secondary to overt cellular damage was evaluated. After 72 hr of incubation with cyclic AMP, lymphocytes exhibited normal cellular morphology, excluded trypan blue, and were not decreased in number as compared with the control culture. Moreover, the inhibition by dibutyryl cyclic AMP was reversible; lymphocytes incubated with inhibitor for 72 hr, washed, and exposed to PHA-P incorporated thymidine-^{3}H to the same extent as control lymphocytes. Thus there was no evidence that irreversible lymphocyte damage had occurred.

The inhibition by dibutyryl cyclic AMP was maximal only if the nucleotide was present during the 1st hour of incubation. When it was added after 24 hr of incubation the inhibition of PHA-P stimulation was much less striking (Fig. 3). It would therefore appear that the inhibitor was acting at a relatively early phase in the transformation process.

The possibility that the inhibition is indirect, being mediated through some cell other than the lymphocyte, was also considered. However, in experiments utilizing highly purified peripheral blood lymphocytes rather than a mixed cell population the inhibitors were equally effective.

The possibility that low concentrations of cyclic AMP might stimulate DNA synthesis was evaluated. At exogenous cyclic AMP levels of 1–10 $\times$ 10^6 moles/liter low grade stimulation of thymidine-^{3}H uptake was demonstrable (Table I). Stimulation of similar magnitude of ^{3}H-labeled thymidine, uridine, and leucine uptake was obtained with 1–2 $\times$ 10^{-8} M concentrations of DU cyclic AMP.

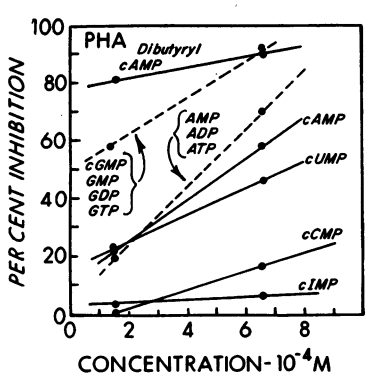

FIGURE 4 Inhibition of DNA synthesis in PHA-stimulated lymphocytes. Peripheral blood leukocytes were incubated with PHA for 72 hr with thymidine-^{3}H present during the final 24 hr. The cyclic and noncyclic nucleotides were added at 0 time. cAMP, cGMP, cUMP, cCMP, and cIMP refer to the respective cyclic nucleotides.

TABLE I

Effect of Low Concentrations of Exogenous Cyclic AMP on DNA Synthesis in Unstimulated (No PHA) Lymphocytes

Final cyclic AMP concentration	Exogenous cyclic AMP, uptake of thymidine-^{3}H		Control (no cyclic AMP added), uptake of thymidine-^{3}H
		cpm	*cpm*
60 $\times$ 10^{-6} mole/liter	280		215
	292	301*	259
	331		367
20 $\times$ 10^{-6} mole/liter	285		229
	118	228*	171
	281		246
6 $\times$ 10^{-6} mole/liter	302		163
	301	307*	156
	318		209
			—
			224*

Results are expressed in counts per minute after a 4 hr pulse beginning at 44 hr.
Peripheral blood leucocytes were incubated for 52 hr with thymidine-^{3}H present during the final 4 hr of the incubation. Cyclic AMP was added at 0 time.
* Numbers represent averages.

PHA-Stimulated protein synthesis. Table II summarizes the effect of various exogenous nucleotides and reagents which elevate endogenous lymphocyte cyclic AMP levels on PHA stimulated uptake of leucine-^{3}H

TABLE II

Per Cent Inhibition of PHA-Stimulated Protein Synthesis at 48 hr

Reagent	6.5 $\times$ 10^{-4} mole/liter	1.6 $\times$ 10^{-4} mole/liter
	%	%
Dibutyryl cyclic AMP	80	61
Cyclic AMP	57	13
AMP	56	20
ATP	66	54
Cyclic GMP	67	61
Isoproterenol	88	37
Aminophylline	100	72
PGA_1	—	100

Peripheral blood leucocytes were incubated for 36 hr with PHA with leucine-^{3}H present during the final 12 hr. Inhibitors were added at 0 time, before PHA. Unstimulated control cells incorporated an average of 8840 counts per 10 min whereas PHA-stimulated cells incorporated an average of 26,760 counts per 10 min.

TABLE III

Per Cent Inhibition of PHA-Stimulated RNA Synthesis at 48 hr

Reagent	6.5×10^{-4} mole/liter	1.6×10^{-4} mole/liter
	%	%
Dibutyryl cyclic AMP	87	66
Cyclic AMP	10	0
AMP	28	0
ATP	26	15
Cyclic GMP	69	30
GMP	84	52
GTP	70	52
Cyclic CMP	95	89
Cyclic TMP	18	17
Cyclic IMP	0	0
Isoproterenol	59	29
Aminophylline	97	61
PGA_1	—	100

Peripheral blood leucocytes were incubated for 36 hr with uridine-^{3}H present during the final 12 hr of the culture. Inhibitors were added at 0 time, before PHA. Unstimulated control cells incorporated an average of 2445 cpm whereas PHA-stimulated cells incorporated an average of 28,120 cpm.

into lymphocyte protein. The inhibitory effects of these reagents on protein synthesis largely parallel their inhibitory effects on DNA synthesis.

PHA-stimulated RNA synthesis. In Table III a similar evaluation of inhibitory effects on PHA-stimulated uptake of uridine-^{3}H into lymphocyte RNA is presented. The adenosine nucleotides were less effective in inhibiting RNA synthesis than DNA synthesis. Contrariwise, cyclic CMP was a much better inhibitor of uridine than of thymidine uptake. Otherwise, the pattern of inhibition is similar to that obtained in the study of DNA synthesis.

Binding of labeled PHA to lymphocytes. Table IV summarizes the results of a study of the binding of ^{125}I-labeled erythroagglutinating PHA to purified human peripheral blood lymphocytes in the presence and absence of several inhibitors of lymphocyte transformation. There is no indication that the inhibitors interfere with cellular uptake of PHA.

DISCUSSION

The results of this study indicate that isoproterenol, norepinephrine, aminophylline, and members of the prostaglandin family (PGE_1, PGE_2, PGA_1, PGA_2 and $PGF_{1\alpha}$) interfere with the incorporation of labeled precursors into the protein, DNA, and RNA of PHA-stimulated human peripheral blood lymphocytes. Studies with radiolabeled PHA largely excluded the possibility that these agents interfere with PHA-lymphocyte binding. As shown in the preceding paper each of the above agents raises the level of endogenous cyclic AMP in human lymphocytes suggesting a possible basis for the inhibition of transformation. In accord with this possibility $PGF_{1\alpha}$, which over the concentration range examined is the least potent of the five prostaglandins in raising lymphocyte cyclic AMP levels, is also the poorest inhibitor of PHA-stimulated incorporation of thymidine-^{3}H into DNA. Admittedly each of the inhibitors might affect intracellular metabolites other than cyclic AMP. For example, phosphodiesterase inhibition by theophylline might be expected to raise intracellular levels of cyclic GMP and other cyclic nucleotides. Taken together, however, the common effects of these agents on intracellular cyclic AMP levels would appear to be the most likely explanation for the inhibition.

If intracellular cyclic AMP is directly involved in the inhibitory effects of prostaglandin, aminophylline, and catecholamines on lymphocyte transformation it might be possible to obtain verification by the addition of cyclic AMP (7) to lymphocyte cultures. However, experiments in which lymphocytes were incubated with various cyclic and noncyclic nucleotides have failed to elucidate this question. To be sure, relatively low concentrations (0.1 mmole/liter) of cyclic AMP and its 2'0, N6 dibutyryl derivative (DU cyclic AMP) did produce significant inhibition of thymidine uptake into nuclear DNA. However, further investigation cast considerable doubt as to the significance of this inhibition in terms of the intracellular effects of cyclic AMP. Noncyclic adenine nucleotides (ATP, ADP, and AMP) and adenosine were equivalent to cyclic AMP as inhibitors. Moreover, members of the guanosine series (cyclic GMP, GTP, and GMP) were shown to be even more effective inhibitors of thymidine uptake than the

TABLE IV

Effect of Cyclic Nucleotides, Aminophylline, and Isoproterenol on PHA-Binding to Lymphocytes

Reagent	Bound PHA
	*cpm**
None (control)	6670
Dibutyryl cyclic AMP (1×10^{-4} mole/liter)	6650
Cyclic GMP (1×10^{-4} mole/liter)	7014
Isoproterenol (7×10^{-4} mole/liter)	6709
Aminophylline (1×10^{-4} mole/liter)	6927

1×10^6 purified lymphocytes were incubated with ^{125}I-labeled erythroagglutinating PHA (10^5 cpm) for 40 min at R.T., washed four times with phosphate-saline containing 0.1% bovine serum albumin and counted. In the absence of cells, tubes contained an average of 160 cpm.

* Each value is corrected for background and is the average of four determinations.

various adenosine derivatives, and as in the adenine series, cyclic GMP inhibited no better than the noncyclic guanine 5'-nucleotides. The inhibitory effects of noncyclic nucleotides did not appear to be due to secondary increases in intracellular cyclic AMP concentrations since guanosine and 5'GMP failed to alter lymphocyte cyclic AMP levels.[1] While it is possible that with more extended studies direct effects of extracellular guanosine on lymphocyte adenyl cyclase might be demonstrable it seems more probable that the inhibition of thymidine-^{3}H uptake into DNA by extracellular nucleotides is unrelated to the inhibition observed when intracellular cyclic AMP levels are elevated.

The basis for the inhibition of lymphocyte transformation by extracellular nucleotides is uncertain. Interference with uridine and thymidine transport into the cell by competition for transport receptors might explain decreased isotope uptake into RNA and DNA. However, the decrease in leucine incorporation into lymphocyte protein is more difficult to explain on this basis. Another point to consider is that cyclic AMP added at 24 hr is relatively ineffective in preventing thymidine uptake into DNA. Since the thymidine-^{3}H is not added until another 24 hr have elapsed interference of cyclic AMP with thymidine transport cannot be the explanation. Whatever explanation is given, the near equivalence of the mono-, di-, and trinucleotides both in the guanosine and adenosine series (Fig. 4) must be considered. Possibly the various adenosine and guanosine phosphates are broken down to common derivatives, most likely adenosine and guanosine, during transport into the cell. In human red cells ATP and ADP are broken down to adenosine by enzymes present on the cell surface. Adenosine is rapidly transported into the cell where it is quantitatively rephosphorylated to 5'-AMP (8).

The diminution in lymphocyte transformation in association with increased lymphocyte cyclic AMP levels must be reconciled with the observation that one of the initial effects of PHA, a potent stimulator of lymphocyte transformation, is to elevate the intracellular cyclic AMP concentration. A possible explanation might be that isoproterenol, aminophylline, and prostaglandins arrest cellular differentiation at a later point in the cell cycle, at a time when cyclic AMP levels in PHA-stimulated cells have fallen below those in control cells. As shown in the preceding paper this fall occurs within 6 hr after exposure to PHA. Inhibition at this point might be nonspecific in the sense that it involves an intracellular effect of cyclic AMP not normally exerted during lymphocyte transformation.

Even with the inhibition exerted by prostaglandins, aminophylline, and catecholamines on late metabolic events in PHA-stimulated lymphocytes, an early stimulatory effect of cyclic AMP needed to be considered. Such an effect might be masked if elevated cyclic AMP levels were maintained over too long a period of time. With this possibility in mind we attempted to manipulate lymphocyte cyclic AMP levels with isoproterenol and other agents so as to appropriate the absolute values and time course of changes produced by PHA itself. However, exposure of cells to various concentrations of isoproterenol for periods of a few minutes up to 6 hr failed to produce late changes in thymidine-^{3}H uptake into nuclear DNA. Similar results were obtained with aminophylline and PGE_1. The failure to stimulate lymphocyte differentiation with these agents despite the production of alterations in lymphocyte cyclic AMP levels similar to those produced by PHA has three possible interpretations. (*a*) While an increase in cyclic AMP concentration may be a necessary early effect of PHA, other early metabolic alterations directly attributable to PHA (changes in cyclic GMP levels for example) may well be equally important. (*b*) PHA may have to act over a period of many hours, and in the absence of late PHA effects the final events in lymphocyte transformation cannot take place. In point of fact studies with antibodies to PHA suggest a need for continued PHA stimulation over a period of at least 6 hr. (*c*) As a less likely possibility lymphocytes could contain two separate adenyl cyclase systems, one responsive to PHA and one responsive to isoproterenol and the prostaglandins. If each of the two adenyl cyclases were to regulate its own intracellular cyclic AMP pool, distinctive metabolic effects depending on the cyclase involved could occur. Under these circumstances isoproterenol and prostaglandins would be unable to produce the major intracellular effects of PHA. However, one might still expect that brief or prolonged exposure of the cells to dibutyryl cyclic AMP (which presumably would have access to both compartments) would result in lymphocyte transformation. DU cyclic AMP did stimulate lymphocyte transformation but the effect was small in comparison with that of PHA. The weakness of this argument is that results obtained with a DU cyclic AMP do not necessarily correspond to what is observed with nonacylated cyclic AMP in cells permeable to both nucleotides (9, 10). Nonetheless, separate pools of cyclic AMP in lymphocytes would appear to be an unlikely explanation for our failure to stimulate lymphocyte transformation during these experiments.

Taken as a whole our data fail to provide evidence that cyclic AMP *alone,* increased as a result of stimulation of lymphocyte adenyl cyclase by PHA, can initiate the complex series of metabolic alterations which culminate in lymphocyte transformation. Indeed the pre-

[1] Purified lymphocytes were incubated for 5 and 60 min with 5'-GMP and guanosine (400, 80, and 20 μmoles/liter).

dominant effect of a *sustained* elevation of lymphocyte cyclic AMP levels is an *inhibition* of macromolecule synthesis both in PHA-stimulated and control cells. The only data which might be considered to be inconsistent with this interpretation is the weak stimulatory effect of low concentrations of added cyclic AMP in lymphocytes not exposed to PHA (Table I). However the increase in thymidine uptake in this circumstance is quite small relative to what is observed with PHA itself.

The influence of exogenous nucleotides on lymphocyte transformation differs in certain details from what was observed by Ryan and Hedrick (11) with malignant mammalian cells in tissue culture. In cultures of mouse L-cell fibroblasts and Hela cells low concentrations of cyclic AMP (11) produced marked inhibition of cell replication. DU cyclic AMP and various noncyclic nucleotides were substantially less inhibitory. With PHA-stimulated lymphocytes DU cyclic AMP was a considerably better inhibitor than cyclic AMP. The basis for this difference is uncertain but it may have to do with an alteration in cell membrane permeability in the neoplastic cells.

Work in progress indicates that agents which elevate intralymphocyte cyclic AMP levels also interfere with antigen-induced transformation of human lymphocytes in vitro. Thus with lymphocytes from subjects with tuberculin sensitivity, prostaglandin E_1, isoproterenol, and aminophylline at concentrations of < 0.5 mmole/liter markedly inhibited purified protein derivative (PPD)-stimulated uptake of thymidine-^{3}H into lymphocyte DNA. The physiological significance of the prostaglandin inhibition is uncertain since the levels required appear to be well above the circulating prostaglandin concentrations normally observed in vivo. Whether the clinical use of theophylline and β-adrenergic agents in bronchial asthma and related diseases ever results in immunosuppression at the level of the secondary cellular response to antigen remains to be established. Benner, Enta, Lackey, Makino, and Reed (12) have studied the effects of epinephrine on the primary and secondary immune response in rats to 2,4-dinitrophenyl-egg albumin. The early administration of epinephrine in a dose of 600 μg/kg for 10 days after initial exposure to antigen reduced antibody formation about 10-fold. Repetition of the epinephrine injections after booster injections of antigen also suppressed the secondary response. On the basis of the in vivo studies and our own results in vitro, drugs capable of producing sustained elevation of intracellular cyclic AMP levels deserve further evaluation as immunosuppressive agents.

Note added in proof. We have recently received a communication from Dr. Margaret Cross, Department of Biochemistry, University of Oxford, Oxford, England, dealing with her own as yet unpublished studies on the transformation of pig peripheral blood lymphocytes. In this system DU cyclic AMP (10^{-7}–10^{-8} mole/liter) caused an immediate burst of RNA synthesis, later DNA synthesis, and eventual transformation indistinguishable in timing and magnitude from that produced by PHA. Her results would appear to strengthen the possibility that cyclic AMP may indeed be an important intracellular messenger in PHA-stimulated lymphocytes.

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