CYTIDINE 3',5'-MONOPHOSPHATE (CYCLIC CMP)

II. INITIATION OF LEUKEMIA L-1210 CELL GROWTH IN VITRO

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SUMMARY: Leukemia L-1210 cells grown in vitro to stationary phase and cooled for 1 hr at 4°, undergo a lag of approximately 2 hr before they resume growth upon addition of 9 volumes of fresh (37°) medium. This lag is abolished by cyclic CMP, and resumption of cell growth is detected within 15 min. Under the same conditions of growth, cyclic AMP, cyclic GMP or cyclic UMP prolong the 2 hr lag by 1.5 - 22 hr; in the presence of cyclic CMP this extended lag is abolished. It is suggested by these observations that cyclic CMP can assume a growth regulatory function.

INTRODUCTION: In a recent communication (1) we reported the presence of cyclic CMP in extracts of leukemia L-1210 cells. Although proof that this cyclic nucleotide is a true cell component and not an artifact of the isolation procedure is still outstanding, it has the capacity, described in this paper, to initiate the growth of stationary phase leukemia L-1210 cells.

EXPERIMENTAL: Leukemia L-1210 cells were grown for 40 hr in a 250 ml spinner flask containing 100 ml of RPMI 1630 medium + 10% dialysed calf serum, 100 u/ml of penicillin and 100 μg/ml of streptomycin. A 50 ml portion of the culture containing 1-3 x 10^6 cells/ml was then cooled at 4° for 1 hr, and 9 volumes of fresh medium (37°) containing 20 mM HEPES buffer were added to a desired portion of the cooled culture. To aliquots of this culture, the various cyclic nucleotides, dissolved in 0.1 ml of saline per 10 ml of culture, were added. The same amount of saline was added to aliquots serving as control. In experiments involving relatively short periods of incubation (up to 8 hr), the cultures were maintained in 100 ml screw-cap culture flasks, and samples were removed from the bottles at the specified intervals. To avoid the repeated exposure of the same culture to air, in experiments where many samples were taken over prolonged periods of incubation, 1 ml aliquots of the culture were introduced into 16 x 125 mm screw-cap culture tubes, which were in-

cubated at 37° for the specified length of time, and were then assayed individually.

To determine cell numbers and cell viability, 0.5 ml aliquots of the cultures were diluted with 0.5 ml of a 0.1% solution of trypan blue. Counting in duplicate, was carried out on a standard hemocytometer (8 WBC sections being counted each time), and viability was determined on the basis of dye exclusion.

For protein determinations, 50 ml cultures contained in 100 ml culture flasks were incubated under the conditions specified, and after removal of two 0.5 ml aliquots for counting, the cultures were centrifuged at approximately 500 x g, washed 5 times with 10 ml amounts of saline, and the protein content was determined by the method of Lowry (2).

RESULTS

Leukemia L-1210 cells grown *in vitro* for 40 hrs to stationary phase (as determined by the absence of any further increase in cell number), and cooled subsequently at 4° for 1 hr, resumed growth only after a lag of approximately 2 hr following the addition of 9 volumes of fresh (37°) medium (Fig 1). When cyclic CMP at 1×10^{-4}M was added to the culture together with the fresh medium, resumption of cell division was detected within 15 min. At this time, the increase in cell number amounted to approximately 3% above control, and after 2 hr of incubation the increase amounted to approximately 22% (Fig. 1). Cell viability in this and all other experiments reported here was approximately 99%, based upon dye exclusion. The initial rate of growth obtained in the presence of cyclic CMP was essentially the same as that of control (Fig. 1) and a decrease in this rate was seen after 1 hr of incubation. If, at this time, a second addition of 1×10^{-4}M cyclic CMP was made, the onset of the decline in rate was postponed for 1.5 hrs.

The extent of growth stimulation was dependent upon the concentration of cyclic CMP used (Table 1), and after 2 hr of incubation ranged from approximately 9% at 1×10^{-8}M cyclic CMP to approximately 22% at 1×10^{-4}M. Since counting of the cells by hemocytometer is subject to some error (with respect to the actual total cell number present), the protein content of the cultures was also determined, and as exemplified by the data in Table 1, the coincidence between the two measurements

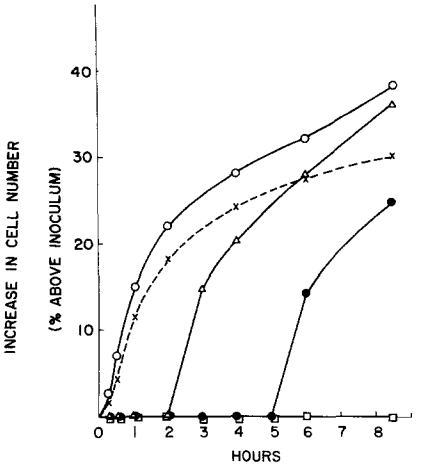

Fig. 1. Effect of cyclic CMP and cyclic AMP on the in vitro growth of leukemia L-1210 cells. The cells serving as the inoculum were grown to stationary phase and cooled at 4°, and the cyclic nucleotides were added together with 9 volumes of fresh (37°) media. △ : control, O : cyclic CMP (10^{-4}M), ● : cyclic AMP (10^{-6}M), □ : cyclic AMP (10^{-4}M), X : cyclic AMP (10^{-4}M) + cyclic CMP (10^{-5}M).

was very good. It should be pointed out that the values given in all instances are averages of at least three determinations. Unlike cyclic CMP, cytidine 2',3'-(cyclic) monophosphate, 5'-CMP, CDP, CTP, cytidine or cytosine did not abolish the 2 hr lag.

Under the same conditions of growth (Fig 1), cyclic AMP, at 1 x 10^{-6}M, prolonged the 2 hr lag of the control culture by approximately 3 hr (total 5 hr), and at 1 x 10^{-4}M, by approximately 22 hr (total 24 hr) (data not shown). At 1 x 10^{-5}M cyclic AMP, the total lag was approximately 12 hr, and at 5 x 10^{-5}M it was 15 hr. Cyclic GMP and cyclic UMP, at 1 x 10^{-5}M, caused a total lag of 3.5 and 4 hr, respectively.

When cyclic CMP at 1 x 10^{-5}M was added to the cooled culture together with 1 x 10^{-4}M cyclic AMP, the 24 hr lag was abolished (Fig 1), but the ensuing rate of growth was somewhat less than the one effected by cyclic CMP alone. Lower concentrations of cyclic CMP (1 x 10^{-6} - 1 x 10^{-7}M) also abolished the lag, but the rates of growth decreased proportionately.

Table 1. Effect of the Concentration of Cyclic CMP on the Extent of Growth of Leukemia L-1210 Cells In Vitro*

Concentration of Cyclic CMP	Percent Increase Relative to Control after 2 hrs of Incubation*	
	Cell Number	Protein
M		
10^{-4}	22.2	22.4
10^{-5}	19.5	20.9
10^{-6}	18.0	17.4
10^{-7}	13.2	12.4
10^{-8}	10.1	9.3

* The cells serving as the inoculum were grown to stationary phase and were then cooled for 1 hr at 4°. Nine volumes of fresh (37°) medium containing the specified concentrations of cyclic CMP were added, and the cultures were incubated for 2 hrs. The cell numbers were determined by hemocytometer, and the amount of protein by the method of Lowry et al (2).

DISCUSSION:

The observation that cyclic CMP can initiate the growth of leukemia L-1210 cells treated by methods which supposedly arrest them in G_1 (3 - 5), might indicate that this cyclic nucleotide is involved in the regulation of cell growth. We have recently detected the presence of cyclic CMP in extracts of L1210 cells (1), but proof that the compound is not an artifact of the isolation procedure is still outstanding. Cyclic CMP may exert its growth stimulatory effect by antagonizing cyclic AMP, which has long been shown to play a role in the density-dependent inhibition of cell proliferation (6-8) and which, when supplied exogenously, has been observed to inhibit the growth of various cell lines (9-12). The fact that cyclic CMP can prevent not only the 2 hr

lag seen upon incubation of the cooled stationary phase culture, but can equally prevent the extended lag resulting upon addition of exogenous cyclic AMP, supports this suggestion. Of course, in the latter case, competition between exogenous cyclic CMP and cyclic AMP for cellular uptake cannot be excluded, although cyclic CMP can prevent the lag even at 1/10 - 1/100 the concentration of cyclic AMP.

Further studies are obviously required to substantiate the suggestion that cyclic CMP has a growth regulatory function, and to elicit the mechanism by which cyclic CMP initiates the growth of the L-1210 cells.

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