Lymphocyte Stimulation by Cyclic AMP, GMP and Related Compounds[1] (36695)

JERRY E. MCCRERY AND PERRY G. RIGBY

Hematology Division, University of Nebraska Medical Center, Omaha, Nebraska 68105

The lymphocyte, the fundamental cell of immune function in man, has recently become the subject of massive investigation. Much is currently known about its morphology, production, turnover, and responsiveness; however, the question "How does the lymphocyte work?" has not been adequately answered. The phenomenon of lymphocyte transformation, triggered by surface interaction with antigens and certain "mitogens" is readily observed in tissue culture and correlates well with cellular immunity *in vivo*; however, the control mechanisms remain for further clarification.

With continuing interest in the effects of added RNA, polynucleotides, and nucleic acids, and considering the development of the "second messenger" concept, studies were initiated to consider the role of cyclic adenosine monophosphate (C-AMP) in lymphocyte transformation (1–3). Previously several investigations have shown effects by this high energy compound on mitotic division in several cell lines *in vitro*. Studies, therefore, were performed utilizing the addition of cyclic-AMP, 5′-GMP (GMP), and related compounds to normal human lymphocytes in tissue culture.

Materials and Methods. The method of lymphocyte cultures is based on that reported by Hirschhorn *et al.* and later by Johnson, Pratt and Rigby (1) from this laboratory. Heparinized human blood was withdrawn and allowed to sediment at 37° in screw cap glass tubes for 1–3 hr. Supernatant plasma was removed, and 1–2 million cells in 0.2 ml plasma were pipetted into tissue culture tubes containing 2 ml of MEM Spinner media, supplemented with 20% fetal calf serum, penicillin, streptomycin and L-glutamine. Cultured lymphocytes were approximately 90–95% pure. Cultures were incubated at 37° in 5% CO_2 and air.

The endpoint of lymphocyte culture was evaluated by the uptake of tritiated thymidine and monitored by morphologic examination. Cell cultures were done in triplicate; cell counts in duplicate. The radioactive counting technique was modeled on that of Makman, Dvorkin, and White (6) utilizing 2–3 mCi of tritiated thymidine added to the tissue culture tube 68 hr after onset. Cells were harvested 24 hr later, and after cell washing, the DNA was precipitated with cold 5% trichloracetic acid, and the material collected on a 0.45 μ Millipore filter. Filters were dried and dissolved in Brays scintillant and counted on a Unilux Model II scintillation counter. The data is expressed as a percentage of the corresponding control. Statistics were computed using the Student's t test.

Results. Basic work seen in Table I, with otherwise unstimulated normal human lymphocytes shows that high concentrations of C-AMP are inhibitory to the tritiated thymidine uptake, while C-AMP in concentrations ranging from 10^{-4} to 10^{-8} appeared to be stimulatory with peak action at 10^{-7} M. The results at 10^{-7} and 10^{-2} M are significant compared to the control; the others are not.

On the other hand, incubation of similar cells with 5′-AMP (AMP) in a dose range of 10^{-4} to 10^{-8} M shows no significant effect upon tritiated thymidine uptake.

However, GMP under similar conditions shows a stimulatory result which is statistically significant at 10^{-8} M concentration,

[1] Presented in part at the Central Soc. Clin. Res. Meet., Chicago, IL, Nov, 1971. Supported by Damon Runyon Grant No. 1112.

TABLE I. The Uptake of Tritiated Thymidine (%) in Normal Human Lymphocytes Incubated with Related Nucleotides.[a]

		Concn (M)				
	Control	10^{-2}	10^{-4}	10^{-6}	10^{-7}	10^{-8}
C—AMP	100	53[b] (9)	132	106	154[b] (15)	127
AMP	100		93	128		80
GMP	100		139	101		179[b] (9)
CMP	100		86	108		157

[a] Number of trials given in parentheses.
[b] Denotes statistical significance.

but no significant change at other concentrations tested.

Preliminary studies with CMP show little effect, except at a concentration of 10^{-8} M where in one experiment we observed a value of 157% of control. This is, however, not significant at this time.

Table II shows results of incubating lymphocytes from 3 patients with the antigen PPD alone and together with C-AMP at 10^{-7} M concentration. It may be seen that tritiated thymidine uptake was consistently increased in each case when C-AMP was supplied to the PPD-stimulated cells.

Discussion. As noted in Table I and reported previously, cells incubated in media containing C-AMP in high concentrations (in the range of 10^{-2} M) show suppressed tritiated thymidine uptake (2). This same phenomenon has been reported by Heidrick and Ryan (7) in several cell lines. Heidrick and Ryan have also observed higher concentrations of C-AMP in strain L cells that have reached confluency, and offered this as a possible explanation for the phenomenon of "contact inhibition." The mechanisms involved in these inhibitory actions are yet to be elucidated.

As has been reported previously from this laboratory and by several authors, the addition of small amounts of C-AMP (in the range of 10^{-6} to 10^{-8} M) to normal lymphocytes in tissue culture results in apparent stimulation with an increase in tritiated thymidine uptake (2, 3). This has been observed by Whitfield *et al.* (14) in rat thymocytes and by Smith *et al.* (10), Hirschhorn, Grossman and Weissmann (11) and others, (8, 9) in human lymphocytes. Smith *et al.* (10) observing an early rise of C-AMP concentrations in PHA-stimulated cells, has proposed that it is ultimately responsible for the PHA response. Prior work from this laboratory has shown that imidazole and caffeine, presumably acting on the enzyme phosphodiesterase in C-AMP catabolism, increase the tritiated thymidine uptake in human lymphocytes with added PHA (3). Furthermore, a longer life span was observed in tumor immunized mice receiving C-AMP injections after challenge with the same live tumor (12).

The involvement of C-AMP in lymphocyte

TABLE II. The Uptake of Tritiated Thymidine (%) by Lymphocytes from Three Patients, Incubated with PPD and C-AMP.

Patients	Control	PPD	C-AMP 10^{-7} M	PPD + C-AMP 10^{-7} M
M.G. (Schmidt's syndrome)	100	66	75	200
R.W.[b] (pulmonary interstitial fibrosis—possible TB)	100	3700	117	4400
P.R.[b] (normal control)	100	190	106	215

[b] Positive response to PPD skin test.

transformation is further implicated by its augmentation of the PPD response shown in Table II. We have reported previously that caffeine has a similar effect when incubated with PPD-stimulated cells (3).

In relationship to the kinetics of cyclic-AMP within the cell, the events at the cell surface of the lymphocyte have been repeatedly implicated. The attachment of mitogens such as PHA, antigens such as PPD, or hormones such as isoproterenol have been implicated in the stimulation of membrane bound adenyl cyclase, as happens in hormone stimulated cells in other systems (13, 14). The activation of adenyl cyclase increases the formation of cyclic-AMP from ATP, in an immediate but probably transitory reaction. The cyclic-AMP formed may be degraded by the enzyme phosphodiesterase to 5′-AMP to enter other metabolic pathways within the cell. It has been shown by several authors (9, 15) that cyclic-AMP, a high energy compound, has an influence on several metabolic systems within the cell, presumably changing the molecular structure and phosphorelating certain enzymes. This may result in changing events such as glycogenolysis, histone release from DNA, or an alteration of the microtubular system as three possibilities. Whether cyclic-AMP has any direct influence on DNA or RNA is not at this time known. There is, of course, the possibility that other intermediates are involved in the control of metabolic events related to mitosis.

In regards to the effect of other nucleotides upon lymphocyte transformation, our data at this time indicated that AMP and CMP probably play no significant role. On the other hand, 5′-GMP did produce a significant response in the tritiated thymidine uptake. This laboratory has previously reported that GMP in higher concentrations is inhibitory to tritiated thymidine uptake (2).

What is the mechanism of this GMP-stimulation? There are at least three possibilities: it may have an action independent of C-AMP that is yet undiscovered; it may stimulate adenyl cyclase raising intracellular C-AMP concentrations; or it may inhibit the action of phosphodiesterase, again raising C-AMP concentration.

Whitfield *et al.* (14), working with rat thymocytes have recently reported increased mitotic activity in the presence of C-GMP in the concentration range of 1×10^{-6} to 5×10^{-6} *M*. They noted an immediate rise in intracellular C-AMP levels secondary to inhibition of the enzyme phosphodiesterase. This mechanism may also be responsible for 5′-GMP activity although they were unable to detect a mitotic increase with the 5′-nucleotide.

How do these findings relate to the full picture of lymphocyte transformation? Is C-AMP indeed the "second messenger" in transformation, or are there other messengers? Is it the peak concentration, the turnover, or timing that is most important? These questions will be the impetus for further investigations.

Summary. C-AMP is low concentrations (10^{-7} to 10^{-8} *M*) increases, and C-AMP in high concentrations (10^{-2} *M*) suppresses tritiated thymidine uptake of normal lymphocytes in tissue culture. GMP (10^{-8} *M*) increases but AMP and CMP do not significantly affect the tritiated thymidine uptake of normal human lymphocytes in tissue culture. C-AMP (10^{-7} *M*) increases the tritiated thymidine uptake of PPD-stimulated human lymphocytes in tissue culture.

1. Johnson, D. M., Pratt, P. T., and Rigby, P. G., Blood **29**, 800 (1967).

2. Rigby, P. G., and Ryan, W. R., Eur. J. Clin. Biol. Res. **15**, 774 (1970).

3. McCrery, J. E., Hall, J. C., and Rigby, P. G., Proc. Leukocyte Cult. Conf., 6th East Sound, Washington (1971).

4. Ryan, W. L., and Heidrick, M. L., Science **162**, 1484 (1969).

5. Hirschhorn, K., Bach, F., Kalodny, R. L., Frichein, I. R., and Hashem, N., Science **124**, 1185 (1963).

6. Makman, M. H., Dvorkin, B., and White, A., J. Biol. Chem. **241**, 1646 (1966).

7. Heidrick, M. L., and Ryan, W. L., Cancer Res. **31**, 1313 (1971).

8. MacManus, J. P., and Whitfield, J. F., Exp. Cell Res. **58**, 188 (1969).

9. MacManus, J. P., Perris, A. D., Whitfield, J. F., and Rixon, R. H., Proc. Leukocyte Cult. Conf., 5th, p. 125 (1970).

10. Smith, J. W., Steiner, A., Newberry, W. M., Jr., and Parker, C. W., Fed. Proc., Fed. Amer. Soc.

Exp. Biol. **28**, 566 (1969).

11. Hirschhorn, R., Grossman, J., and Weissmann, G., Proc. Soc. Exp. Biol. Med. **133**, 1361 (1970).

12. Rigby, P. G., Cancer Res. **32**, 455 (1972).

13. Ling, N. R., "Lymphocyte Stimulation," Vol. 1. Wiley, New York (1968).

14. Whitfield, J. F., MacManus, J. P., Franks, D. J., Gillan, D. J., and Yondale, T., Proc. Soc. Exp. Biol. Med. **137**, 453 (1971).

15. Feingold, D. S., and Parris, E. E., N. Engl. J. Med. **279**, 1378 (1968).

Received April 10, 1972. P.S.E.B.M., 1972, Vol. 140.