Regulation of Growth of Mouse Mastocytoma Cells

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SUMMARY

N6,O2'Dibutyryladenosine cyclic 3',5'-phosphate plus theophylline inhibited the growth of the mouse mast cell tumor line PY 815 both in vivo and in vitro. The inhibitory effect on growth in vitro was rapidly reversed following removal of the drugs. Growth inhibition was accompanied by reduced cell surface activity and increased cell-cell adhesion. The drug-treated cells accumulated distinct membrane-bound granules, which are characteristic of more mature mast cells. Treated cells also developed increased amounts of surface-associated acidic mucopolysaccharides. These results suggest that increased intracellular cyclic adenosine 3':5'-monophosphate causes mouse mastocytoma cells to decrease growth and elicits the expression of a more differentiated mast cell phenotype.

INTRODUCTION

Recent evidence has implicated cyclic AMP and cyclic GMP in the regulation of events that control the cell cycle in eukaryotes (see, for example, Refs. 1 and 22). For example, an inverse relationship between cyclic AMP concentration and cell doubling time was reported by Otten et al. (28), while Sheppard (38) found that the cyclic AMP content in normal, density-inhibited 3T3 cells was twice that of spontaneously or virally transformed 3T3 cells. Dibutyryl cyclic AMP plus theophylline blocked mitosis in synchronized S phase populations of 3T3 cells (44) and slowed the growth of virally transformed cells, causing a partial phenotypic reversion of their morphology (38). Similar observations have been made on the effects of dibutyryl cyclic AMP and theophylline on transformed mouse fibroblasts (19), neuroblastoma (30), hepatoma (43), and melanoma (20) cells in culture. Overall, such research suggests that increased intracellular cyclic AMP slows growth and permits or induces cells to express their differentiated functions. We report here the effects of dibutyryl cyclic AMP and theophylline on the growth of the mouse mastocytoma cell line PY 815 both in vivo and in vitro. We have also examined the effect of m-AMSA on the cyclic AMP and ATP content of mastocytoma cells. This drug is a potential antileukemia agent (7), an immunosuppressive agent (3), and a potent inhibitor of the growth of PY 815 mastocytoma cells both in vivo and in vitro.

MATERIALS AND METHODS

Chemicals

Dibutyryl cyclic AMP was obtained from the Sigma Chemical Co., St. Louis, Mo. Theophylline was a product of Calbiochem, Los Angeles, Calif. All radiochemicals were purchased from the Radiochemical Centre, Amersham, England.

Cells

PY 815 mouse mastocytoma cells were passaged in vivo in DBA × C3H F1 hybrid mice by serial i.p. injection of approximately 10⁶ cells. In vitro mouse mastocytoma cells were grown in suspension in RPMI tissue culture Medium 1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal calf serum and buffered with bicarbonate. Cell suspensions were incubated at 37° in an atmosphere of 5% CO₂ and 95% air in a National CO₂ incubator. Cell densities were determined with a hemocytometer and with a Coulter counter. Cell viability was demonstrated by trypan blue exclusion.

Assays

For assaying cyclic AMP, cells were recovered by centrifugation (900 × g, 5 min) at 0°, washed 3 times at 0° with 10 ml phosphate-buffered saline (0.01 M phosphate buffer, pH 7.25:0.14 M NaCl:0.003 M KCl), and then extracted by the procedure of Cooper et al. (10). Cyclic AMP in the extracts was determined with the Radiochemical Centre cyclic AMP assay kit, using their standard procedure. ATP was assayed using the firefly luciferin-luciferase procedure devised by Addanki et al. (2), as modified by Stanley and Williams (39).
Microscopy

For examination by light microscopy (Reichardt), cells were stained with 5% Giemsa or with 0.5% Alcian Blue in 3% acetic acid, followed by 0.25% safranin O in 0.125 M hydrochloric acid specifically to stain surface acid mucopolysaccharides.

For electron microscopy, cells were fixed at 20°C in 6% glutaraldehyde in phosphate buffer (pH 7.5), isosmotic for animal tissues (24), and washed at 4°C several times with phosphate buffer. The cells were then either processed, stained with lead citrate:uranyl acetate, and embedded using standard procedures for staining and thin sectioning or freeze fractured using a standard type II freeze-fracture device (6). Thin sections and replicas were examined in a Phillips EM200 electron microscope.

Velocity Sedimentation

Four mice were each given i.p. inoculations of 10⁴ mastocytoma cells. Two days later, 2 of the mice were each given i.p. injections of [5- methyl-3H]thymidine (2 µCi; 26 Ci/m mole) in phosphate-buffered saline (0.2 ml), and the other 2 mice were given injections of [2-¹⁴C]thymidine (1µCi; 62 mCi/m mole) in phosphate-buffered saline (0.2 ml). After 1 hr, the latter 2 mice were given injections of 7.5 x 10⁻⁸ M dibutyryl cyclic AMP and 7.5 x 10⁻⁸ M theophylline (0.2 ml) to give estimated in vivo concentrations of 1 x 10⁻⁴ M and 1 x 10⁻² M, respectively. All 4 mice were sacrificed 5 hr after drug injection. The peritoneal cells were recovered in phosphate-buffered saline (3 ml) and cell densities were determined using the Coulter counter. Aliquots (2.5 ml) of cells from each mouse were pooled, and the mixture (10 ml; 1 x 10⁶ cells approximately) was placed on a 7 to 25% gradient of calf serum for velocity gradient fractionation, according to the procedure of Miller and Phillips (23). Ten-ml fractions were recovered from the gradient and a sample was taken from each fraction for determination of cell number with the Coulter counter. The remainder of the fractions was precipitated in 5% trichloroacetic acid and filtered onto Whatman GF/C glass fiber filters. After washing with ice-cold 5% trichloroacetic acid, the filters were dried and the ³H and ¹⁴C radioactivity associated with the individual filters were determined in a Packard liquid scintillation spectrometer. The results for each fraction were calculated and are expressed as percentage of radioactivity in the fraction relative to that in the fraction with maximum activity (see Chart 1). Corrections were made for slight variations in the size of the individual fractions collected.

Growth Studies

Cyclic AMP. Four identical suspension cultures of PY 815 mastocytoma cells were grown in RPMI Medium 1640 (20 ml) to a density of 1.2 x 10⁶ cells/ml. Dibutyryl cyclic AMP (10⁻⁴ M) and theophylline (10⁻² M) were then added to 2 of the cultures. After 26 hr further growth, the cells in each of the drug-treated and untreated cultures were recovered separately by centrifugation and resuspended in fresh medium containing 10% fetal calf serum with or without dibutyryl cyclic AMP (10⁻⁴ M) and theophylline (10⁻² M) to give final cell densities of 2.5 x 10⁶ cells/ml. Aliquots (2 ml) of the 4 resulting cell suspensions were then added to a series of small Petri dishes that were returned to the CO₂ incubator. Three dishes from each series were removed from the incubator after 1, 2, 3, and 4 hr, and the cell densities and mitotic indices were determined. The morphological characteristics of the cells were also examined by light microscopy (oil immersion), and the viability of the cells was checked by trypan blue exclusion. Viability was 95 to 100% at all times. Where necessary, cell numbers were corrected for resedimentation.

m-AMSA. To study the effects of the antileukemia drug m-AMSA on intracellular cyclic AMP and ATP of mastocytoma cells, the cells were seeded at 5 x 10⁶/ml in a series of flasks containing 20 ml of RPMI Medium 1640 supplemented with 10% fetal calf serum. After 3 doublings, 0.2 ml of m-AMSA solution (final concentration, 0.04 or 0.08 µg/ml) was added to triplicate flasks, and 0.2 ml sterile water was added to control cultures. Cell density was followed using a Coulter counter. Cyclic AMP and ATP were measured in extracts prepared from 8-ml aliquots of the cells at 4, 8, 15, and 25 hr after drug addition.

In separate experiments it was shown that 0.04 µg of m-AMSA per ml prevented cell replication but did not cause cell death, whereas 0.08 µg of m-AMSA per ml was toxic to about 30% of the cells.

Because it was obvious by light microscopy that cells treated with m-AMSA were much larger than were untreated cells, the size distribution of cells in control and drug-treated cultures was also examined by comparing 3 size classes arbitrarily differentiated by Coulter counter discrimination as 20 (total cell count), 60 (large cell count), and 80 (very large cell count).

The 80:20 ratio of cells was then expressed as a percentage to indicate increasing cell size.

RESULTS

In Vivo Growth Studies. The effect of dibutyryl cyclic AMP and theophylline on the growth of mastocytoma cells in vivo was examined by treating mice carrying peritoneal log phase mastocytoma cells first with [¹⁴C]thymidine for 1 hr to label S phase cells and then with dibutyryl cyclic AMP and theophylline for 5 hr. Cells from the treated mice were then removed and mixed with mastocytoma cells taken from similar mice that had been given [³H]thymidine for 1 hr but no drugs during the subsequent 5 hr, and the mixture (1 x 10⁶ total cells approximately) was fractionated by the velocity gradient sedimentation technique developed by Miller and Phillips (23) to compare cell sizes. This radioactive labeling and velocity gradient procedure has been used by Chin and Marbrook (personal communication) to determine the doubling time and cell cycle phases of mastocytoma cells in vivo. They found as follows: G1, 1.5 hr; S, 5 hr; G2 + M, 2.5 hr.
As shown in Chart 1, after 6 hr S phase cells that had incorporated [3H]thymidine for 1 hr had all passed through G2 and M to produce a relatively homogeneous population of smaller [3H]-labeled cells, whereas the drug-treated [3H]-labeled cells still mostly sedimented as a distinct population of larger cells, consistent with the interpretation that the drugs had blocked or delayed the division of the actively replicating S phase cells in vivo. There was no detectable loss of cell viability in either population of cells added to the original mixture prior to the fractionation (for exact details of the procedure, see "Materials and Methods")

In Vitro Growth Studies. Mastocytoma cells growing in suspension culture in RPMI Medium 1640 exhibit log phase growth over 3 to 4 days, and over this period they will multiply from a density of 10⁴ cells/ml to 1 to 2 x 10⁶ cells/ml, with an average cell cycle time of approximately 9 hr. At the latter density, cell multiplication ceases and a rapid decrease in cell density soon follows, due to cell lysis. The reduced growth rate observed at late log phase may partially reflect a growth-inhibitory effect due to increased cyclic AMP in the cells as serum growth factors become depleted from the medium (26), since we found that the cyclic AMP content of mastocytoma cells doubled at late log phase, while the ATP content of the cells decreased steadily throughout the period of growth. Results of such an experiment are presented in Table 1. In separate experiments, values for cyclic AMP of between 6 to 12 pmoles/10¹⁰ cells were found during early log phase, whereas late log phase cells contained 20 to 22 pmoles/10⁷ cells. Fluctuation in individual estimations did not exceed 20%. The protein content of treated cells increased during log phase growth from 120 to 180 μg/10⁶ cells, although there was no dramatic change in the distribution of cell sizes between normal and drug-treated cultures when cell size distribution was examined using the Coulter counter.

Addition of dibutyryl cyclic AMP (10⁻⁴ M) and theophylline (10⁻³ M) to early log phase mastocytoma cells growing in vitro caused a marked reduction in growth rate, and the average doubling time of the cells increased from 9 hr to about 18 hr (Chart 2A). The intracellular cyclic AMP content of the cells was elevated compared to control cultures, while at late log phase a further rise in cyclic AMP occurred (Table 1). The effect on growth was rapidly reversible, since mid-log phase cells, pretreated with the drugs for 26 hr and then transferred to fresh medium without drugs, grew immediately at the original uninhibited rate (Chart 2). However, transfer of the cells after 26 hr to fresh medium containing dibutyryl cyclic AMP and theophylline increased the growth inhibition. There was no major change in the number of mitotic cells after transfer of drug-treated cells to fresh medium with or without drugs, at least over the 1st 4 hr following transfer, suggesting that the cells were not all delayed in G2 just prior to mitosis.

Whereas fresh medium containing 10% fetal calf serum increased cell surface ruffling, blebbing, and the production of spidery outgrowths by mastocytoma cells, the addition of dibutyryl cyclic AMP and theophylline immediately "quieted" the membranes and decreased ruffling and blebbing. Electron micrographs of fixed and sectioned untreated log phase blebbing cells showed that the blebs contained mitochondria, endoplasmic reticulum, and ribosomes (Fig. 1a). In contrast, blebs on the surface of cells treated with dibutyryl cyclic AMP (5 x 10⁻⁶ M) and theophylline (10⁻³ M) for 72 hr appeared to be mostly filled with polyribosomes (Fig. 1b).

Microscopy. When untreated mastocytoma cells were stained with Alcian Blue and safranin O, 60 to 80% of the cells stained blue, indicating a preponderance of neutral surface mucopolysaccharides. However, 90% of the cells treated for 72 hr with dibutyryl cyclic AMP (10⁻⁴ M) and theophylline (10⁻² M) stained red with the same stain, suggesting that these carried increased surface acidic mucopolysaccharides (4, 9). Intermediate proportions of red- and blue-staining cells were observed at lower concentrations of dibutyryl cyclic AMP. The drug-treated cells appeared to clump more than did untreated cells, indicating increased adhesiveness.

Cells treated for 72 hr with dibutyryl cyclic AMP (5 x 10⁻⁶ M) and theophylline (10⁻³ M) were also examined by electron microscopy following thin sectioning or freeze fracture. After 72 hr of drug treatment, the surface contours of treated and untreated cells appeared similar in the electron microscope. However, the drug-treated cells had accumulated numerous progranules, which could be seen by freeze fracture to contain many membrane-bound particles (Fig. 2). Compaction of the progranules had also occurred to form mature mast cell-type granules. Mature granules were absent in untreated cells, although occasional progranules were seen (Fig. 3). There was no evidence of alterations in, or aggregation of, membrane proteins in the freeze fractures of drug-treated cells. Mitochondria packed with well-ordered cristae were a characteristic feature of the drug-treated cells.

Effects of m-AMSA. When log phase mastocytoma cells were treated with 0.04 μg of m-AMSA per ml, growth ceased and the cyclic AMP in the cells increased from 6 to 7 pmoles/10⁶ cells to 12 to 14 pmoles/10⁶ cells during the time (9 hr) required for untreated cells to double in number.
Table 1

Amounts of cyclic AMP and ATP in mastocytoma cells at various times during growth in culture

The initial inoculum contained 3 × 10⁴ cells/ml and drug additions were begun after 1 cell doubling had occurred.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time after drug addition (hr)</th>
<th>Cyclic AMP (pmoles/10⁶ cells)</th>
<th>ATP (pmoles/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no drugs)</td>
<td>4</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>12.8</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>11.8</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>67</td>
<td>19.2</td>
<td>53</td>
</tr>
<tr>
<td>+ Dibutyryl cyclic AMP</td>
<td>4</td>
<td>130</td>
<td></td>
</tr>
<tr>
<td>(10⁻⁴ M) and theophyll-</td>
<td>24</td>
<td>29</td>
<td>128</td>
</tr>
<tr>
<td>line (10⁻⁴ M)</td>
<td>48</td>
<td>20.2</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>67</td>
<td>40.8</td>
<td>61</td>
</tr>
</tbody>
</table>

* Cyclic AMP estimations were carried out in triplicate. Individual estimations of cyclic AMP fluctuated less than 20% about the mean.

cyclic AMP/cell between treated and untreated cultures was almost eliminated (control, 22 pmoles/10⁶ cells; treated, 25 pmoles/10⁶ cells). The percentage of larger cells in cultures treated with 0.04 µg of m-AMSA per ml increased up to a peak at 15 hr after commencing drug treatment (i.e., Coulter counter 80:20 ratio) but had decreased to normal after 24 hr culture. With 0.08 µg of m-AMSA per ml, the peak was attained after 8 to 9 hr and declined more slowly.

The ATP concentration increased from 285 pmoles/10⁶ cells to 1276 pmoles/10⁶ cells in cultures treated with 0.08 µg of m-AMSA per ml for 6 hr, and subsequently declined so that by 8 hr the values were 285 pmoles/10⁶ cells for control and 666 pmoles/10⁶ cells for treated culture. These results suggested that the effects of m-AMSA were not directly on cyclic AMP or ATP production but rather via inhibition of cell division and growth.

**DISCUSSION**

Combs (8, 9) has studied the development of mast cells in some detail. He found that embryonic mast cells were round, lymphocyte-like cells with large nuclei and high mitotic activity. The embryonic cells contained few granules, and those detected appeared to contain proteolytic enzymes but no histamine. The cells stained blue with Alcian Blue-safranin 0 combination, indicating a neutral outer mucopolysaccharide coat. In contrast, mature mast cells were not mitotically active and they contained numerous dense, compacted granules. The mature cells stained red with Alcian Blue-safranin O, indicating an acidic mucopolysaccharide surface. Combs also described 2 intermediate stages of differentiation in which a fine fibrous material amassed within progranules and subsequently coalesced to form compact granules containing histamine, heparin, and basic proteins. As noted in the results presented above, the action of dibutyryl cyclic AMP and theophylline on PY 815 mouse mastocytoma cells in culture slowed their growth and changed the morphological features of the cells so that they resembled more mature mast cells. Tomita et al. (40) have reported that dibutyryl cyclic AMP increases the histamine content of PY 815 mastocytoma cells 3- to 5-fold. Thus,
increased cyclic AMP permits further differentiation of mastocytoma cells, which, if untreated, tend to resemble immature lymphocyte-like mast cells. The drugs did not affect cell viability at the concentrations we selected, and the inhibitory effect of the drugs on growth was immediately reversed following transfer of cells to fresh medium without drugs. Thus, the effect of dibutyryl cyclic AMP and theophylline was not permanent but rather permissive. The mastocytoma cells clearly retained the capacity to respond to the cyclic AMP growth signal and consequently to express their differentiated function. Therefore, failure to mature to the differentiated state seems to lie in the absence or reduced production of the cyclic AMP signal. Our cyclic AMP determinations (see below) tend to suggest that reduced adenyl cyclase activity is involved. Altered cell membranes may affect adenyl cyclase activity and prevent the production of sufficient cyclic AMP to slow cell growth and permit cell maturation. Similar suggestions have been made by others. Nevertheless, the nature of the membrane changes must be relatively subtle since we could not detect any alteration in, or aggregation of, membrane proteins in freeze fractures of treated cells (cf. Refs. 14 and 34).

Puck et al. (17, 18, 29, 32, 33) have described the effects of dibutyryl cyclic AMP and hormones on cell adhesiveness and differentiation of Chinese hamster ovary cells. Several of the effects that they observed resemble those reported here, since their cells showed reduced membrane activity and greater differentiation in the presence of the drugs. The effect of cyclic AMP was also rapidly reversible; however, the drugs themselves did not decrease the growth rate of Chinese hamster ovary cells, although they altered cell morphology and reduced membrane activity (17, 18). The authors suggested that cyclic AMP increases and aligns microfilaments, thereby inducing the observed changes (29, 33).

We did not observe great morphological changes in the form of mastocytoma cells treated with cyclic AMP, other than effects on surface activity and cell–cell adhesiveness. In our electron micrographs there were no obvious accumulations of microtubules before or after drug treatment, although a more detailed study would be necessary before firm conclusions could be made. The various differences may be related to the culture methods, since the mastocytoma cells were grown in suspension whereas the Chinese hamster cells were grown on surfaces.

The cyclic AMP concentration in early log phase mastocytoma cultures was particularly low (6 to 12 pmoles/10^7 cells) compared, for example, with 3T3 cells (6 to 20 pmoles/10^6 cells) (34). The 20 pmoles/10^6 cells found in late log phase mastocytoma cells compared favorably with the same figure reported for mastocytoma cells by Henney et al. (16). After treatment with dibutyryl cyclic AMP and theophylline, the apparent cyclic AMP concentrations of treated cells doubled. However, our assay procedure does not distinguish between N^6-butyryl cyclic adenosine 3':5'-phosphate, the active metabolite in cells (21), and cyclic AMP, so that the “effective” concentration in drug-treated cells was probably composed of both compounds. Nevertheless, a mere doubling of the effective cyclic AMP level appears to suffice to slow growth and permits further maturation of mastocytoma cells in culture. This suggests that relatively slight perturbations of the production of cyclic AMP in cells have quite dramatic consequences for growth and differentiation. We concluded, from staining cells and from their altered adhesiveness (cf. Refs. 14 and 45), that the alteration of the cyclic AMP level in mastocytoma cells also changes the properties of the surface mucopolysaccharides. If the altered cell surface in its turn affected the activity of membrane-associated adenyl cyclase, an interlocked and coupled system would exist that might normally balance the production of cyclic AMP and thereby control growth. This control system would retain sensitivity to external signals through their interaction with the cell surface, while disturbing or disrupting the cell surface receptor(s) could destabilize the coupled system, leading to reduced cyclic AMP production and stimulated growth. Many of the known mitogens interact with or modify the polysaccharides of the cell surface and stimulate cell growth. For example, the mitogen lectin (41), neuraminidase and galactose oxidase (25), and trypsin (27, 37) all affect cell surface polysaccharides. In contrast, exogenous cyclic AMP is known to increase secretion of acidic mucopolysaccharides by 3T3 cells, and it decreases growth (13), possibly by restoring functional polysaccharides to the cell surface. These and numerous other observations suggest that, in the normal cell cycle, growth may be controlled through changes in carbohydrate metabolism, especially as they relate to the synthesis of cell surface polysaccharides. This certainly seems to be true for cells transformed by oncogenic viruses, where several enzymes concerned with the synthesis of cell surface polysaccharides have been shown to be altered by virus infection (5, 11, 12, 15, 31, 35, 36).

ACKNOWLEDGMENTS

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REFERENCES


Fig. 1. Electron micrographs of thin-sectioned mastocytoma cells. a, untreated log phase blebbing cells; b, late log phase cells after treatment with dibutyryl cyclic AMP (5 × 10⁻⁴ M) and theophylline (10⁻³ M) for 72 hr. Cells were stained for electron microscopy by conventional methods, using lead citrate:uranyl acetate. Bars, 1 μm. MITO, mitochondrion; CYTO, cytoplasm; N, nucleus; PR, polyribosomes. × 23,000.

Fig. 2. Electron micrographs of thin-sectioned and freeze-fractured mastocytoma cells cultured for 72 hr with dibutyryl cyclic AMP and theophylline. Bars, 0.5 μm. In a, thin section showing progranule (P) packed with “bags” (B) near an irregular surface (M). × 66,000. In b, thin section of another progranule (P) showing initial stages of compaction. M, irregular surface. × 66,000. In c, freeze fracture showing membrane-enveloped particles (arrow) within progranules. Cells were stained with lead citrate:uranyl acetate. × 60,000.

Fig. 3. Electron micrographs of late log phase mastocytoma cells cultured with or without dibutyryl cyclic AMP and theophylline. Bars, 1 μm. In a, untreated cell lacking progranules. In b, cells treated for 72 hr with drugs and showing accumulated cytoplasmic progranules (CG). N, nucleus; CYTO, cytoplasm. × 11,500.
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