Increased cellular levels of spermidine or spermine are required for optimal DNA synthesis in lymphocytes activated by concanavalin A

[methylglyoxal bis(guanylhydrazone)/cell proliferation]

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Communicated by Arthur B. Pardee, July 17, 1975

ABSTRACT There are large increases in cellular levels of the polyamines spermidine and spermine in lymphocytes induced to transform by concanavalin A. The anti-leukemic agent methylglyoxal bis(guanylhydrazone) (MGBG) blocks synthesis of these polyamines by inhibiting S-adenosylmethionine decarboxylase. Previous results showed that when cells are activated in the presence of MGBG the synthesis and processing of RNA, as well as protein synthesis, proceed as in the absence of the drug. In contrast, the incorporation of \( \text{[methyl}^3\text{H]} \) thymidine into DNA and the rate of entry of the cells into mitosis are inhibited by 60% in the presence of MGBG. Several experiments suggest that MGBG inhibits cell proliferation by directly blocking polyamine synthesis and not by an unrelated pharmacological effect: (1) the inhibitory action of MGBG is reversed by exogenously added spermidine or spermine; (2) inhibition of DNA synthesis by MGBG shows the same dose-response curve as does inhibition of spermidine and spermine synthesis; and (3) if MGBG is added to cells which have been allowed to accumulate their normal complement of polyamines, there is no inhibition of thymidine incorporation. MGBG-treated and control cultures initiate DNA synthesis at the same time and show the same percentage of labeled cells by autoradiography. Therefore, it appears that in the absence of increased cellular levels of polyamines, lymphocytes progress normally from G1 through S and into G2. Furthermore, these experiments suggest that the increased levels of spermidine and spermine generally seen in rapidly proliferating eukaryotic systems are necessary for enhanced rates of DNA replication.

Stimulation of the rate of biosynthesis of the aliphatic polyamines spermidine and spermine appears to be an invariable correlate of increased cell proliferation. Striking increases in the levels of the enzymes of polyamine biosynthesis and, to a lesser extent, enhanced cellular levels of these compounds have been observed in a variety of systems (1–5). Although the general phenomenon has been well described, and a variety of hypotheses have been advanced to explain the role of increased polyamine levels, few experiments have been designed to directly test the role of these compounds. Recently, with the discovery of specific inhibitors of polyamine synthesis, a new avenue of experimentation has become accessible. Williams-Ashman and Schenone demonstrated methylglyoxal bis(guanylhydrazone) (MGBG) to be a potent inhibitor of S-adenosylmethionine decarboxylase (6). More recently, Snyder and his coworkers have shown that \( \alpha \)-hydroxyornithine inhibits the conversion of ornithine to putrescine, the biosynthetic precursor of spermidine and spermine, both in vitro and in vivo (7, 8). If these inhibitors are specific for polyamine biosynthesis, and exhibit no other pharmacological actions, they will be powerful tools to examine the cellular function of the polyamines.

We have used MGBG to study the role of polyamines in lymphocytes which have been stimulated by concanavalin A (Con A) to grow and divide. MGBG rapidly brings about complete inhibition of the biosynthesis of spermidine and spermine when added to activated lymphocytes (9, 10). From the studies to date, it is clear that normal transcription and translation continue after polyamine accumulation is blocked with MGBG. We have therefore concluded that the increased cellular levels of polyamines seen in proliferating cells are not involved in protein synthesis or in the synthesis, processing, and accumulation of RNA (10). Hence, in defining a role for the enhanced polyamine levels, our attention has been directed to later events in the process of lymphocyte activation. Specifically, we have been examining the effects of MGBG on DNA replication and cell division (11). We demonstrate in this paper that blocking polyamine accumulation with MGBG leads to decreased rates both of \( \text{[methyl}^3\text{H]} \) thymidine incorporation into DNA and cell division. Evidence is presented which strongly argues that these effects of MGBG are due directly to inhibition of spermidine and spermine accumulation and not to a pharmacological action of MGBG unrelated to polyamine biosynthesis.

EXPERIMENTAL PROCEDURES

All procedures for the preparation and culture of lymphocytes from bovine suprathoracic lymph glands, for isolation and assay of DNA, and for polyamine analysis have appeared (10, 12, 13). For microscopic examination of the cells after colcemid treatment, about \( 1 \times 10^6 \) cells were concentrated by centrifugation, resuspended in 2 drops of newborn calf serum, and dispersed using a syringe with a 27 gauge needle. A sample was then fixed on a microscope slide with glacial acetic acid/95% ethanol (1:3 v/v) and stained with standard Wright's stain (Fischer Scientific Co.). For autoradiography after \( \text{[methyl}^3\text{H]} \) thymidine incorporation, the cells were washed, fixed as above, and exposed to Kodak nuclear track emulsion.

RESULTS

By 40 hr after the addition of Con A to bovine lymphocytes, the rates of DNA synthesis and cell division are nearly maximal. In the experiments shown in Table 1, MGBG was added at 12 hr after Con A, thus blocking spermidine and spermine synthesis before significant accumulation had taken place (ref. 10; Fig. 3). The rate of incorporation of \( \text{[methyl}^3\text{H]} \) thymidine into DNA was inhibited by about 60% in the presence of MGBG. This degree of inhibition was

Abbreviations: MGBG, methylglyoxal bis(guanylhydrazone); Con A, concanavalin A.

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identical when the concentration of exogenous thymidine was varied from 0.75 μM to 10 μM and was also unchanged by blocking endogenous thymidine synthesis with 10 μM amethopterin in the presence of hypoxanthine. The proportion of cells undergoing division, as measured by the appearance of mitotic figures in the presence of colcemid, was also decreased by 60%. On the other hand, the proportion of cells labeled with [methyl-3H]thymidine, as detected by autoradiography, did not significantly change in the presence of MGBG. Thus, the rate of thymidine incorporation per S-phase cell was reduced about 60%. Furthermore, inhibition of thymidine incorporation by MGBG remained the same between the time of initiation of DNA synthesis (24 hr) and 60 hr after stimulation with Con A (Fig. 1). It can also be seen that cultures initiate DNA synthesis at the same time in the presence or absence of MGBG. We conclude that MGBG does not alter the rate of entry of activated lymphocytes into S-phase, but inhibits the rate of DNA replication. This leads to a prolongation of S-phase and the observed decrease in number of cells entering mitosis.

In order to relate these effects of MGBG on DNA synthesis and cell division to inhibition of polyamine synthesis, it was necessary to rule out other pharmacological effects of MGBG unrelated to polyamine synthesis. We have taken three experimental approaches to this problem of drug specificity: (a) we have asked whether the effects of MGBG could be reversed by exogenously added spermidine; (b) we have tested the effect of MGBG on cells which have accumulated their full endogenous complement of polyamines; and (c) we have compared the dose-response curves for inhibition of polyamine accumulation and of [methyl-3H]thymidine incorporation.

The first approach, addition of exogenous polyamines, suffers from a technical difficulty. Many sera, among these the newborn calf serum used in our culture medium, contain high levels of an amine oxidase active on spermidine and spermine (14). The oxidized products of the polyamines are known to be toxic (15-17) and it was, therefore, not surprising to find that addition of spermidine or spermine to our standard culture medium resulted in rapid cell death. However, horse serum contains low levels of polyamine oxidase (17, 18) and replacement of newborn calf serum in our culture medium with horse serum allowed the experiment described in Fig. 2. When MGBG was added 12 hr after Con A and [methyl-3H]thymidine incorporation was measured from 36 to 39 hr, typical inhibition was observed. Addition of exogenous spermidine or spermine at 24 hr after Con A produced essentially complete reversal of the inhibition of [methyl-3H]thymidine incorporation.

Table 1. Influence of MGBG on [3H]thymidine incorporation and cell division

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<thead>
<tr>
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<th>-MGBG</th>
<th>+MGBG</th>
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<tbody>
<tr>
<td>[3H]Thymidine incorporation*</td>
<td>27,600</td>
<td>11,100</td>
</tr>
<tr>
<td>Labeled cells†</td>
<td>28%</td>
<td>26%</td>
</tr>
<tr>
<td>Mitotic figures‡</td>
<td>2.3%</td>
<td>0.9%</td>
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</table>

* Counts per min per μg of DNA; 2 hr incorporation [3H]thymidine (6.7 Ci/mmol, 5 μCi/ml) beginning at 48 hr after Con A addition. The control in the absence of Con A gave 500 cpm/μg.
† Percent of total cells; 2 hr pulse of [3H]thymidine beginning at 48 hr after Con A addition.
‡ Percent of total cells; 3 hr of colcemid (0.2 μg/ml) beginning at 42 hr after Con A addition.
§ 8 μM MGBG added 12 hr after Con A.

If MGBG was added at the same time as [methyl-3H]thymidine, rather than 12 hr after Con A as above, no inhibition of incorporation was observed. Fig. 3A shows the time course of acquisition of resistance to MGBG, when [methyl-3H]thymidine incorporation was measured from 45 to 47 hr after Con A. MGBG inhibition of thymidine incorporation was maximal when the inhibitor was added at early times. Beginning at 18 hr inhibition became progressively less and addition of MGBG after approximately 40 hr resulted in no inhibition of thymidine incorporation. This behavior coincided with the accumulation of spermidine and spermine (Fig. 3B), and suggests that MGBG becomes less effective as intracellular polyamines accumulate.

The last piece of evidence in support of the specificity of MGBG action is presented in Fig. 4, where dose-response
FIG. 3. Comparison of the influence of the time of MGBG addition on inhibition of [methyl-3H]thymidine incorporation (A) with the accumulation of polyamines (B). Con A was added to all cultures at zero time. MGBG was added at the indicated times (A) and all cultures were labeled with [methyl-3H]thymidine (6.7 Ci/mm, 5 μCi/ml) from 45 to 47 hr. Thymidine incorporation is presented as percent of maximal incorporation, with 100% equal to 29,500 cpm/μg of DNA. The data on polyamine accumulation (B) were taken from ref. 10. MGBG was added at 2 μM.

curves for MGBG inhibition (added at 24 hr after Con A) of both [methyl-3H]thymidine incorporation and spermidine accumulation are illustrated. Both curves are similar, displaying half-maximal responses at roughly 0.2 μM MGBG, and maximal inhibition at 2 μM. Similar results were obtained for spermine accumulation (19). S-adenosylmethionine decarboxylase from bovine lymphocytes was also inhibited by MGBG over this same concentration range, with half-maximal inhibition at 0.2 μM (19).

DISCUSSION

Two potential problems arise in attempting to interpret these results. The first, which was dealt with above, concerns the question of specificity of MGBG action. Three results taken together argue that the action of MGBG on DNA synthesis, at the concentrations used, is mediated solely through inhibition of polyamine synthesis: (1) reversal by exogenously added polyamines; (2) diminished inhibition as endoge-

ous polyamines accumulate; and (3) similar dose-response curves for inhibition of polyamine accumulation and of [3H]thymidine incorporation. Thus, we interpret the inhibition of [3H]thymidine incorporation by MGBG to be an accurate reflection of intracellular polyamine function. A second problem lies in the interpretation of the 60% inhibition of thymidine incorporation. One interpretation is that this reflects an inhibition of DNA synthesis. On the other hand, a change in the characteristics of labeling of the dTTP pool by exogenous [3H]thymidine in the presence of MGBG could easily produce 60% inhibition of incorporation. These two possibilities cannot be distinguished simply on the basis of [3H]thymidine incorporation into DNA. However, two results favor the former interpretation. First, increasing exogenous thymidine more than 10-fold from our usual concentration (0.75 μM) to 10 μM does not alter the extent of MGBG inhibition of incorporation. Second, treatment with amethopterin, which would cause a drastic reduction in the intracellular dTTP pool, produces no effect on inhibition of exogenous thymidine incorporation by MGBG. Furthermore, the finding that the entry of cells into mitosis is inhibited to an identical extent as inhibition of [3H]thymidine incorporation, taken together with the fact that the cultures initiated DNA synthesis at the same time in the presence or absence of MGBG is consistent with a prolongation of S-phase and hence inhibition of DNA replication.

The interpretation of the physiological effects of MGBG is complicated by continued accumulation of putrescine in the presence of the inhibitor (9, 10). At the time of initiation of DNA synthesis, 24 hr after addition of Con A, the anticipated cellular level of putrescine would be 2- to 3-fold higher than in the absence of MGBG. One cannot rigorously exclude either positive or negative effects of this increased level of putrescine on DNA synthesis. Thus, the 40% residual rate of thymidine incorporation observed in the absence of spermidine and spermine accumulation could be due to these elevated levels of putrescine (see below). Conversely, it might be argued that the excess cellular putrescine in the presence of MGBG inhibits DNA synthesis. Very clearly, these potential effects of putrescine on DNA synthesis should be investigated, for example, using an inhibitor of putrescine biosynthesis (see Introduction).

A possible involvement of polyamines in DNA synthesis has been suggested from experiments in prokaryotic systems. In putrescine-deficient mutants of Escherichia coli, there appear to be abnormalities in both chromosome replication (20) and in the synthesis of phage DNA (21). In a cell-free system from the same organism, Kornberg and his colleagues have found a striking stimulation of phage DNA replication by spermidine (reviewed in ref 22), thus providing even stronger evidence for a role of polyamines in this process.

Given the interpretation that blocking spermidine and spermine accumulation with MGBG leads to inhibition of DNA replication, what does this tell us about the role of polyamine increases in proliferating tissues? As indicated in the Introduction, stimulation of the rate of polyamine biosynthesis has been observed in all proliferating systems that have been examined to date. On the basis of the data presented here, we would propose that these increased polyamine levels are necessary for optimal DNA synthesis and could conceivably play a role in the regulation of the progression of a cell through S-phase. The fact that cultures initiate DNA synthesis at the same time in the presence or absence of MGBC indicates that the progression of cells from
G₀ through G₁ and into S-phase is not affected by blocking the synthesis of spermidine and spermine. This is consistent with our previous conclusion that spermidine and spermine accumulation in this system is unnecessary for normal RNA and protein synthesis (10). A point which needs further comment is that DNA replication is inhibited by only 60% in the complete absence of spermidine and spermine accumulation. One interpretation of this finding is that polyamines are not absolutely required for DNA synthesis in mammalian cells. Another possibility is that the basal levels of polyamines present in unstimulated cells are sufficient to allow DNA synthesis to proceed at 40% the normal rate. Either of these interpretations imply that polyamine levels cannot be playing an absolute regulatory role in initiating or terminating DNA synthesis, but can only modulate the rate of progression through S-phase in lymphocytes by a little more than 2-fold. A third possibility arises from the fact that putrescine continues to accumulate at a greater than normal rate in the presence of MGBG (see above). Thus, at 48 hr after Con A addition, the total micromoles of cellular putrescine, spermidine, and spermine are similar in the presence and absence of MGBG, although the relative proportions are radically altered by the inhibitor (9, 10). It is possible that the expanded cellular levels of putrescine could fulfill the proposed function of spermidine and spermine in DNA replication, albeit at 40% efficiency. The lower efficiency of putrescine would presumably be due to the different spatial orientation of positive charge in this molecule as compared with spermidine and spermine. If this is the case, blocking the synthesis of putrescine, and hence of spermidine and spermine, should have much more profound effects on DNA replication than those observed with MGBG. Thus, the possibility is still open that polyamines may be playing an all or none role in the regulation of DNA synthesis in proliferating eukaryotic cells.


This work was supported by a research grant (GM13997) and a training grant (GM00052) from the National Institute of General Medical Science and by a research grant from the National Science Foundation (BMS75-05581). Support was also received from the University of Washington institutional grant from the American Cancer Society and the University of Washington Graduate School Initiative 171 Funds. We would like to thank Drs. Richard Palminteri and Joyce C. Knutson for comments on the manuscript, and Dr. A. Button and P. D. and J. Meats for supplying the lymph glands.