Growth Regulatory Effects of Cyclic AMP and Polyamine Depletion Are Dissociable in Cultured Mouse Lymphoma Cells

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ABSTRACT Treatment of mouse lymphoma S49 cells with D,L-α-difluoromethylornithine (DFMO), an inhibitor of ornithine decarboxylase, depleted cellular polyamine levels and stopped cell growth. The cells were arrested predominantly in G_1. Thus, polyamine depletion may lead to a regulatory growth arrest in S49 cells. We tested two hypotheses regarding the relationship of growth arrest mediated by polyamine limitation to that mediated by cyclic AMP (cAMP). The hypothesis that cAMP-induced arrest results from polyamine depletion is not tenable, because the arrest could not be reversed by addition of exogenous polyamines, and because cellular polyamine levels do not drop in dibuturyl cyclic AMP (Bt_2cAMP)-arrested cells. The hypothesis that polyamine-mediated growth arrest is effected via modulation of cAMP levels or cAMP-dependent protein kinase activity was also shown to be incorrect, because a S49 variant deficient in cAMP-dependent protein kinase was arrested by DFMO. The activities of the polyamine-synthesizing enzymes ornithine decarboxylase (ODC) and S-adenosyl methionine decarboxylase (SAMD) are both reduced in Bt_2cAMP-treated cells to about 10% of that in control populations, as shown previously. DFMO diminishes ODC activity and augments SAMD activity in both untreated and Bt_2cAMP-treated cells, leading to polyamine depletion in both cases.

Numerous studies indicate that the polyamines putrescine, spermidine, and spermine influence cell proliferation. Two sorts of evidence support this: first, polyamine levels and the activities of their synthesizing enzymes, ornithine decarboxylase (ODC) and S-adenosyl methionine decarboxylase (SAMD), vary with the proliferative state of the cell (1), and second, pharmacologically induced depletion of cellular polyamines leads to reduced cellular proliferation (2–11). Polyamines may simply be necessary constituents for proliferating cells or they may be regulatory molecules, signaling the cells to initiate or cease proliferation.

We ask here whether polyamines are necessary for growth of mouse lymphoma S49 cells, whether they behave as regulatory molecules with respect to cell cycle specificity of arrest, and finally whether causal connections exist among regulatory systems controling cyclic AMP (cAMP), polyamines, and cell growth.

ODC is the enzyme responsible for synthesis of putrescine from ornithine. Spermidine and spermine are generated from putrescine by addition of one and two aminopropyl groups, respectively. SAMD generates the aminopropyl donor molecules for this reaction and is the rate-limiting enzyme for the synthesis of these higher polyamines. We show here that treatment of S49 cells with α-difluoromethylornithine (DFMO), an enzyme-activated irreversible inhibitor of ODC, depletes cells of polyamines and halts cell proliferation. Because regulators of cell growth act primarily to change the duration of the G_1 phase of the cell cycle (12) we assume that a G_1 arrest is suggestive of a regulatory growth arrest. S49 cells show a predominantly G_1 phase arrest with DFMO treatment. This indicates that in these cells polyamines are potentially regulatory molecules. Previous work has demonstrated that treatment of S49 cells with biologically active cAMP analogues such as dibutyryl cAMP (Bt_2cAMP) arrests cells in the G_1 phase (13) and extinguishes the activities of ODC and of SAMD (14, 15). Both responses are mediated via the cAMP-dependent protein kinase, as both are abolished in S49 mutant cells devoid of that kinase (13–15).

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On the basis of these data, a causal model can be drawn; cAMP levels rise, cAMP kinase is activated, ODC and SAMD are inactivated, polyamine levels decrease, and G_1 arrest results from the depletion of polyamines. We show here that this model is wrong. A second model that connects polyamines and cAMP may be considered: DFMO reduces ODC activity, polyamine levels decrease, this leads to increased cAMP levels, cAMP kinase is activated, and G_1 arrest ensues. We show that this model also is incorrect and conclude therefore that cAMP and polyamines influence cell growth through mechanisms whose initial steps, at least, are distinct.

MATERIALS AND METHODS

Data in all figures and tables are from representative experiments, each of which was repeated multiple times.

Cell Culture: In these studies we have used mutant S49 cells (deathless variant, clone 211.1.2), which have all the growth regulatory responses to elevated cAMP levels of wild type cells but which, unlike wild type S49 cells, do not die in response to elevated cAMP (14, 16). This is necessary to distinguish cAMP's effects on polyamines due to growth arrest from those due to cellular death. Cells were maintained in Dulbecco's modified Eagle's (DME) medium, 3 gm/l glucose, and 10 percent heat-inactivated horse serum at cell densities from 10^5 to 10^6 per ml. Cell numbers were determined with an electronic particle counter (Coulter model ZBI, Coulter Electronics Inc., Hialeah, FL).

Enzyme Assays: ODC and SAMD assays were performed as described (14) except that the released $^{14}CO_2$ was trapped on a piece of filter paper (Whatman 3 mm, Whatman Inc., Paper Div., Clifton, NJ) spotted with 10 μl 1 M hyamine hydroxide. In both assays enzyme activities were linear with time and proportional to the amount of protein added. Cellular lysates for enzyme assays were prepared from cells washed once with cold phosphate-buffered saline (PBS) and resuspended in hypotonic buffer (5 mM Tris HCl pH 7.5, 2 mM dithiothreitol) at a concentration of 4×10^7 cells/ml. Cells were quick-frozen in a dry ice-ethanol bath and stored in liquid nitrogen. Cells were lysed by a total of three cycles of freeze-thawing. Homogenates were centrifuged at 100,000 *g* for 60 min and the supernatants assayed. In some cases the supernatants were again stored in liquid nitrogen until assayed.

Polyamine Measurements: Cells were washed one time with PBS and centrifuged. The PBS was decanted and the tubes were wiped to remove all excess solution. Cell pellets were resuspended in 8% fresh 5-sulfosalicyclic acid (250 μl/pellet or 3.5×10^7 cells/250 μl, both methods gave the same results). Polyamine levels were measured as described (9).

Flow Cytometry: The distribution of cell populations in the cell cycle was determined by staining ethanol-fixed cells with chromomycin and analyzing them with a flow cytometer (FCM) at the Lawrence Livermore Laboratory as described (17).

Experimental Design: Cells were fed by a threefold dilution, allowed to grow 1 d, and again fed, this time by fivefold dilution with medium containing the appropriate drug. Polyamine levels, cell numbers, and FCM distribution were measured at indicated times following drug addition.

RESULTS

Effects of Polyamine Depletion on Cell Growth

Treatment of S49 cells with DFMO stopped cell growth (Fig. 1). This effect was completely prevented by adding 10 μM putrescine along with the drug. Putrescine's ability to abolish DFMO's growth inhibitory effect indicates that the inhibition is due to polyamine depletion and not to some nonspecific effect of the drug. Cells arrested by 3 d of drug treatment could be made to resume growth by refeeding with fresh medium containing putrescine. DFMO treatment of S49 cells was not very toxic; it reduced their cloning efficiency in medium containing putrescine to 76% in 2 d and 70% in 3 d, relative to control cells. Refeeding with fresh medium alone did not reverse the growth arrest. This result further supports the specificity of DFMO by ruling out the possibility that putrescine abolishes the effect of DFMO by reducing its uptake into cells.

The kinetics of DFMO arrest were the same regardless of whether cultures were treated at initial cell densities as low as 10^4 per ml or as high as 10^6 per ml (data not shown). Initial cell densities lower than 10^4 per ml could not be tested, as S49 cells do not grow exponentially under these conditions. Therefore, we have no evidence for or against the hypothesis that arrest is mediated by a factor released by DFMO-treated S49 cells, as has been demonstrated for CHO cells (18).

Measurement of intracellular levels of polyamines (Table I) showed that DFMO treatment led to a depletion of spermidine. Putrescine addition replenished spermidine pools. Putrescine levels in S49 cells were too low to be reliably measured with the number of cells used, except when cells were treated with exogenous putrescine. In those experiments in which the amount of endogenous putrescine could be measured, its modulation paralleled that of spermidine. These data imply that depletion of spermidine and perhaps putrescine leads to cessation of cell proliferation.

Cell Cycle Specificity of DFMO Arrest

To determine the cell cycle specificity of DFMO arrest, we analyzed control and DFMO-treated cells by flow cytometry (FCM), a procedure that measures DNA content per cell and thus determines the distribution of the cell population among the G_1, S, and G_2 + M phases of the growth cycle. FCM measurements are shown of cells treated with DFMO for 24, 48, and 72 h, and of untreated and DFMO plus putrescine-treated cells at 24 h (Fig. 2). FCMs of untreated control cells and DFMO plus putrescine-treated cells at 48 and 72 h are not

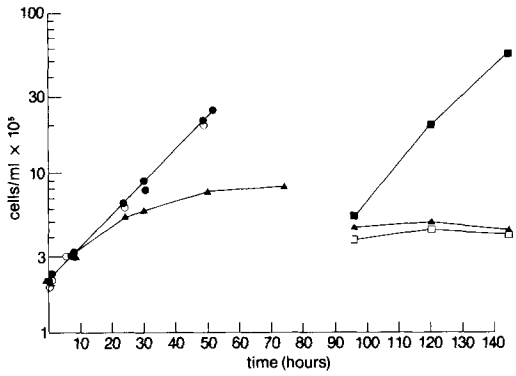

FIGURE 1 Effect of DFMO on cell growth and its reversal by putrescine. S49 cells were grown without drugs (●) or in the presence of 0.1 mM DFMO (▲), DFMO plus putrescine (○), or DFMO followed by 74 h with medium alone (□) or with medium containing putrescine (■), as described in Materials and Methods. After removal from medium containing DFMO, S49 cells were resuspended at 3×10^5 cells/ml and treated with 0.1 mM DFMO or with 10 μM putrescine as indicated. Data are summarized from two experiments. For reversal of DFMO treatment, S49 cells were counted with a hemocytometer.

TABLE I

Measurement of Intracellular Levels of Polyamines

Treatment	Spermidine	Spermine
	nm/10⁶ cells	
None	0.69	0.84
DFMO	0.017	0.30
DFMO + Putrescine	1.20	0.59

Effect of DFMO and putrescine on cellular polyamine pools. S49 cells were treated as in Fig. 1 and harvested for polyamine measurements after 49 h of drug treatment.

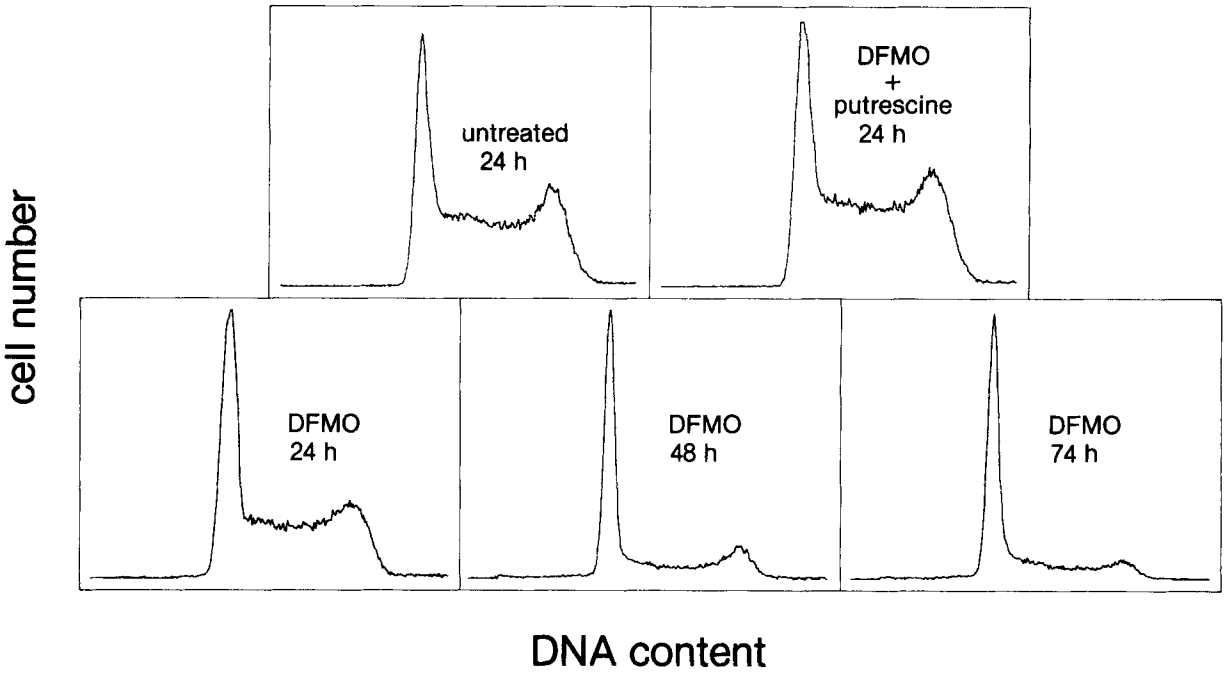

FIGURE 2 Cell cycle distribution of DFMO treated cells. Cells were treated as in Fig. 1 and FCM measurements taken at 24, 48, and 72 h. FCM measurements of DFMO-treated cells are shown at all time points. FCM measurements of untreated and DFMO plus putrescine-treated cells are shown at 24 h only, as discussed in the text. Data are displayed as fluorescence per cell on the abscissa and as cell number on the ordinate, both in arbitrary units.

included as exponentially growing cultures have reached very high cell densities by 48 h. When cells were plated at lower densities, they did not show FCM pertubations after 48 and 72 h but did when similarly high cell densities were reached (data not shown). In these experiments, DFMO- and Bt_2cAMP-arrested cells at no time reached cell densities at which untreated cells show FCM pertubations. The fraction of cells in the G_1 phase was slightly increased after 1 d of treatment with DFMO; after 3 d the effect was pronounced. This implies that the growth inhibition produced by DFMO results from a prolongation of G_1 relative to other phases of the cell cycle. Such a G_1-specific growth arrest is consistent with a potentially regulatory role for polyamines. DFMO's effect on cell cycle distribution was completely prevented by the addition of 10 μM exogenous putrescine. Treatment of cells with DFMO in medium containing dialyzed horse serum rather than unmodified horse serum changed neither the kinetics nor the cell cycle specificity of arrest (data not shown). Therefore, the amount of polyamines in the horse serum is insufficient to affect the response.

Interrelation between cAMP and Polyamine Growth Regulatory Systems in S49 Cells

We tested in a number of ways the hypothesis that Bt_2cAMP-mediated arrest of S49 cells occurs via depletion of intracellular polyamines. This hypothesis predicts that the arrest should be reversed by replenishing polyamine pools. There was no effect on the kinetics of Bt_2cAMP arrest when exogenous putrescine was added at a concentration that completely prevents growth inhibition by DFMO (Fig. 3). Nor did the addition of putrescine have any effect on the FCM distribution of Bt_2cAMP arrested cells (Fig. 4). In order to show that the exogenous putrescine entered the cells and effectively replenished pools, we measured intracellular polyamines. Fig. 5 shows that exogenous putrescine entered Bt_2cAMP-treated cells and was metabolized to spermidine; spermidine pools were elevated above control levels in cells treated with both putrescine and Bt_2cAMP. Putrescine pools were also elevated to easily measurable levels, i.e., above control levels (data not shown). Therefore, Bt_2cAMP arrest is not reversed by elevation of polyamine pools. This implies that the arrest is not mediated via depletion of polyamine pools.

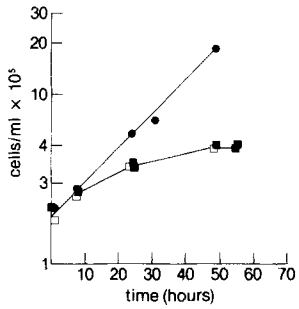

FIGURE 3 Effect of putrescine and Bt_2cAMP on growth of S49 cells. S49 cells were untreated (●) or treated with either 0.5 mM Bt_2cAMP (■) or Bt_2cAMP plus 10 μM putrescine (□) and cell number was measured.

Intracellular polyamine pools were measured as a function of time in untreated cultures and in cultures treated with DFMO, DFMO plus putrescine, Bt_2cAMP, Bt_2cAMP plus putrescine, and Bt_2cAMP followed 24 h later with DFMO (Fig. 5). All cultures were refed with fresh medium at the time of drug addition. As previously discussed, putrescine levels were too low to measure reliably but appeared to vary directly with spermidine levels (data not shown). Spermine pools stayed relatively constant under all conditions (data not shown), as reported for other cell types (5–10, 18, 19). Spermidine pools rose about twofold in untreated control cells during the 50-h

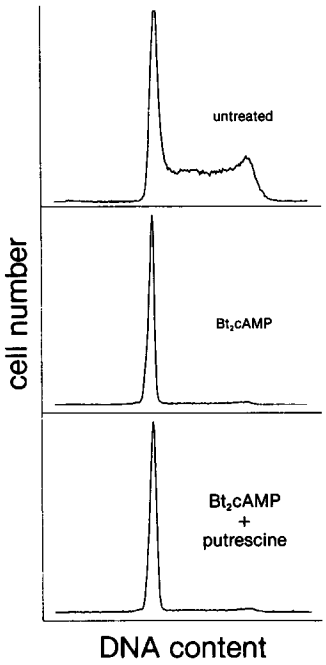

FIGURE 4 Effect of putrescine on the cell cycle distribution of Bt_2cAMP-arrested S49 cells. S49 cells were treated as in Fig. 3 and FCM measurements taken at 26 h. Data are displayed as in Fig. 2.

course of the experiment, rose even more rapidly in putrescine-treated cells, fell precipitously in DFMO-treated cells, and remained constant in Bt_2cAMP-treated cells. Since polyamine levels do not drop in Bt_2cAMP-treated cells we can again conclude that the arrest is not mediated through modulation of polyamine levels. This result was unexpected as ODC and SAMD activities drop to about 10% of control values under these conditions. Clearly, that reduced activity is sufficient to maintain polyamine pools during growth arrest. Further treatment of Bt_2cAMP-treated cells with DFMO at 24 h led to a decrease in spermidine levels, indicating that the remaining ODC activity can be pharmacologically inhibited and is indeed required to maintain polyamine pools. Similar experiments that included longer time courses showed that DFMO continues to deplete spermidine levels in Bt_2cAMP-treated cells to 10% of values in cultures treated with Bt_2cAMP alone (data not shown).

Fig. 6 shows the activities of ODC and SAMD under the same conditions as used in the experiments shown in Fig. 5. Cultures refed with medium alone exhibited increases in both ODC and SAMD activities. This stimulation accounts for the increase in spermidine pools seen in Fig. 5. DFMO treatment of cells rapidly extinguished ODC activity and produced a slight augmentation of SAMD activity. This enhancement of SAMD activity was more prominent in other experiments. DFMO induction of SAMD has been reported in other cell systems, and it was suggested that SAMD induction was stimulated by reduced polyamine levels (7, 11, 20–23). Bt_2cAMP treatment of cells led to a small early rise followed by a precipitous drop in both ODC and SAMD activities, as previously reported (14); each fell to ~10% of its initial value by 10 h. Subsequent treatment of Bt_2cAMP-treated cells with DFMO dropped ODC activity further and induced SAMD activity. The induction of SAMD was abolished by the addition of putrescine along with DFMO, suggesting that the reduction of polyamine pools is the signal for the induction.

Studies in this and in other laboratories have shown that the kin^- S49 cell mutant is devoid of measurable cAMP-dependent protein kinase activity and lacks all responses to elevated cAMP levels (14, 24, 25). We tested the hypothesis that DFMO arrest is modulated through the cAMP kinase by using this mutant. Deathless (kin^+) and kin^- cells were treated in parallel with DFMO, and cell growth and FCM measurements were made. Both cell types were arrested, and the kinetics of the arrest were identical (data not shown). FCM measurements made 72 h after treatment with DFMO were identical in the two cell types, indicating that the change in cell cycle distribution of the arrested cells is also not mediated via the cAMP kinase (Fig. 7). Therefore the arrest and change in cell cycle distribution induced by DFMO cannot be modulated via cAMP kinase, as the arrest and redistribution occur identically in cells having or lacking that kinase.

DISCUSSION

The major conclusions we draw from these data are that polyamine depletion arrests the growth of S49 cells, that the cell cycle specificity of the arrest is characteristic of a regulatory response, and that this arrest is dissociable from cAMP-mediated arrest.

We have shown that DFMO-mediated arrest is due to polyamine depletion. It is possible that the arrest is due to a secondary effect of polyamine depletion rather than being a direct response to lowered polyamine levels. For example, the increased SAMD activity accompanying polyamine depletion could lead to decreased levels of S-adenosylmethionine and/or increased levels of decarboxylated S-adenosylmethionine. This possibility has been discussed in other reports (26, 27).

S49 cells arrested by DFMO are predominantly in G_1. We recognize that this suggests a G_1-specific block but does not

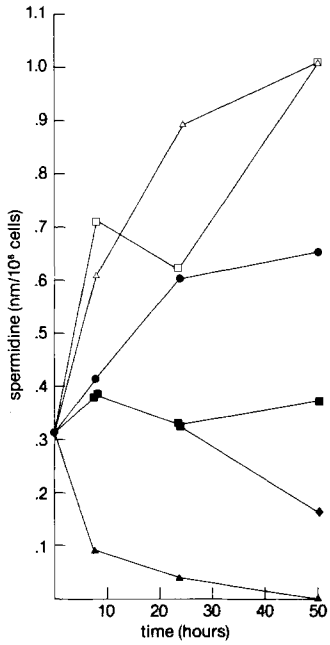

FIGURE 5 Spermidine levels in S49 cells treated with various combinations of DFMO, putrescine, and Bt_2cAMP. Cells were treated as described in Materials and Methods and harvested for polyamine measurements. Drug concentrations are the same as those in previous figures. Control (●), DFMO (▲), Bt_2cAMP (■), DFMO plus putrescine (△), Bt_2cAMP plus putrescine (□), and Bt_2cAMP followed at 24 h with DFMO (◆).

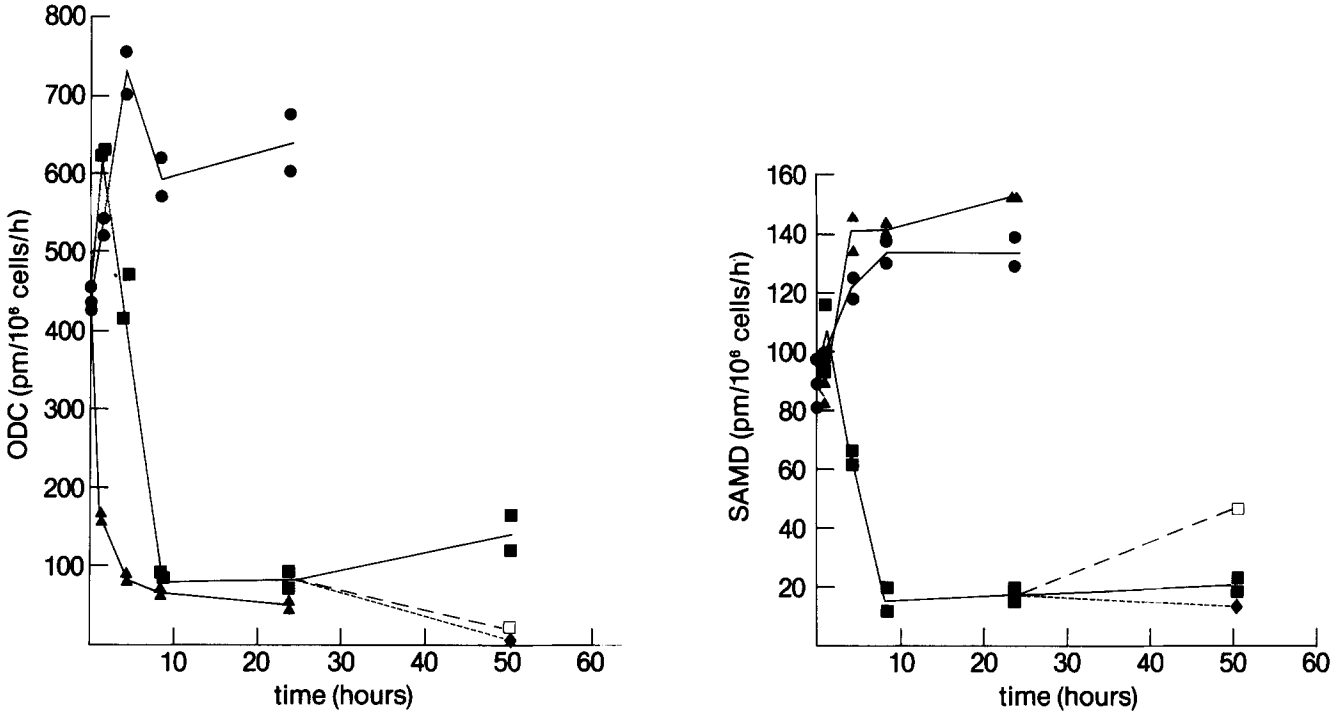

FIGURE 6 Time course of ODC and SAMD activities in S49 cells treated with various combinations of DFMO, putrescine, and Bt_2cAMP. S49 cells were treated with drugs, lysates prepared, and assayed for ODC and SAMD. Control (●), DFMO (▲), Bt_2cAMP (■), Bt_2cAMP followed at 24 h with DFMO (□), and Bt_2cAMP followed at 24 h with DFMO and putrescine (◆). Drug concentrations are the same as in previous figures.

prove that progression through other phases of the cell cycle is unaffected. Further studies are needed to resolve this. The growth inhibition induced by polyamine depletion in S49 cells is neither so prompt nor so complete as that induced by activation of cAMP-dependent protein kinase. Whether and, if so, under what conditions intracellular depletion of polyamines is of physiologic significance in regulating cell growth remains to be established.

In all cases the activities of polyamine synthetic enzymes are consistent with the modulations in polyamine pools. Bt_2cAMP treatment leads to a coordinate lowering of ODC and SAMD activities; the polyamine pools are little perturbed. DFMO lowers ODC and induces SAMD; putrescine levels are reduced, and the remaining spermidine is converted to spermine and/or other products. This occurs either when cells are treated with DFMO alone or when DFMO treatment follows Bt_2cAMP treatment. Thus the ODC activity that remains in Bt_2cAMP-treated cells is functional in maintaining polyamine levels. The kinetics of modulation of ODC activity and cell division by DFMO and Bt_2cAMP do not differ greatly, yet DFMO drastically reduces spermidine levels, whereas spermidine levels are little perturbed in Bt_2cAMP-treated cultures. Therefore, Bt_2cAMP-treated cells must maintain their spermidine pools largely by reducing SAMD activity, thus reducing the rate of conversion of spermidine to spermine, or possibly by reducing the rate of spermidine's metabolic conversion through other pathways, such as oxidation or acetylation. It appears that ODC and SAMD activities are modulated in a way that retains normal polyamine levels during Bt_2cAMP arrest.

Russell et al. (28) reported polyamine levels, ODC activities, and SAMD activities in S49 cells treated with Bt_2cAMP. Their data differ from ours in that they found that polyamine levels had dropped in cells treated with Bt_2cAMP for 24 h. This difference may be attributable to the fact that they used wild

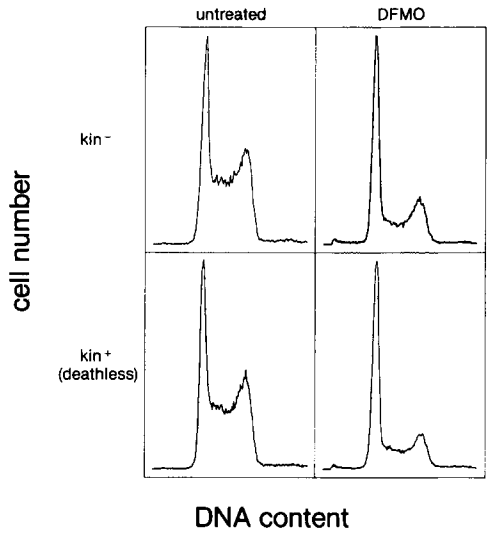

FIGURE 7 Effect of DFMO on the cell cycle distribution of kin^+ and kin^- S49 cells. Kin^- and deathless (kin^+) S49 cells were grown with and without 0.1 mM DFMO for 72 h and analyzed by FCM.

type S49 cells, whereas we used the cAMP deathless variant. This variant, although identical to the wild type in its growth response to DFMO (unpublished) and Bt_2cAMP (16), is resistant to the cytolytic effect of Bt_2cAMP. Interpretation of their results is also made difficult by the fact that their control cultures were neither growing exponentially nor maintaining stable ODC activities during the course of the experiments reported. Our results, however, support and extend their conclusion that, in viable cells, cAMP has little effect on spermidine pools.

We have tested two models that connect cAMP- and poly-

amine-mediated arrests of S49 cells: first, that cAMP arrest is mediated by lowering polyamine pools, and second, that polyamine arrest is mediated via cAMP-dependent kinase. Both models are incorrect. Therefore, the initial steps for arrest mediated by cAMP and polyamines are distinct.

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