13. M. E. Haney and M. M. Hoeny, *Antimicr. Agents Chemother.* **1967**, 349 (1968); J. Berger, A. I. Rachlin, W. E. Scott, L. H. Sternbach, M. W. Goldberg, *J. Am. Chem. Soc.* **73**, 5295 (1951).
14. H. A. Turner, R. J. Raleigh, D. C. Young, *J. Anim. Sci.* **44**, 338 (1977).
15. C. J. Van Nevel and D. I. Demeyer, *Appl. Environ. Microbiol.* **34**, 251 (1977).
16. For testing in vivo, monensin was administered as Rumensin (60 g of monensin sodium per pound of Rumensin).
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Rise and Fall of Cyclic AMP Required for Onset of Lymphocyte DNA Synthesis

Abstract. *The adenosine 3′,5′-monophosphate (cyclic AMP) levels of mouse lymphocytes rose and fell sharply 10 hours after stimulation with concanavalin A. Treatment of the cells with indomethacin reversibly prevented the increase in cyclic AMP and the subsequent onset of DNA synthesis. When the heightened cyclic AMP before S phase was maintained by either inhibiting phosphodiesterase or by adding the 8-bromo derivative of cyclic AMP, DNA synthesis was also blocked. Both the increase and decrease in cyclic AMP appear to be required for progression of lymphocytes into the S phase of growth.*

Numerous studies have attempted to define the relation between adenosine 3′,5′-monophosphate (cyclic AMP) metabolism and cellular proliferation. Results from several experimental systems have led to the widely held concept that cyclic AMP acts primarily as a negative regulator of cell division (*1, 2*). Cyclic AMP analogs or agents that elevate intracellular cyclic AMP inhibit the multiplication of a variety of cultured cells (*3*). Direct measurement of intracellular cyclic AMP has also shown that proliferating, growth-regulated fibroblasts have low amounts of cyclic AMP that increase as the cells establish contact and cease dividing (*4*). Finally, transformed cells, exhibiting unregulated growth, have relatively low concentrations of cyclic AMP that are independent of cell density (*4, 5*). Because of these studies, little attention has been paid to evidence that indicates cyclic AMP plays a positive role in cellular proliferation (*6*).

An increase in cyclic AMP before the S phase of growth in certain cell types has indicated a positive role for cyclic AMP in cell proliferation and led to the idea that a brief rise in cyclic AMP is part of the series of events leading to DNA synthesis (*7*). Investigation of the relation between the cyclic AMP increase and DNA synthesis has been confined to regenerating liver cells, and the results have not been consistent; preventing the rise in cyclic AMP before S phase did not always inhibit DNA synthesis (*8, 9*) and, in one study, the drug indomethacin prevented S phase, but did not block the increase in cyclic AMP (*10*). Additional supportive evidence has come from cell cycle analyses. In several systems including regenerating liver cells in vivo, elevated cyclic AMP in the G_1 phase of growth preceded low levels found in S phase, again suggesting a positive role for the cyclic nucleotide in cell proliferation (*11, 12*).

Mouse spleen lymphocytes remain quiescent (G_0 phase) until triggered by a mitogen such as concanavalin A (con A) to enter a proliferative cycle. We now report systematic measurements of cyclic AMP in such mitogen-stimulated lymphocytes and have investigated the significance of any observed changes. Our results indicate that cyclic AMP has a complex role in lymphocyte proliferation; we found that a sharp rise and fall in cyclic AMP preceded DNA synthesis, and we provide evidence that both the increase and decrease are required for the progression of these cells into S phase.

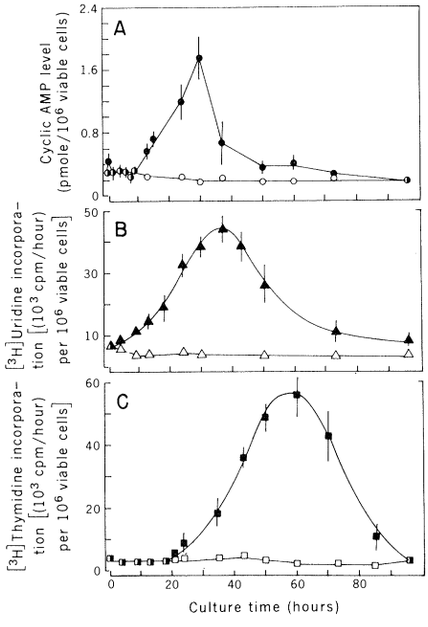

Fig. 1. Change in (A) intracellular cyclic AMP (○, ●) and (B) rates of [³H]uridine (△, ▲) and (C) [³H]thymidine incorporation (□, ■) during proliferative response of con A–stimulated lymphocytes (*13*). Open symbols represent unstimulated control cultures and closed symbols represent con A–stimulated cultures. Each time point signifies the means (± standard error of the mean) from four experiments.

Intracellular cyclic AMP levels were determined at various intervals after con A stimulation (*13, 14*) (Fig. 1). Similar results were obtained whether the cyclic AMP levels were calculated as a function of cell number (Fig. 1) or cell protein content (data not shown). Within the first 30 minutes after addition of mitogen, intracellular cyclic AMP increased by a factor of 1.5 compared to control cells. This rise was transient; the concentration returned to the control value within 1 hour and remained constant until 10 hours after the addition of con A; at that time, cyclic AMP again increased and continued to rise until approximately 30 hours after stimulation, when a sixfold increase over control was reached. Thereafter, the concentration of cyclic AMP fell rapidly and returned to near baseline by 50 hours. The cyclic AMP increase at 10 hours preceded the beginning of S phase, and its decline occurred before the onset of DNA synthesis; however, the cells were not well synchronized, causing some overlap between the two curves. Thus, both a rise and fall in cyclic AMP seemed to occur before the onset of S phase. The cyclic AMP concentration did not change in any obvious way later in the proliferative cycle, but synchrony was lost by 60 hours and later changes may have been masked.

In view of the complexity of RNA metabolism, only a very general comparison can be made between uridine incorporation and cellular cyclic AMP levels (Fig. 1). RNA synthesis began to increase between 2 and 4 hours after exposure to con A and increased over a long time period. This increase occurred before the major rise in the cellular cyclic AMP. Thus, the major cyclic AMP peak did not seem to be related to changes in RNA metabolism; in subsequent experiments, manipulation of the cyclic AMP level did not affect the rate of uridine incorporation into RNA.

Although intracellular cyclic AMP levels reflect the balance between its synthesis, degradation, and export, it is likely that the increase we observed was

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Table 1. The effect of the 8-bromo analog of cyclic AMP (0.5 m*M*) on DNA synthesis and cell cycle phase distribution in lymphocytes stimulated by con A for 48 hours (*20, 21*).

Time of 8-bromo cyclic AMP addition (hours)	[^{3}H]Thymidine incorporation (cpm/hour) per 10^6 viable cells	Percentage of cells in		
		G_1	S	G_2 + M
*	56,518	43	35	22
2	4,838	92	5	3
20	10,625	81	11	8
30	31,250	60	25	15
40	54,060	45	32	23

*No analog added.

Table 2. The effect of Ro-20/1724 on intracellular cyclic AMP levels and cell cycle phase distribution in con A–stimulated lymphocytes (*14, 21*).

Hours after con A	Ro-20/1724 (μM)	Cyclic AMP (pmoles per 10^6 viable cells)	Percentage of cells in		
			G_1	S	G_2 + M
30	0	1.1	76	15	9
60	0	0.5	46	41	13
60	2	1.5	76	11	13
60	5	2.4	70	18	12

caused primarily by synthesis. We attempted to block the presumed increase in adenylate cyclase activity with indomethacin, an inhibitor of prostaglandin synthesis (*15*). This approach was based on observations that prostaglandins are potent stimulators of adenylate cyclase and that they elevate cyclic AMP in lymphocytes (*16, 17*); in addition, an increase in immunologically positive prostaglandin-like material has been reported in mitogen-stimulated lymphocytes (*18*). When indomethacin (0.2 m*M*) was added 2 hours after con A stimulation, it blocked both the cyclic AMP increase and DNA synthesis (Fig. 2); no effect on RNA synthesis was apparent (data not shown).

After removal of indomethacin, the concentration of cyclic AMP rose, becoming similar to that of the untreated cells (Fig. 2). About 10 hours after the beginning of this cyclic AMP increase, thymidine incorporation began; this interval was the same as that for cells not exposed to indomethacin, indicating that an elevated intracellular cyclic AMP concentration is required for progression to S phase. This result supports a positive role for cyclic AMP in lymphocyte proliferation and implicates the participation of prostaglandins in these events. Moreover, because both the rise in cyclic AMP levels and later thymidine incorporation took place after removal of the indomethacin and without readdition of mitogen, it appears these cells were committed to DNA synthesis.

To determine whether a fall in intracellular cyclic AMP has to precede S phase, we artificially elevated cyclic AMP in con A–stimulated lymphocytes and studied the consequences of this elevation on DNA synthesis. In these experiments, we employed either the nonmetabolized 8-bromo analog of cyclic AMP, or an inhibitor of cyclic AMP phosphodiesterase, Ro-20/1724 (*19*). The addition of the 8-bromo analog (0.5 m*M*) before the onset of DNA synthesis effectively blocked the progression of

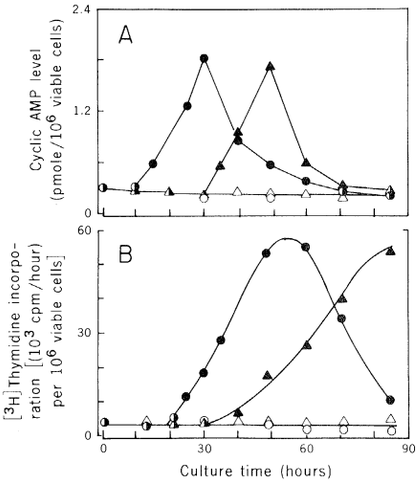

Fig. 2. The effect of addition and removal of indomethacin on (A) cyclic AMP and (B) [^{3}H]thymidine incorporation in con A–stimulated lymphocytes. Indomethacin (0.2 m*M*) was added 2 hours after mitogen to parallel cultures of con A–stimulated lymphocytes. Indomethacin was removed at 30 hours from one group of cultures (▲) by washing twice with RPMI 1640 medium and resuspending the cells in complete medium. The other group (△) was handled similarly, except that indomethacin (0.2 m*M*) was again added after washing. The changes in these parameters were compared with con A–stimulated cells not inhibited by indomethacin (●) and in unstimulated lymphocytes (○).

stimulated lymphocytes into S phase, measured by [^{3}H]thymidine incorporation (*20*) or flow microfluorometry (*21*) (Table 1), but did not affect incorporation of uridine (data not shown). If the analog was added later than 20 hours after stimulation, less inhibition of DNA synthesis was observed. At 40 hours after con A stimulation, a time when DNA synthesis was in progress, the 8-bromo analog of cyclic AMP caused no inhibition. These observations suggest that once lymphocytes are in S phase they are no longer susceptible to the effects of the analog. Similar results were obtained with Ro-20/1724, which led to increased cyclic AMP levels and blocked the flow of cells from G_1 to S phase (Table 2). From these data we concluded that the intracellular cyclic AMP must fall before S phase begins.

Our results suggest that a complex relationship exists between cyclic AMP levels and DNA synthesis. A prompt but transient biphasic change in cyclic AMP in lymphocytes after mitogen stimulation has been reported (*22*). We also found a slight change that occurred within the first 30 minutes after con A addition (Fig. 1). It has been proposed that cyclic AMP serves as a mitogenic signal in lymphocyte proliferation (*23*); however, attempts to trigger resting lymphocytes to enter proliferation by experimentally increasing the cyclic AMP have generally been unsuccessful. Furthermore, lymphocyte proliferation induced by exposure to mitogens can be effectively reversed between 8 and 10 hours later by agents presumably acting at the cell surface on the mitogen receptor. Concanavalin A stimulation can be blocked by the haptenic competitor α-methylmannoside and other mitogens such as periodate or neuraminidase, plus galactose oxidase treatments are reversed by cysteine or sodium borohydride (*24*). Consequently, the significance of the early changes in cyclic AMP is unclear at the present time.

We have shown, however, that a later, major rise and fall in cyclic AMP is necessary for the onset of S phase in lymphocytes. The cyclic AMP level began to rise 10 hours after con A addition, at which time the cells were in a later portion of G_1 phase and apparently committed to DNA synthesis. Our study also shows that a subsequent fall in cyclic AMP must occur for the lymphocytes to leave G_1 phase and begin DNA synthesis. These results are consistent with the concept that cyclic AMP acts as a modulator of more than one event during the cell cycle and that both the increase and decrease participate in the progression

(*7*). It is possible that a similar sequence of events operates in cells other than lymphocytes.

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References and Notes

1. C. W. Abell and T. M. Monahan, *J. Cell. Physiol.* **59**, 549 (1973); F. J. Chlapowski, L. A. Kelly, R. W. Butcher, *Adv. Cyclic Nucleotide Res.* **6**, 245 (1976); D. L. Friedman, *Physiol. Rev.* **56**, 652 (1976).
2. I. H. Pastan, G. S. Johnson, W. B. Anderson, *Annu. Rev. Biochem.* **44**, 491 (1975).
3. R. R. Burk, *Nature (London)* **219**, 1272 (1968); W. L. Ryan and M. L. Heidrick, *Science* **162**, 1484 (1968); G. S. Johnson and I. H. Pastan, *J. Natl. Cancer Inst.* **47**, 1357 (1971); J. R. Sheppard, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1316 (1971).
4. M. L. Heidrick and W. L. Ryan, *Cancer Res.* **31**, 1313 (1971); J. Otten, G. Johnson, I. Pastan, *Biochem. Biophys. Res. Commun.* **44**, 1192 (1971); S. Bannai and J. R. Sheppard, *Nature (London)* **250**, 62 (1972).
5. J. R. Sheppard, *Nature (London) New Biol.* **236**, 14 (1972).
6. J. F. Whitfield, R. H. Rixon, J. P. MacManus, S. D. Balk, *In Vitro* **8**, 257 (1973); T. Hovi and A. Vaheri, *Nature (London) New Biol.* **245**, 175 (1973); S. Schor and E. Rozengurt, *J. Cell. Physiol.* **81**, 339 (1974); M. E. Cross and M. G. Ord, *Biochem. J.* **124**, 241 (1971).
7. J. F. Whitfield, G. P. MacManus, R. H. Rixon, A. L. Boynton, T. Yondale, S. Swierenga, *In Vitro* **12**, 1 (1976).
8. J. P. MacManus, B. M. Braceland, T. Yondale, J. R. Whitfield, *J. Cell. Physiol.* **82**, 157 (1973).
9. S. Thrower and M. Ord, *Biochem. J.* **144**, 361 (1974).
10. J. F. Whitfield, A. L. Boynton, J. P. MacManus, R. H. Rixon, P. R. Walker, U. Armato, in *Cyclic Nucleotides and the Regulation of Cell Growth*, M. Abou-Sabe, Ed. (Dowden, Hutchinson and Ross, Stroudsburg, Pa., 1976), pp. 97–130.
11. J. R. Sheppard and D. M. Prescott, *Exp. Cell Res.* **75**, 293 (1972); W. E. Seifert and P. S. Rudland, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4920 (1972); A. J. T. Millis, G. A. Forrest, D. A. Pious, *Exp. Cell Res.* **83**, 335 (1974); D. H. Russell and P. J. Stambrook, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1482 (19 → C. E. Zeilig and N. D. Goldberg, *ibid.* **74**, 1052 (1977).
12. J. P. MacManus, D. J. Franks, T. Yondale, B. M. Braceland, *Biochem. Biophys. Res. Commun.* **49**, 1210 (1972).
13. Splenic lymphocytes from (C57Bl1/6 × DBA/2) F_1 hybrid mice (8 to 12 weeks old, Jackson Laboratory) were stimulated with con A (2 μg/ml) (*25*). RNA and DNA syntheses were assessed by incorporation of [^{3}H]uridine and [^{3}H]thymidine, respectively, into trichloroacetic acid–precipitable material during a 1-hour pulse (*25*). Cells were counted on a hemocytometer. Cell viability, assessed by trypan-blue exclusion, decreased from 100 percent to 80 percent during the first 24 hours and stabilized at 70 percent for the rest of the culture time. The suspensions consisted chiefly of small lymphocytes (> 95 percent) as judged by morphology; included in the remainder were macrophages. Concanavalin A stimulates T lymphocytes to proliferate, but B lymphocytes would also be present, and the possibility that the cyclic AMP and proliferative responses originated from different cell populations cannot be excluded.
14. Intracellular cyclic AMP was determined by an adaptation of the method of Brown (*26*). Duplicate 10-ml samples of lymphocyte cultures were centrifuged at 650*g* for 10 minutes; the pellets were resuspended in 1 ml of ice-cold 5 percent trichloroacetic acid and stored at −20°C. For the assay, the cell sample was thawed and centrifuged at 1000*g* for 10 minutes. The pellet was saved for protein determination (*27*), and the supernatant was extracted five times with three volumes of water-saturated diethyl ether acidified with hydrochloric acid. The aqueous fraction was incubated at 50°C for 60 minutes to remove any residual ether. A series of unlabeled cyclic AMP standards ranging from 0 to 10 pmole were extracted in parallel with the cell samples. The assay mixture consisted of 0.1 ml of bovine adrenal protein kinase, 0.1 ml of unlabeled cyclic AMP standard or sample, 0.2 ml of buffer (0.1*M* tris-HCl, *p*H 7.4, 8 m*M* theophylline, and 6 m*M* mercaptoethanol), and 2 pmole of cyclic [^{3}H] AMP (36.6 Ci/mmole) in 0.05 ml of distilled water. After the mixture was incubated at 4°C for 3 hours, 0.3 ml of assay buffer with 10 percent (weight to volume) charcoal and 2 percent (weight to volume) bovine serum albumin was added, and the mixture was centrifuged at 4°C in a Sorvall GLC-1 centrifuge for 10 minutes at 3000*g*. A sample (0.4 ml) of the supernatant was mixed with 5 ml of Bray's solution and the radioactivity was determined. Samples and standards were assayed in triplicate. For each assay, appropriate blanks containing 0.1 ml of water were included, and a standard curve was constructed by plotting the amount of cyclic AMP standards against radioactivity in the supernatant. Intracellular cyclic AMP was expressed as the number of picomoles per 10^6 viable cells or per milligram of protein. The authenticity of the cyclic AMP in the sample was confirmed by its susceptibility to degradation by phosphodiesterase.
15. J. R. Vane, *Nature (London) New Biol.* **231**, 232 (1971); J. B. Smith and A. L. Willis, *ibid.*, p. 235; S. H. Ferreira, S. Moncada, J. R. Vane, *ibid.*, p. 237.
16. J. W. Hinman, *Annu. Rev. Biochem.* **41**, 161 (1972); B. Samuelson, E. Granstrom, K. Green, M. Hamberg, S. Hammarstrom, *ibid.* **44**, 669 (1975).
17. J. W. Smith, A. L. Steiner, C. W. Parker, *J. Clin. Invest.* **50**, 442 (1971); C. W. Parker, M. L. Baumann, M. G. Huber, *ibid.* **52**, 1336 (1973); D. J. Franks, J. P. MacManus, J. F. Whitfield, *Biochem. Biophys. Res. Commun.* **44**, 1177 (1971).
18. V. A. Ferraris and F. R. DeRubertis, *J. Clin. Invest.* **54**, 378 (1974).
19. H. Sheppard and G. Wiggan, *Biochem. Pharmacol.* **20**, 2181 (1971).
20. The cyclic AMP analog, 8-bromo cyclic AMP, was added to parallel cultures of con A–stimulated lymphocytes at the times indicated. At 48 hours after con A addition, DNA synthesis was assessed by [^{3}H]thymidine incorporation. The rate of incorporation in unstimulated control lymphocytes was 3620 count/min per hour per 10^6cells. Net incorporation was calculated by subtracting the control value from total incorporation.
21. Cell cycle phase distribution was measured by flow microfluorometry. Samples (2 ml) of the culture were centrifuged and the lymphocytes were resuspended in 2 ml of a solution of sodium citrate (1.2 mg/ml) containing 50 μg of propidium iodide per milliliter (*28*). After staining for 10 minutes, cell fluorescence, proportional to the amount of cellular DNA, was measured by a Cytofluorograf (model 4801 Bio/physics Systems).
22. J. W. Smith, A. L. Steiner, W. M. Newberry, Jr., C. W. Parker, *J. Clin. Invest.* **50**, 432 (1971).
23. C. W. Parker, T. J. Sullivan, H. J. Wedner, *Adv. Cyclic Nucleotide Res.* **4**, 2 (1974).
24. A. E. Powell and M. A. Leon, *Exp. Cell Res.* **62**, 316 (1970); A. Novogradsky and E. Katchalski, *ibid.* **97**, 1 (1976); L. Furcht and S. Gentry, *Cell. Immunol.* **22**, 225 (1976). Cysteine (2 m*M*) inhibits lymphocyte proliferation completely if added up to 8 hours after neuraminidase–galactose oxidase (NAGO) treatment. Thereafter cells gradually become irreversibly stimulated and are totally insensitive to cysteine by 15 hours after NAGO stimulation.
25. T. Wang, C. Marquardt, J. Foker, *Nature (London)* **261**, 702 (1976).
26. B. L. Brown, *Biochem. J.* **121**, 561 (1971).
27. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
28. J. Fried, A. G. Perez, B. D. Clarkson, *J. Cell Biol.* **71**, 172 (1976).

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Microcirculation of the Spleen: An Open or Closed Circulation?

Abstract. *By injecting plastic microspheres of a specific size (3 to 4 micrometers) into the circulation and following their movement and distribution in the spleen, it was revealed how blood travels from the arterial capillaries to the venous sinuses. This method demonstrated that both open and closed circulation exist in the spleen and that about 90 percent of the blood takes the open route of circulation in the normal unanesthetized rabbit.*

Although the microcirculation of the spleen is central to its function as a blood filter, the intermediary vascular connection between the arterial capillary and the venous sinus has remained a subject of debate for the last 100 years. Three major theories about intermediary splenic circulation exist: (i) The open circulation theory states that the arterial capillaries open directly into the cordal meshwork of the red pulp and the blood is then forced into the venous sinuses (*1*); (ii) the closed circulation theory states that the arterial capillaries connect directly to the venous sinuses (*2*); (iii) the third theory holds that both open and closed circulation exist in the spleen (*3*). These differences in interpreting the nature of splenic circulation suggest that the methods for previous studies may have been inadequate, since both morphological observation of fixed spleen and direct observation of the living spleen produce conflicting results and are unable to settle the question. In addition, the ambient temperature of the exteriorized spleen, anesthesia, and manipulation of the spleen during experimentation affect the organ's microcirculation (*4*). We now describe a new method for studying the splenic circulation. This method demonstrates that both open and closed circulation exist in the spleen, and that about 90 percent of the blood takes the open route in normal unanesthetized rabbits. This method does not require direct observation of the vascular connection between the arterial capillary and the venous sinus and avoids invasive manipulation of the spleen and anesthesia.

Blood cells in the spleen pass through the reticular meshwork of the cords and pores (the interendothelial spaces) of the sinus wall. These pores measure be-

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