Polyamines and Biosynthetic Enzymes in the Rat Intestinal Mucosa and the Influence of Methylglyoxal-bis(guanylhydrazone)1

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ABSTRACT

The use of methylglyoxal-bis(guanylhydrazone) (MGBG) in the clinical treatment of myeloid and lymphoid disorders has been limited by severe host toxicity to renewing tissues, particularly the intestinal mucosa. Since the drug is a potent inhibitor of spermidine biosynthesis, the distributions of ornithine and S-adenosylmethionine decarboxylases and polyamine pools have been characterized in the rat intestinal mucosa in an attempt to discern the basis for MGBG toxicity. A method of epithelial cell isolation in which fractions of cells are sequentially collected in a villus tip-to-crypt gradient was used. Ornithine decarboxylase activity was highest in the villus tip region and unexpectedly lowest in the crypts, while S-adenosylmethionine decarboxylase activity showed the opposite pattern. Intracellular polyamine pools were uniform along the gradient corresponding to the villus length and increased appreciably in the crypt region. The relative concentrations of the individual polyamines were highest in the crypts, with spermidine and spermine being nearly equivalent in all regions. Twenty-four hr after a single i.p. injection of MGBG (50 mg/kg), S-adenosylmethionine decarboxylase activity increased markedly, especially in the crypt region (~50-fold), while ornithine decarboxylase activity also increased but to a lesser extent. Putrescine pools were most affected by MGBG and were elevated 5- to 6-fold, especially in the crypt region. The results are consistent with an alteration of polyamine biosynthesis by MGBG being involved in the antiproliferative toxicity of the drug.

INTRODUCTION

The clinical use of MGBG4 in the treatment of myeloid and lymphoid proliferative disorders has been limited by profound toxicities though the proliferating tissues of the patient. Gastrointestinal toxicity and bone marrow depression are among the most dramatic side effects (21). At present, the molecular basis for the antiproliferative action of MGBG is not understood. It is known that both the antileukemic action and the antiproliferative toxicities can be prevented in animals by the simultaneous administration of the polyamine, spermidine (19, 20). This prevention is most probably a consequence of competition by spermidine for uptake of MGBG since both share the same carrier (6, 8). Alternatively, it may represent a restoration of intracellular spermidine pools depleted by MGBG which is a potent inhibitor of AMeDC, a key enzyme in the biosynthesis of spermidine and spermine (4, 33).

In order to further evaluate the relationship between MGBG and spermidine, polyamine biosynthesis has been examined in the intestinal epithelium of untreated and MGBG-treated rats. Intestinal epithelial cells were isolated nonenzymatically from the mucosa by a method devised by Weiser (32). It allows for the sequential elution of epithelial cells as a villus tip-to-crypt gradient that corresponds to their location along the villus and hence to their stage of differentiation. Thus, in addition to probing the basis for MGBG toxicity in the small intestine, this study offers an added opportunity to investigate the role of polyamines and their biosynthetic enzymes in the maturation process of intestinal epithelial cells.

MATERIALS AND METHODS

Materials. MGBG was obtained as the dihydrochloride salt from the Drug Development Unit, National Cancer Institute, Bethesda, Md., and was verified to be 99% pure by thin-layer chromatography. Mature Sprague-Dawley rats weighing 300 to 400 g were given a single i.p. injection of either sterile 0.85% NaCl solution or MGBG in 0.85% NaCl solution at a dose of 50 mg/kg. After 12 to 24 hr, control and MGBG-treated rats were sacrificed by cervical dislocation, and the entire small intestine was excised.

Isolation of Intestinal Epithelial Cells. The intestine was treated exactly as described by Weiser (32) except that 10 rather than 9 cell fractions were collected following incubation times of 4, 2, 2, 3, 4, 5, 7, 10, 10, and 10 min with the eluting buffer. The technique relies on dissociation with citrate and removal of cells in the presence of buffered 1.5 mM EDTA. No degradative enzymes were used. The 10 cell fractions obtained form a villus tip-to-crypt gradient of epithelial cells representative of the intestinal mucosa.

Measurement of Alkaline Phosphatase and Sucrase Activities. In order to ensure that the methodology for sequential elution of epithelial cells was functioning as expected in these experiments, the brush border marker enzymes, sucrase and alkaline phosphatase, were measured on the cell fractions eluted from the intestine of an untreated rat. Sucrase was assayed as described by Dahlqvist (5), and alkaline phosphatase was assayed as described by Weiser (32). Protein deter-
mensions were performed according to the method of Lowry et al. (16) on aliquots of cells having known cell densities.

Measurement of ODC and AMeDC Activities. Cell fractions taken from the intestinal mucosa of untreated and MGBG-treated rats were washed twice in PBS, and 10^7 cells were removed for determination of ODC and AMeDC activities. The cells were pelleted, dried gently with a cotton swab, and resuspended in 0.4 ml of buffer containing 25 mM Tris (pH 7.5), 0.1 mM EDTA, and 5 mM dithiothreitol at 4°C. The cell suspensions were then quick frozen in a dry ice- acetone bath for 1 min and warmed to 37°C for 1 min 3 times. The freezing and thawing was repeated twice. The broken-cell suspension was centrifuged at 12,000 x g for 3 min using a microcentrifuge (Brinkman Instruments, Inc., Westbury, N. Y.). The supernatant served as the enzyme source for the following assays.

The methods used for the determination of ODC and AMeDC activity rely on the quantitation of catalytically released carbon dioxide from ornithine in the presence of pyridoxal phosphate as described by Heby and Russell (10) or from S-adenosylmethionine in the presence of putrescine as described by Pegg and Williams-Ashman (27). For measurement of ODC activity, 50 µl of cell extract were added to 150 µl of incubation mixture containing 0.2 mM pyridoxal phosphate, 5 mM dithiothreitol, 100 mM glycylglycine buffer (10 mM glycylglycine, 5 mM EDTA, 5 mM dithiothreitol, and distilled water to 100 ml, pH 7.2), and 1 mM 32P-ornithine with 32P-[1-14C]ornithine (58 mCi/mmol; New England Nuclear, Boston, Mass.). The incubation mixture for AMeDC contained 2.5 mM putrescine, 5 mM dithiothreitol, 100 mM sodium phosphate buffer, and 0.2 mM S-adenosyl-L-methionine with S-[carboxyl-14C]adenosyl-L-methionine (51 mCi/mmol; New England Nuclear). Reactions were carried out with continuous shaking for 1 hr at 37°C in 18- x 102-mm glass tubes each fitted with a rubber stopper and a disposable polypropylene center well (Kontes Co., Vineland, N. J.) containing 100 µl of 0.1 N NaOH. The reaction was terminated with an injection of 500 µl of 20% H2SO4 through the rubber stopper into the reaction mixture using a 22-gauge needle attached to a 1-ml syringe. The tubes were incubated for an additional 30 min to trap remaining carbon dioxide. The contents of the center well were neutralized with 100 µl of 0.1 N HCl, and the entire well was plunged into a scintillation vial containing scintillation cocktail [5.74 g of Permablend (Packard Instrument Co., Downers Grove, Ill.) per liter of toluene containing 15% by volume of BBS-3 (Beckman Instrument, Inc., Fullerton Calif.). Vials were counted in a Packard Model 3320 scintillation counter.

Determination of Intracellular Pools of Polyamines. The cell fractions representing the intestinal mucosa from drug-treated or untreated rats were analyzed for polyamine content by HPLC. Cell samples were washed twice in cold PBS, and an aliquot of 10^7 cells was removed for polyamine determination. The cells were pelleted, and the PBS supernatant was carefully removed with a cotton swab. The remaining cell pellet was extracted with 0.5 ml of 0.6 M perchloric acid for 30 min at 4°C and then centrifuged for 3 min at 12,000 x g using a microcentrifuge. The supernatant was frozen at -70°C until analysis by HPLC. Polymamines in a 1 50-µl sample of the perchloric acid extract were separated on an HPLC assembly containing a 2.8 mm-diameter glass microbore column with a 2-cm column height packed with cationic exchange resin DC-4A (Durrum Chemical Corp., Palo Alto, Calif.). The chromatogram was developed at 500 lb/sq in with a flow rate of 8 ml/hr using a sodium borate buffer system. The eluate containing amines was mixed with 0.05% o-phthalaldehyde (Durrum Chemical Corp.) in 0.4 M borate buffer (pH 10.4) containing 1 mM 2-mercaptoethanol and 0.9% Brij 35. The o-phthalaldehyde reagent was also pumped at 8 ml/hr. The mixture was passed through a flow cell of a Fluoro-Monitor (American Instrument Co., Silver Spring, Md.). Its fluorescence was measured by activating it with UV passing through a primary filter (Corning No. 7-51) and recording the emitted light response through a secondary filter (Watten No. 8) in a Hewlett-Packard Model 3385A Automation System. A typical chromatogram of polymamines representing a known mixture of putrescine, spermidine, and spermine hydrochlorides is shown in Chart 1. The percentage of error for such standards is less than 5%. The sensitivity of the HPLC system is in the range of 0.1 nmol/50-µl sample.

RESULTS

It had been shown previously (32) that the sequential elution methodology isolates only epithelial cells and excludes the cells of the serosa and interstitium. The cells were collected in 10 fractions beginning with those from the villus tips (Fractions 1 to 3) and ending with the cells of the crypt region (Fractions 7 to 10). By light microscopy, the cells appeared to be singular and morphologically intact. Those in Fractions 9 and 10 had a tendency to aggregate if allowed to sit and had to be agitated prior to counting to ensure single-cell suspensions. Viability by trypan blue exclusion was approximately 90% for all cell fractions from either untreated or MGBG-treated rats.

The effectiveness of the methodology for separating villus from crypt cells was confirmed by the distribution of sacrose and alkaline phosphatase among the cell fractions. The maximum enzyme activity for both enzymes was located in Fractions 1 and 2 and declined progressively in subsequent fractions. This is consistent with the findings of others (1, 31, 32) and with the concept that these enzymes are associated with the brush border of the columnar cells located at the villus cells. The undifferentiated cells located in the crypt are essentially lacking in these enzymes. Similar gradients were obtained using animals treated with MGBG.

Both polyamine and enzyme data obtained from the eluted cell gradient of the intestinal mucosa can be represented either according to the gradient fraction or according to the percentage of total gradient protein present in each fraction. The former is more convenient for comparison of drug-treated
versus untreated animals while the latter gives a more accurate indication of the actual location of enzyme or polyamine distribution in the intestinal mucosa. This is because each gradient fraction contains a different number of cells and hence a different amount of protein. For example, approximately 75% of the total protein is removed in the first 5 of 10 gradient fractions. Accordingly, polyamine and enzyme data for control animals are presented in both ways (Charts 2 and 4) while data for MGBG-treated animals are presented as gradient fractions to facilitate comparisons (Charts 3 and 5).

The activities for the 2 polyamine-biosynthetic enzymes showed a paradoxical distribution along the villus tip-to-crypt cell gradient (Chart 2). ODC activity was highest in the villus tip region and fell steadily, being one-fifth to one-sixth of that of the cells of the crypt region. In contrast, AMeDC activity was lowest at the villus tip and highest in the crypt region, differing at the extremes by a factor of 5 to 6. In both cases, the enzyme activity gradient was nearly linear along the cell gradient so that the 2 median activities for both AMeDC and ODC tended to occur in Fractions 4, 5, or 6 (Chart 2A). This median point is located closer to the crypt region when the enzyme activities are plotted according to percentage of total protein (Chart 2B).

The possibility that the nonparallel distribution of ODC activity with respect to AMeDC activity was due to degradation of the former enzyme by hydrolytic enzymes was considered. AMeDC would be protected by the presence of putrescine inherent in the tissue and in the incubation medium. The inclusion of 20 μM pyridoxal phosphate in the eluting buffer resulted in a sizable increase in overall ODC activity (Chart 3A), but the asymmetric distribution along the gradient was still preserved. AMeDC activity was unchanged under these conditions (Chart 3B).

Following treatment with a single i.p. injection of MGBG (50 mg/kg) for 24 hr, the ODC activity increased by about 2-fold in all cell fractions, and the same gradient was essentially maintained (Chart 4A). The effect of MGBG treatment on AMeDC activity was considerably more dramatic (Chart 4B). In the villus region (Fractions 1 to 3), there was a 20- to 50-fold increase in enzyme activity, and the gradient in enzyme activity was lost. As in control cell fractions, the crypt region contained the greatest AMeDC after MGBG treatment. It increased progressively from Fraction 7, the beginning of the crypt region, to Fraction 10 where a maximum activity was found. In one animal treated for only 12 hr with MGBG (50 mg/kg), the increases in enzyme activities were intermediate between control and 24-hr-treated animals.

HPLC analysis of intracellular polyamine pools gave good separation of the 3 species and high reproducibility (see Chart 1). The column recovery of radioactive polyamine standards added to cell fractions prior to precipitation by perchloric acid was 80 to 85% of the total radioactivity added. When standards were added to the perchloric acid extract of cell fractions, recovery increased to 90 to 95%.

The intracellular polyamine pools for the epithelial cells in untreated animals were quite similar throughout most of the villus cell fractions (Chart 5), but increased appreciably in the
crypt region. The individual polyamine pools essentially paralleled one another throughout the gradient. The spermine and spermidine levels of the intestinal epithelia were similar and consistently higher than those of putrescine. Treatment with MGBG failed to alter the spermidine or spermine pools but increased those of putrescine by about 3-fold in the villus region and up to 5- to 6-fold in the crypt region (Chart 6). This latter effect by MGBG paralleled its effect on AMeDC activity (Chart 4).

**DISCUSSION**

The epithelial cells of the intestinal mucosa undergo a morphological segregation as they migrate from the crypt region to the villus tip. Those located at the bottom of the crypts are mitotically active but poorly differentiated. These multipotent cells give rise to villus columnar mucous, enteroendocrine and Paneth cells which, excepting the Paneth cells, experience a differentiation process as they move towards the villus tip (3). The most common of these, the columnar cells, reach full maturity at the upper third of the villus and are eventually shed into the lumen of the intestine. In the rat, this migration and maturation process requires 36 to 72 hr (15). Weiser (32) has developed a nonenzymatic means for isolating these various cells in a gradient representative of their location along the villus or crypts of the intestinal mucosa. That this sequential elution methodology does in fact reflect an *in situ* villus tip-to-crypt cell gradient has already been established (32). Most convincing in this regard is the distribution of the marker enzymes of the various epithelial cell types and the time-dependent progression of[^H]thymidine along the gradient in a pattern comparable to that observed by autoradiography (2).

In this study, this methodology has been used to characterize polyamine biosynthesis and distribution in the epithelial cells of the intestinal mucosa as a villus tip-to-crypt gradient of differentiation. Of the various parameters examined, the distribution of the 2 polyamine-biosynthetic enzymes, ODC and AMeDC, was the most striking. ODC, the enzyme which catalyzes the biosynthesis of putrescine from ornithine, is located mainly in the mature, nondividing cells of the villus tip. This distribution is not altered by stabilization of the enzyme during cell isolation procedures with pyridoxal phosphate. The enzyme activity in the crypts is negligible, being one-fifth to one-sixth of that detected in the villus tips. This finding confirms an earlier report by Baylin et al. (1), who found that both ODC and diamine oxidase (histaminidase) were highest in this same area. This distribution is unexpected since high levels of ODC have always been associated with rapidly proliferating cell populations (13) such as those of the crypt region. In particular, increases in ODC activity have been correlated with increases in DNA, RNA, and protein synthesis. In the intestinal mucosa, however, no such correlations seem to exist since DNA synthesis is confined to the cells of the crypts (2, 15, 32), maximum protein synthesis...
Chart 6. Effect of a single i.p. injection of MGBG (50 mg/kg) after 24 hr on the intracellular polyamine levels. Data obtained from samples taken from the same gradients used for enzyme assays (Chart 4). Control data for this experiment are presented in Chart 5.

occurs in the midgradient region, and presumably maximum RNA synthesis occurs proximal to that region. Rather, the gradient of enzyme activity seems to correlate with morphological and functional differentiation of the epithelial cells as they migrate along the villus walls. Baylin et al. (1) have raised the possibility that the high levels of ODC found in rapidly proliferating tissues could be more involved with rapid maturation of recently formed cells rather than with actively proliferating ones. On this basis, one would not expect inhibitors of ODC such as α-difluoromethylornithine (17, 18) to inhibit cell proliferation in the crypts but rather their maturation.

The other polyamine-biosynthetic enzyme, AMeDC, is a key enzyme in the synthesis of spermidine and spermine. The aminopropyl group generated by decarboxylation of S-adenosylmethionine is subsequently transferred to putrescine and spermidine to give spermidine and spermine, respectively. The latter reactions are accomplished by synthases. When compared with ODC, the distribution of AMeDC activity in the intestinal mucosa seems paradoxical. It assumes an enzyme activity gradient which is opposite to that for ODC, being 5 to 6 times higher in the crypt region and decreasing progressively along the villus tip-to-crypt gradient. The distribution of AMeDC activity is consistent with a requirement for spermidine in cell proliferation. However, since it is highest in the regions where ODC activity is lowest, the source of putrescine for spermidine synthesis is an enigma. Conceivably, the ODC distribution may be related to that of diamine oxidase which is similar (1).

The distribution of the various intracellular polyamine pools along the villus tip-to-crypt gradient was essentially uniform except for an increase in the crypt region. The spermidine: spermine ratio of about 1 was somewhat surprising especially since it persisted all along the gradient. It was expected that the ratio would increase markedly in the crypts where cell proliferation occurs and decrease in the villus region where cells become differentiated (29). The fact that the spermidine: spermine ratio is comparatively low may reflect the age of the rats used (~10 months), since Janne et al. (14) have found that, in the small intestine, this ratio is about 1.7 at 3 months and decreases with age. Overall, the polyamine pools failed to correlate with the biosynthetic enzymes or with diamine oxidase, a degradative enzyme for putrescine which has been localized to the villus tip region (1). These findings imply that there is no major shift in polyamine levels of intestinal epithelial cells during either cell proliferation or differentiation in the intestinal mucosa. However, these values represent total cellular polyamine pools and do not recognize possible compartmental shifts in polyamines related to these cell processes. The lack of correlation between polyamines and the enzymes may again be related to the availability of free polyamines to these cells by absorption from the intestinal contents.

The effects of MGBG on the polyamines and their biosynthetic enzymes were entirely as predicted from other systems. Both AMeDC and ODC activities were elevated by drug treatment. The profound increase in AMeDC activity has been noted in a number of tissues exposed to MGBG and is known to be partly attributable to an extended enzyme half-life as a consequence of MGBG binding (7, 9, 11, 12, 26) and partly to enhanced enzyme synthesis (7). The molecular basis for increases in ODC (11, 12) is less well understood but may relate to a feedback mechanism relevant to increases in putrescine pools. The increase in putrescine levels reflects MGBG inhibition of AMeDC activity and enhancement of ODC activity. Similar findings have been noted in liver, kidney, prostate, and thymus of adult rats (12, 25, 26). That there does not occur any significant diminution in intracellular spermidine and spermine concentrations is in line with the remarkably slow rates of degradation of these polyamines once they have been synthesized in vivo (30).

It is interesting that the greatest effects of MGBG with respect to increased polyamine pools and AMeDC activity occurred primarily in the crypt region of the mucosa. This is consistent with the antiproliferative properties of the drug and with pathological findings in rats treated with MGBG (22). Specifically, animals treated for 24 hr with MGBG (120 mg/kg) show a suppression of mitosis in the crypt region.

Recent evidence suggests that MGBG may have more than one site of action in the cell (24). In particular, it has been shown that MGBG at therapeutic doses causes profound ultrastructural and functional damage to the mitochondria of L1210 cells treated in vivo or in culture (24, 28) and that this damage is dependent on cell proliferation (23). Thus, a direct causation for antiproliferative activity by inhibition of polyamines cannot be inferred. Moreover, it has been found recently (17, 18) that inhibitors of polyamine biosynthesis which lack other intracellular sites of action, such as α-methylornithine and α-difluoromethylornithine, tend to be cytostatic in action. By contrast, MGBG is known to be cytotoxic, again suggesting the significance of a second intracellular site of action, possibly the mitochondrion. In this regard, MGBG may resemble a combined drug regimen such that one drug action, that involving polyamines, halts cells in cycle and the second drug action, that involving mitochondria, results in cytotoxicity.

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