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Cyclic AMP, A Nonessential Regulator of the Cell Cycle

(lymphoma/mutants/flow microfluorimeter)

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ABSTRACT **Flow-microfluorimetric analysis has been carried out on populations of exponentially growing S49 mouse lymphoma cells treated with dibutyryl cyclic AMP. The drug produces a specific concentration-dependent block in the G_1 phase of the cell cycle while other phases of the cycle are not perceptibly altered. The cell cycle of a line of mutant cells lacking the cyclic AMP-dependent protein kinase is not affected by the drug. Since these mutant cells have been shown to maintain a normal cell cycle, even in the presence of high levels of cyclic AMP, periodic fluctuations in the levels of the cyclic nucleotide cannot be required for or determine progression through the cell cycle.**

There is considerable evidence that adenosine 3':5'-cyclic monophosphate (cAMP) has a regulatory effect on the growth of cells in culture. The levels of the cyclic nucleotide increase as untransformed cells approach confluency (1, 2), although this is contradicted by others (3). Intracellular cAMP concentrations are negatively correlated with growth rate among a variety of fibroblast cell lines (4). Transformed cells have a lower cAMP content than untransformed cells (1, 5). Exogenous cAMP analogs or the induction of endogenous cAMP slows or stops growth of some cells (6–10). Proliferation of contact-inhibited cells, induced by refeeding or proteolytic treatment, is prevented by cAMP analogs (7, 11). The cAMP level changes during the cell cycle and is specifically low in mitosis (11, 12).

While these results make it clear that cAMP can cause marked effects on the cell cycle, important questions remain unanswered.

(*i*) Is cAMP merely a negative regulator of the cycle, or is variation of cellular cAMP levels a necessary signal that entrains the cell cycle? Burger *et al.* (11) have proposed, for instance, that a fall in cAMP is the signal that necessarily precedes DNA synthesis.

(*ii*) What is the locus of the growth-inhibitory action of cAMP? This has been variously reported as lying in G_1 (7, 13, 14), in G_2 (8), or in multiple discrete portions of the cycle (15, 16). These studies have in most cases used cell populations synchronized by techniques that possibly cause unbalanced growth, which might raise questions regarding the use of these materials for examining the effect of a growth regulatory substance.

To answer these questions we have studied growth regulation in cultured S49 mouse lymphoma cells. These cells are advantageous because growth of wild-type populations is inhibited by cAMP, and cAMP-insensitive mutants can be derived that are defective in the cAMP binding protein and its associated protein kinase (17, 18). In the present studies we have used the flow-microfluorimeter (19) and other techniques to examine the effects of cAMP on growth regulation in exponentially growing populations and have compared the growth inhibitory effect on populations of mutant and wild-type cells. We conclude that, although in wild-type S49 cells cAMP exerts a specific block in the G_1 phase of the cell cycle, this inhibition is not required for the normal timing of the cycle.

Abbreviation: Bt_2cAMP, $N^6,O^{2'}$-dibutyryl adenosine 3':5'-cyclic monophosphate.

MATERIALS AND METHODS

$N^6,O^{2'}$-Dibutyryl adenosine 3':5'-cyclic monophosphate (Bt_2-cAMP) was purchased from Sigma, theophylline from Calbiochem, and [^{3}H]thymidine from New England Nuclear Corp.

S49 cells (20) were grown in stationary suspension culture in Dulbecco's medium with 10% heat-inactivated horse serum in a humidified atmosphere containing 10% CO_2. Growth experiments were done with cells in 75 cm^2 Falcon flasks containing 20–50 ml of medium. To assure asynchronous growth, cells were maintained without addition of fresh medium for at least 24 hr before an experiment was begun. A mutant subline resistant to Bt_2cAMP was obtained by cloning the cells in soft agar containing the drug (18).

Cells were counted in a Coulter Counter model B. Viable cells excluding trypan blue were determined with a hemocytometer. For autoradiography, cells were labeled for 30 min in [^{3}H]thymidine, 1 μCi/ml (specific activity, 21 Ci/mol), washed with cold phosphate-buffered saline (pH 7.4, air-dried on slides, coated with Kodak NTB-2 photographic emulsion, developed, stained, and scored by conventional methods. Between 500 and 1000 cells were examined per sample; positive nuclei labeled densely.

To determine the distribution of cells in the cycle, samples containing 2 to 5 $\times$ 10^6 cells were washed once with cold

TABLE 1. *Effect of Bt_2cAMP, 0.1 mM, + theophylline, 0.2 mM, on growth of wild-type and cyclic AMP-resistant (cA^R) S49 cells*

Cells	Bt_2cAMP + theophylline	% Cells in G_1	Doubling time (hr)
Wild type	−	34*	18.0
	+	88	
cA^R	−	30	16.5
	+	29	16.5

* Measured 24 hr after drug addition.

phosphate-buffered saline and fixed in phosphate-buffered saline containing 10% Formalin. After DNA was stained with acriflavin (21), the cells were analyzed in the Lawrence Livermore Laboratory flow-microfluorimeter; the apparatus is described in detail elsewhere (22). Briefly, stained cells are hydrodynamically focused in the flow-microfluorimeter, so that they travel in single file, at rates up to 10^4/sec, through the intense beam of exciting light from an argon ion laser. The acriflavin in each cell is thus stimulated to fluoresce; this light is detected by a photomultiplier and converted into an electrical pulse, which is amplified and subsequently stored in a multichannel analyzer. After analysis of a large number (about 10^5) of cells, the contents of the analyzer represents the DNA distribution of the population.

The distribution of cells among G_1, S, and G_2 + M can be calculated from the DNA histograms (23). The duration of each phase of the cycle can then be measured from the doubling time of an exponentially growing population whose growth fraction is unity, assuming that the doubling time equals the generation time and that the population age distribution decreases exponentially with age (24). Computer modeling of exponential steady-state cell growth and kinetic analysis of perturbed populations were done as described by Gray (25).

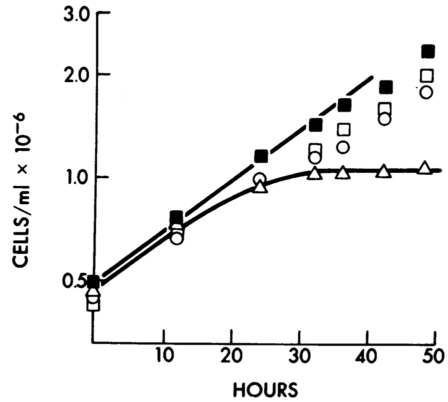

FIG. 1. Effect of Bt_2cAMP on the growth of S49 cells treated with theophylline, 0.2 mM, alone (■) or theophylline at that concentration together with Bt_2cAMP, 0.1 mM (△), 0.03 mM (○), or 0.01 mM (□).

RESULTS

Cell Growth Inhibited by Bt_2cAMP. Cloned S49 cells in exponential growth were treated with 0.2 mM theophylline and the indicated concentrations of Bt_2cAMP, and the cell density was measured as a function of time (Fig. 1). The untreated

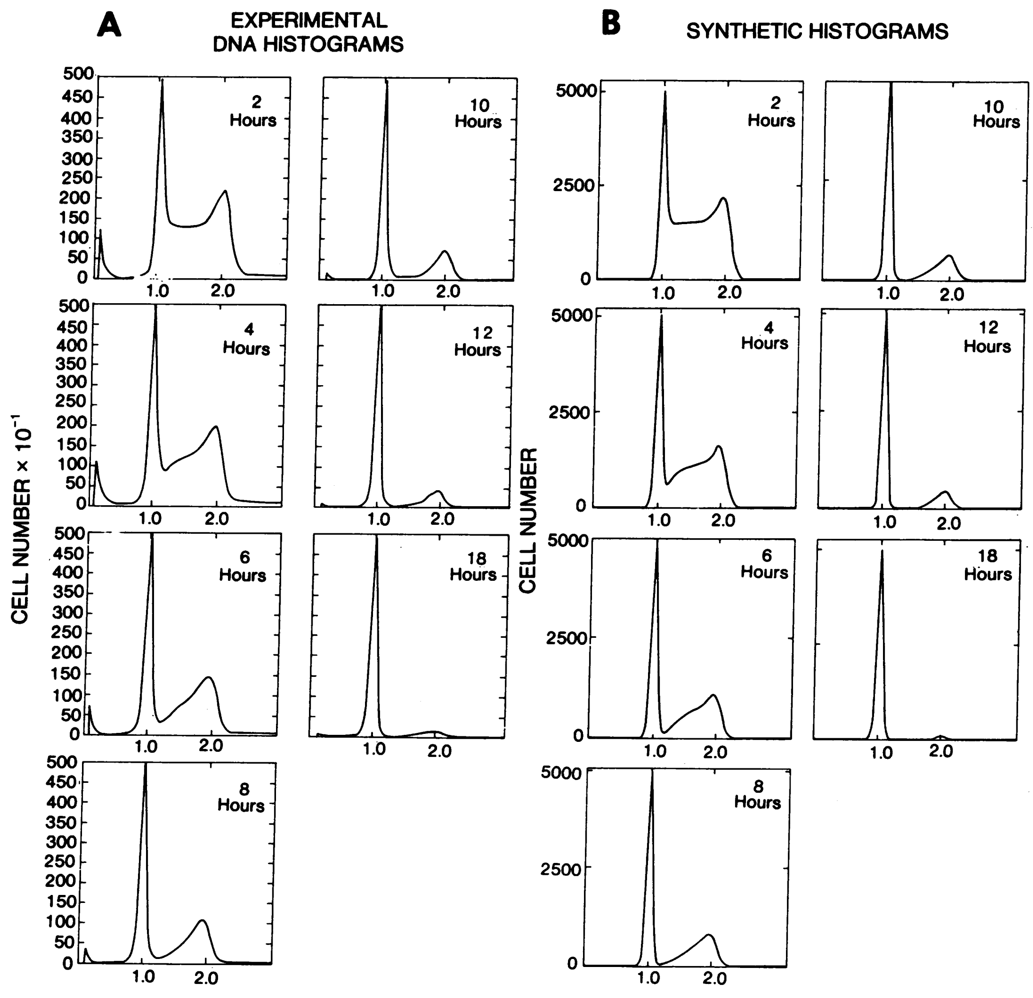

FIG. 2. Kinetics of change in cell cycle distribution after treatment with Bt_2cAMP. (See *text* for experimental details.)

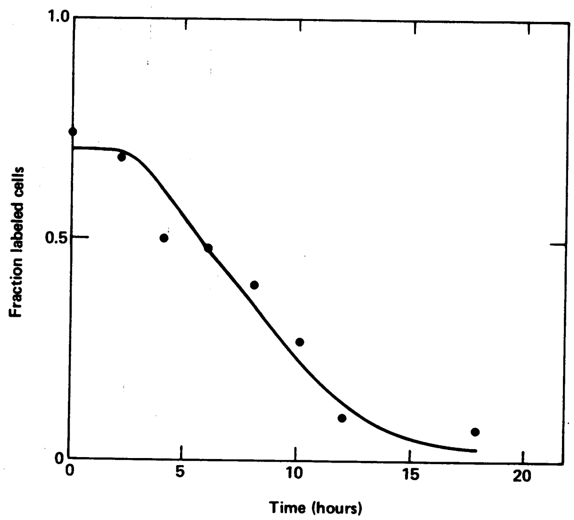

FIG. 3. Fraction of cells labeled with [^{3}H]thymidine after treatment with theophylline, 0.2 mM, and Bt_2cAMP, 0.5 mM. Points represent experimental data; the line represents data generated by a computer model that assumes an early G_1 block (see *text*).

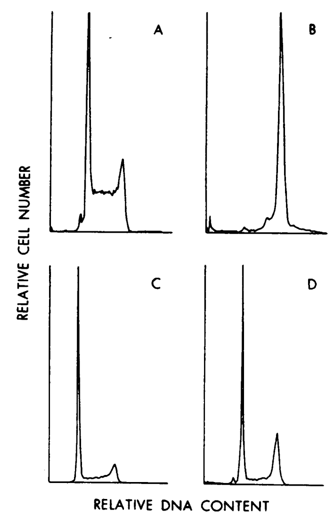

FIG. 4. Effect of colcemid on the cell cycle distribution of Bt_2cAMP-arrested cells. Samples were analyzed by flow-microfluorimeter after the following treatments: A, none; B, colcemid, 1 μg/ml for 10 hr; C, Bt_2cAMP, 0.1 mM, and theophylline, 0.2 mM, for 20 hr; D, Bt_2cAMP and theophylline for 20 hr with addition of colcemid for a further 10 hr.

control cells grew with a doubling time of 17–18 hr. After addition of theophylline and 0.1 mM Bt_2cAMP, the cell density continued to increase at the same rate as the control for approximately one generation time and then remained constant. The growth arrest occurred when the cell number was double that present at the time of drug addition, regardless of the initial cell density, so long as the culture began in exponential, i.e., asynchronous, growth. After 24 hr of treatment the cells were fully viable, as measured by trypan blue exclusion and the resumption of growth upon resuspension in fresh growth medium. After 48 hr, the viability was about 20–50% and declined rapidly thereafter. Lower concentrations of Bt_2cAMP reduced the growth rate after a lag time of about one cell generation (Fig. 1). These results suggest that Bt_2-cAMP inhibits growth in a specific phase of the cell cycle.

To study this question, we analyzed treated and control cells with the flow-microfluorimeter to determine the distribution of the cell population among the G_1, S, and G_2 + M phases of the cycle. Application of this method revealed that the phase durations were 2.1, 12.0, and 3.0 hr for T_{G1}, T_S, and T_{G2+M}, respectively, in wild-type S49 cells. Table 1 shows the doubling time and fraction of cells in G_1 for both the wild-type and a mutant subline unresponsive to cAMP due to a defect in cAMP-dependent protein kinase (17, 18). After treatment with Bt_2cAMP and theophylline for 24 hr, the mutant cells were unaffected in doubling time or cell cycle distribution. The wild-type cells, however, had ceased to grow and the fraction of cells in G_1 had increased from 34 to 88%. It should be noted that the kinetic parameters of the mutant cells differed slightly from those of the wild-type cells.

G_1 Prolonged by Bt_2cAMP. To follow the kinetics of this perturbation in cell cycle distribution, we treated exponentially growing cells as in Fig. 1 with 0.1 mM Bt_2cAMP and 0.2 mM theophylline and, periodically, samples were collected for flow-microfluorimeter analysis (Fig. 2). After 2 hr of treatment, the cell cycle distribution seen in the flow-microfluorimeter (Fig. 2A) did not differ significantly from that in the control untreated population (not shown). By 4 hr a relative decrease in the early S population was apparent, which became more marked at 6 hr and extended progressively to the late S population at 8 and 10 hr. At 12 hr, the number of G_2 cells began to fall; by 18 hr more than 90% of the cells were in G_1.

It is apparent from these data that Bt_2cAMP imposes a G_1 block, and this impression is confirmed by computer-generated DNA histograms (Fig. 2B). These were derived by modeling (25) an exponentially growing population using cell cycle parameters obtained by flow-microfluorimeter analysis of untreated cells. The model was perturbed only by imposing a block in early G_1 and following the change, with respect to time, of the cell cycle distribution of the model population. Computer models assuming a block in late G_1 did not reproduce the experimental data. Comparison of Fig. 2A and B shows that the match is good between experimental and computer-generated data.

Other Cell Cycle Parameters Not Affected by Bt_2cAMP. Experiments were done to exclude the possibility that Bt_2cAMP has another site of action in the cell cycle, and to show that cells that have not yet reached the block, or have escaped from it, cycle normally. The modeling results in Fig. 2 support this hypothesis since they were generated assuming only a block in G_1. To test this idea further, cells were pulse-labeled with [^{3}H]thymidine at intervals after treatment with Bt_2-cAMP and theophylline, and autoradiograms prepared to determine the fraction of labeled cells (Fig. 3). These data were compared to the fraction of cells in S generated by the cell cycle model, again assuming only a block in early G_1. The good agreement between experimental and computer-generated data provides an independent confirmation of the conclusions from the flow-microfluorimeter results. Hence, S is of normal duration for at least one generation after drug addition.

Dose-dependent Partial Block Imposed by Bt_2cAMP. As shown in Fig. 2, there was a residual S and G_2 + M population after 18 hr of treatment with 0.1 mM Bt_2cAMP and 0.2

mM theophylline; these cells are present even after 48 hr of treatment with 0.5 mM Bt_2cAMP and 0.2 mM theophylline. This minor cell population not blocked in G_1 could represent a distinct subclass not responsive to cAMP. This is unlikely because the cells had been recently cloned and only about one in 10^5 cells are mutants resistant to the growth-inhibitory and cytolytic effects of Bt_2cAMP (18).

Other explanations for the presence of cells not blocked in G_1 include the possibility that they are dead, that they are cells subject to a secondary block in other parts of the cycle, or that cells infrequently but regularly escape the G_1 block. All but the last of these would be excluded if it could be shown that the residual S and G_2 + M populations are cycling normally after prolonged drug exposure.

To test this, cells were grown with Bt_2cAMP and theophylline for 20 hr and colcemid was then added to the culture for a further 10 hr, to accumulate cells in c-mitosis (Fig. 4). A control culture, in exponential growth, was treated with colcemid alone for 10 hr. Flow-microfluorimeter samples were prepared before and after colcemid addition. As expected, the control culture after treatment with colcemid contained only a small late S population and a large population of cells with G_2 DNA content, representing cells arrested in mitosis. If the Bt_2cAMP-treated culture contained cycling S and G_2 cells, an increase should be seen in the G_2 + M peak as cells transit into mitosis and are arrested there. This was observed. Indeed, there was an approximately 3-fold increase in the area of the G_2 + M peak, as expected for a population with G_2 + M duration of 3.0 hr accumulating mitotic cells for 10 hr. The DNA distributions of control cells treated with Bt_2cAMP and theophylline did not change between 20 and 30 hr. This confirms as well that the duration of G_2 + M is unaffected by the block.

Therefore, since even high Bt_2cAMP concentrations induce a "leaky" block, experiments were done to investigate the relationship between drug dose and the effectiveness of G_1 arrest. Cells were exposed to different concentrations of Bt_2-cAMP in the presence of 0.2 mM theophylline for 36 hr, so that a nearly steady-state cell cycle distribution was achieved. The fraction of the cell population in G_1 was determined by flow-microfluorimeter as an index of the effectiveness of the block (Fig. 5). The fraction of cells in G_1 was linearly related to the exogenous Bt_2cAMP concentration in the range 10^{-5}–10^{-4} M. The effect of added 10^{-5} M Bt_2cAMP was small but significant compared with the control treated with theophylline alone.

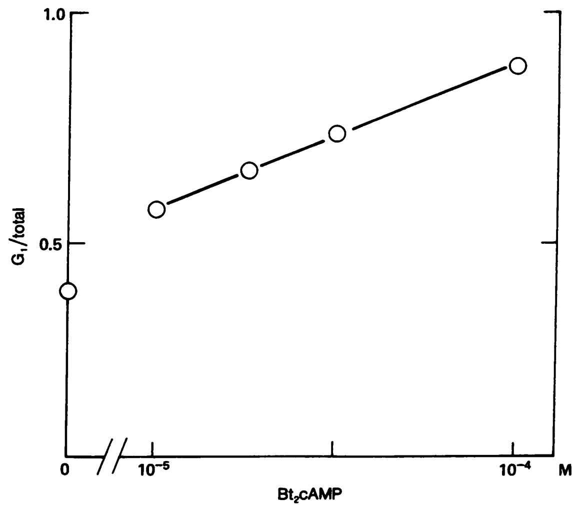

FIG. 5. Fraction of cells in G_1 36 hr after treatment with varying Bt_2cAMP concentrations. All cultures contained 0.2 mM theophylline. The fraction of cells in G_1 was determined by flow-microfluorimeter analysis as described in the *text*.

DISCUSSION

Previous work has shown that cAMP induced endogenously by hormones or added exogenously as the dibutyryl analog inhibits the growth of S49 cells and eventually kills them (17, 18). The present experiments show that growth inhibition is caused by prolonging the mean duration of G_1 without significantly affecting the length of S, G_2, or M. Although high concentrations of Bt_2cAMP prolong G_1 to a degree simulating complete growth arrest, the cells that emerge from G_1 traverse the rest of the cycle with kinetics similar to those of exponentially growing cells. Thus, cells that escape the block are not members of a distinct subpopulation.

Is the effect demonstrated here due directly to cAMP? The G_1 inhibitory effect of Bt_2cAMP can be reproduced by raising the endogenous cAMP level in S49 with choleratoxin, a stimulator of adenylate cyclase (26), and RO 20-1724, a phosphodiesterase inhibitor (27) structurally unrelated to theophylline (data not shown). Theophylline potentiates the growth-inhibitory effect of Bt_2cAMP. To these usual criteria of specificity may be added a genetic one: in an S49 mutant deficient in cAMP-dependent protein kinase, and therefore unresponsive to cAMP with respect to cytolysis, growth inhibition, and phosphodiesterase induction, Bt_2cAMP and theophylline have no effect on cell cycle parameters (Table 1).

The G_1 specificity of the growth regulatory effect of cAMP is not surprising in view of the evidence that cAMP is a significant growth-inhibitory substance in a variety of cultured cell lines, and that growth regulation *in vitro* and *in vivo* is commonly mediated by mechanisms operative in G_1. Indeed, evidence that cAMP or its analogs induce G_1 arrest in cultured fibroblasts has been previously adduced (7). The present work supports these findings and extends them by demonstrating through detailed kinetic analysis of an exponentially growing population that the arrest is specific for G_1 and that cAMP-mediated growth regulation does not require cell attachment to a solid substrate but is seen in suspension culture as well. Growth-inhibitory effects of cAMP have been reported to occur in G_2 in some cases. This may indicate that in certain tissues G_2 is a significant growth control point or that it may become so as a result of malignant transformation.

The failure of Bt_2cAMP to act in a kinase-deficient mutant strongly supports the idea that cAMP itself acts specifically as a G_1 regulator and that it does so through activation of the kinase. The maintenance of an essentially normal cell cycle in the mutant, as measured by growth rate and flow-microfluorimeter, indicates that fluctuation in cAMP levels cannot be the fundamental determinant of progression through the cell cycle, although the cyclic nucleotide may act as a negative modulator of that progression.

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