Correlation of Ultrastructural and Functional Damage to Mitochondria of Ascites L1210 Cells Treated in Vivo with Methylglyoxal-bis(guanylylhydrazone) or Ethidium Bromide

Carl W. Porter, F. Mikles-Robertson, D. Kramer, and C. Dave

Department of Experimental Therapeutics, Grace Cancer Drug Center, Roswell Park Memorial Institute, New York State Department of Health, Buffalo, New York 14263

ABSTRACT

Mitochondria of cultured L1210 leukemia cells undergo extensive swelling and structural disruption when exposed to the anticancer agent, methylglyoxal-bis(guanylylhydrazone) (MGBG). Similar damage has now been observed in ascites L1210 cells treated in vivo with a single dose (>50 mg/kg) of either MGBG or ethidium bromide (EB). After 24 hr, the mitochondria of treated cells swell significantly, lose their inner structure, and, in the case of EB treatment, develop numerous electron densities within the matrix. Analysis of the ribonucleotide pools of these cells by high-pressure liquid chromatography reveals that treatment with either MGBG or EB depletes the adenosine triphosphate pools and markedly reduces the overall adenylate energy charge of the cell. Thus, in addition to being structurally damaged, the mitochondria are functionally impaired by both drugs. When MGBG-treated cells are harvested, washed, and placed in untreated mice, all cells recover near to normal ultrastructure after 48 hr, whereas EB-treated cells do not, even after 96 hr. Analysis of the ribonucleotide pools of the MGBG-treated cells indicates that the mitochondria have recovered their functional capabilities as well. The adenylate energy charge for these cells is essentially the same as that for untreated cells. If allowed to remain in the untreated mice, the cells previously treated with MGBG display unaltered leukemogenicity, killing the animals at nearly the same average time as untreated cells. The significance of the contribution of MGBG-induced mitochondrial damage to the antiproliferative action of the drug and its relationship to inhibition of spermidine and spermine biosynthesis by the drug remains to be established.

INTRODUCTION

Bisguanylhydrazones are a group of compounds in which common terminal amidine groups are separated by variable aliphatic or aromatic structures frequently containing interposed nitrogen groups. Although a number of these compounds are known to possess significant antitumor activity in various experimental systems (15), only the aliphatic derivative, MGBG, 1 has attained clinical usefulness in the treatment of human cancers, in particular, acute myelocytic leukemia (12). Despite the long-standing recognition of its potent antiproliferative activity (15), its mode of action has remained an enigma. Unlike aromatic bisguanylhydrazones, which seem to act by binding nuclear DNA, MGBG binds only weakly to DNA and has little effect on DNA polymerase (6, 7). Recently, it was discovered that the drug causes a profound disturbance in polyamine biosynthesis by inhibiting S-adenosylmethionine decarboxylase, a key enzyme in the synthesis of spermidine and spermine (5, 30). However, attempts to correlate this effect definitively with the antiproliferative action of MGBG have provided suggestive but inconclusive results (7, 10, 18, 19, 24).

To understand further the mode of MGBG action, the ultrastructure of cultured cells treated with the drug was examined recently (16, 20). At growth-inhibitory concentrations of MGBG, the mitochondria of L1210 cells and a variety of other murine and human cell lines were selectively damaged, whereas other cellular organelles appeared normal. The mitochondria were markedly swollen, and the cristae were either distorted or lost. The extent of the damage seemed sufficient to render the mitochondria nonfunctional. The damage was progressive with time and preceded detectable growth inhibition by about 12 hr, suggesting a cause-and-effect relationship between the 2 events. Conceivably, both phenomena could be mediated by a drug disturbance of spermidine or spermine biosynthesis.

The present study was undertaken to determine whether the same mitochondrial damage observed in cultured cells also occurs in ascites L1210 cells treated in vivo with the drug. An attempt was also mad to correlate the ultrastructural damage with impairment of mitochondrial function as assessed by intracellular adenosine ribonucleotide pools. Since the mitochondrial damage induced by MGBG closely resembles that produced by EB (11, 27) and since the 2 molecules bear some structural resemblance to spermidine (1, 3, 7, 29), the effects of EB were also studied for comparison. In addition, the ability of cells treated with either MGBG or EB to recover both structurally and functionally from drug-induced damage was examined. The results indicate that both drugs produce structural damage to the mitochondria of cells treated in vivo and that this damage correlates with impairment of mitochondrial function. In the case of MGBG, the damage is reversible after the cells are removed from the drug. Portions of this work were the subject of a preliminary communication (22).

MATERIALS AND METHODS

Drug Treatment. Murine leukemia L1210 cells were grown in the peritoneum of host DBA/2J mice. An initial inoculum of 10^6 cells in 0.2 ml sterile RPMI 1640 was injected i.p. into each

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2 To whom requests for reprints should be addressed, at Department of Experimental Therapeutics, Grace Cancer Drug Center, Roswell Park Memorial Institute. New York State Department of Health, Buffalo, N.Y. 14263.


4 The abbreviations used are: MGBG, methylglyoxal-bis(guanylylhydrazone), also known as methyl-G or methyl GAG; EB, ethidium bromide; RPMI 1640, Roswell Park Memorial Institute Medium 1640; HPLC, high-pressure liquid chromatography.

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Mitochondrial Damage with MGBG Treatment

On Day 3 of cell growth, the mice were given i.p. injections of EB (Sigma Chemical Co., St. Louis, Mo.) or MGBG (obtained as the dihydrochloride salt from Dr. H. Wood, Drug Development Unit, National Cancer Institute, Bethesda, Md.) 25 to 100 mg/kg, or sterile RPMI 1640. Twenty-four hr later, the cells were harvested from the ascites fluid and washed twice. Cell viability was assessed by means of trypan blue dye exclusion. Cell counts were determined by electronic particle counting (Model-ZF Coulter counter; Coulter Electronics, Hialeah, Fla.), and the samples were adjusted to cell densities appropriate for further processing for one of the following studies: (a) electron microscopy, (b) HPLC, or (c) recovery and leukemogenicity studies.

Electron Microscopy. Treated and control cell samples (~10^7 cells) were washed with cold RPMI 1640 and suspended for 2 hr at 4°C in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Cell pellets were formed by centrifugation and fixed for an additional 2 hr. The fixed pellets were washed overnight in phosphate buffer, postfixed in 1% phosphate-buffered osmium tetroxide for 3 to 4 hr at 4°C, dehydrated in a graded alcohol series, and embedded in Epon-Araldite plastic resin. Semithin sections (500 nm) were prepared on a Porter-Blum MT-1 ultramicrotome (Sorvall Corp., Norwalk, Conn.) and stained with 1% toluidine blue in 1% aqueous sodium borate. Thin sections (~90 nm) were stained with uranyl acetate-lead citrate and examined with a Siemens-Elmiskop 101 electron microscope.

HPLC. Analysis of intracellular nucleotide pool sizes was performed by HPLC. Washed control and drug-treated cell samples (~10^7 cells) were centrifuged at 1800 x g for 2 min, dried with a cotton swab, resuspended in 100 μl of cold 6% perchloric acid, and allowed to stand for 5 min at 4°C. The sample was centrifuged at 2900 x g for 3 min, and the acid-soluble supernatant was neutralized with 50 μl of 2 N KOH. The sample was centrifuged again at 2900 x g for 3 min, and the acid-soluble extract was frozen at −70°C. HPLC analysis was performed on 10-μl aliquots of these samples.

The separation of the various intracellular ribonucleotides, in particular, those of adenosine, was carried out on a HPLC system, described in detail elsewhere (25). A DuPont Model 830 instrument equipped with a dual cell system and U-shaped ABX column (1 mm x 2 mm) was used. Eluents were monitored with 254- and 280-nm detectors, and the area under each nucleotide peak was automatically integrated with a Spectro Physics Minigrator. Identification of components was made on the basis of their elution position on a calibrated column, on the basis of their A260/A254 ratios, and by the use of internal radiolabeled markers.

Recovery and Leukemogenicity Studies. The ability of in vivo-treated ascites L1210 cells to recover normal mitochondrial ultrastructure and functional capabilities was examined. Washed, drug-treated, and control L1210 cell samples (5 x 10^7 cells/0.5 ml 0.9% NaCl solution) were reinfected i.p. into DBA/2J mice and harvested 24, 48, and 72 hr later. After each harvesting, cells were washed twice with RPMI 1640, and the cell density was adjusted. Cell samples (~10^7 cells) from each mouse were either fixed and processed for electron microscopy or precipitated with 6% perchloric acid for HPLC analysis of the acid-soluble fraction.

The ability of drug-treated cells to recover was also assessed on the basis of leukemogenicity in untreated mice. Ascites L1210 cells were first treated in vivo with either MGBG or EB as described above. Washed control cells and cells treated in vivo with a single dose (75 mg/kg) of either MGBG or EB for 24 hr were reinjected i.p. into untreated mice at an inoculum of 10^6 cells/mouse. Groups of 5 mice were used for each drug-treated and control cell experiment. Each group was checked twice daily for dead mice.

RESULTS

Ultrastructural Effects of MGBG Treatment. In vivo treatment of ascites L1210 cells with MGBG resulted in morphological changes nearly identical to those previously seen in cultured L1210 and other types of cells treated in vitro with the drug (17, 20). When Epon thick sections were examined by light microscopy, cells treated with a single dose of MGBG (50 mg/kg for 24 hr) were found to contain numerous small patent vacuoles throughout the cytoplasm. All of the cells were affected similarly by the drug. Despite the extent of the damage and the uniformity of the effect within the cell population, the cell viability prior to fixation was 98 to 100% as measured by trypan blue dye exclusion. The vacuoles were present at concentrations of MGBG greater than 50 mg/kg after 24 hr but were not present in cells treated with 25-mg/kg doses of the drug or in control cells. Host toxicity was encountered with MGBG at 75 mg/kg; at 100 mg/kg, about 30% of the mice died as a consequence of drug treatment.

Cells treated with concentrations of EB greater than 50 mg/kg for 24 hr were similar in appearance by light microscopy to those treated with MGBG. Vacuoles in addition to those containing lipid were present in the cytoplasm of treated cells. In addition, the overall cell size was increased by about 30%. Although a slight increase in cell size was apparent in MGBG-treated cells, it did not approach the proportions seen with EB treatment.

In electron micrographs of untreated ascites L1210 cells (Fig. 1), the mitochondria appeared as small round or elongated structures with ordered lamellar cristae. The electron density of the matrix material was similar to that of the cytosol. Also present in the cytoplasm were several large lipid vacuoles which are characteristic of ascites but not cultured L1210 cells.

Ultrastructural examination of either MGBG- or EB-treated cells (50 mg/kg) revealed that the cytoplasmic vacuoles observed with the light microscope were actually swollen mitochondria (Figs. 2 to 5). Although not swollen to the extent that is often seen with L1210 cells treated in vitro, the mitochondria were definitely pathologically affected. The mitochondrial matrix was electron lucent and, in the case of EB, frequently contained a densely stained mass (Fig. 5) which was not apparent in MGBG-treated cells. The inner and outer membranes of the mitochondria were distended, and the cristae were either absent or markedly distorted. As noted by light microscopy, the cells were affected by drug treatment; in addition, all of the mitochondria in each cell seemed uniformly affected. The lipid vacuoles present in the cytoplasm did not appear to be increased in either size or number as a consequence of treatment with either drug. Other cellular organelles, including the Golgi apparatus and the nuclear substructure, were not significantly altered by treatment with either drug.

Intracellular Ribonucleotide Pool Analysis. HPLC chromat-
ograms of perchloric acid extracts from control L1210 cells were of sufficient resolution to detect mono-, di-, and triphosphate esters of guanosine, adenosine, uridine, and cytosine. Of these, the adenosine phosphate pools were consistently the largest, being about 10-fold greater than any other ribonucleotide. Because of their relative large size and particular relevance to the mitochondrion, the adenosine phosphate pools were selected as the nucleotide indicator of choice for mitochondrial function.

Adenosine ribonucleotides, namely AMP, ADP, and ATP, were measured and quantitated in untreated cells or in those treated with either MGBG or EB (Table 1). In general, the drug-induced alterations were similarly reflected in the pools of other ribonucleotides, but, because of their relatively small size, the changes were more variable than those for the adenosine phosphates.

An assessment of the relative pool sizes for AMP, ADP, and ATP was obtained from automated integration of the area beneath each of the peaks on the HPLC chromatogram. These were converted to nmol/10⁷ cells for AMP, ADP, and ATP, using authentic ribonucleotide standards as discussed elsewhere (25). The various adenosine nucleotide concentrations were used to calculate the energy change of the experimental and control cell samples, using the following formula:

\[
\frac{1}{2} \left[ [ADP] + 2[ATP/AMP] + [ADP] + [ATP] \right]
\]

When all of the adenosine phosphate is in the form of ATP, the energy charge is 1.0.

The HPLC analysis of the adenosine phosphate pools of control and drug-treated cells correlated well with morphological findings. This indicates that the mitochondria are indeed functionally impaired as well as structurally damaged. Cells treated with MGBG, 75 mg/kg, for 24 hr consistently showed a shift in the adenosine phosphate pools toward the less phosphorylated nucleotides (Table 1). The total nucleotide pool for adenosine remained essentially the same as control. Treatment with EB also resulted in a decrease in ATP pools. The total adenosine pool for the cell, however, decreased dramatically. These findings are otherwise apparent from energy charge calculations. Treatment with MGBG or EB lowered the energy charge of the cell from ~0.80 to ~0.50.

Recovery and Leukemogenicity Studies. Despite the extent of the ultrastructural damage to mitochondria, MGBG-treated cells were able to recover a nearly normal cell ultrastructure after being placed in an untreated animal for 48 hr. Recovered cells appeared similar to control cells, except that the mitochondria appeared smaller and deformed (Fig. 6). There was no evidence of vacuoles in the cytoplasm which could represent the originally drug-damaged mitochondria. After 24 hr in the untreated animal, mitochondria of the cells were still slightly swollen, and, in fact, appeared intermediate between the 24-hr treated cells with MGBG and the 48-hr recovered cells. All of the cells in the 24-hr recovery sample were similar in appearance, indicating that restoration of normal morphology was, in fact, a recovery process and not a repopulation of the cells by a drug-resistant type of cells.

Morphological recovery of EB-treated cells was not achieved. After 72 hr in an untreated animal, the cells still appeared damaged to some extent. Some of the mitochondria remained swollen, whereas others appeared small and condensed, suggesting that repopulation by new mitochondria might be taking place (27). The extent of the damage varied considerably from cell to cell, leaving open the possibility that an EB-resistant cell population might have arisen.

Reinjection of MGBG-treated cells into untreated mice for 48 hr also resulted in a recovery of the functional capabilities of the cell. Recovered cells displayed adenosine phosphate pools which were nearly identical to control cells. EB-treated cells failed to recover normal adenosine nucleotide pool profiles when reinjected into untreated mice and remained similar to those for cells after treatment with EB alone (Table 1). The findings are consistent with morphological results in which MGBG-treated cells, but not EB-treated cells, were able to recover normal cell ultrastructure (Fig. 6).

The leukemogenicity studies for cells treated with either MGBG or EB are consistent with the morphological findings (Table 2). An inoculum of 10⁶ L1210 ascites cells treated with MGBG gave median life spans (11.1 days) which were only slightly longer than those for untreated cells (11.6 days), indicating that resistant cell line repopulation was unlikely. Cells treated with EB gave a median life span that was 4 days beyond that for control cells. On the basis of logarithmic progression of cell division, this extension is long enough to allow for a possible repopulation of the original inoculum by a drug-resistant cell population following EB treatment.
Ultrastructural findings reported here indicate that the mitochondrial damage first observed in cultured L1210 cells and a variety of other cell types treated in vitro with MGBG (16, 20) is not a peculiarity of cell culture systems. A nearby identical lesion develops in L1210 cells grown in the ascites fluid of syngeneic mice treated in vivo with a single dose (>50 mg/kg) of the drug. After 24 hr, the mitochondria of treated cells appear swollen and their inner structure is markedly disrupted. As with the in vitro studies, the damage appears to be selective for mitochondria, since other organelles including the nuclear substructure are unaffected, although the overall cell volume seems to be slightly increased over that of control cells.

The same advantages apply to studies into the mechanism of action of EB. Although a number of studies have reported the ultrastructural effects of EB on the mitochondria cultured cells (11, 13, 14, 27), this report is, to our knowledge, the first observation of such damage being produced within the host animal. In vivo treatment of ascites L1210 cells with a single dose (>50 mg/kg) of EB for 24 hr results in mitochondrial swelling and structural damage similar to that observed following treatment of cultured L1210 cells. In the cultured cells, we have observed that the mitochondria proceed through a stage in which the mitochondria swell and the cristae change from a lamellar to a vesicular configuration. Such changes were not noted in vivo, although cells were not examined at the early stages of drug treatment.

The resemblance of the mitochondrial damage produced by EB to that produced by MGBG is striking, as is the similarity in drug dosages required to elicit qualitatively similar morphological damage. However, the mitochondrial damage induced by EB was distinctive, in that conspicuous electron-dense precipitates were present in many of the swollen mitochondria (Fig. 5). These have been previously noted in cultured cells treated with EB (14) and have been found by energy-dispersive X-ray analysis and electron microscope autoradiography to consist, probably, of a complex of drug and mitochondrial DNA (13). Such structures are frequent in NALM-1 human chronic myelocytic leukemia cells treated with MGBG but are not characteristic in most types of cells exposed to the drug (16).

It is tempting to speculate that the close similarity in ultrastructural damage produced by EB and MGBG might indicate similar molecular actions. The possibility is further strengthened by structural resemblances between the EB and the polyamine spermidine (1, 3, 4, 7, 29), although the drug has no effect on polyamine biosynthesis (5). At low concentrations, EB is known to intercalate preferentially into closed circular DNA of mitochondria (28). Although MGBG binds only weakly to linear DNA (7), its ability to bind to closed circular DNA has never been tested. Recently, we found that MGBG is capable of selectively inhibiting the incorporation of thymidine into mitochondrial DNA but not to the extent that EB could (9). It was not determined, however, whether this was due to a direct action on DNA or to another action by the drug, such as interference with oxidative phosphorylation (21) or inhibition of polyamine biosynthesis (5, 30).

HPLC analysis of intracellular ribonucleotide pools (in particular, those of adenosine) indicated that the ultrastructural damage produced by MGBG is sufficient to impair the function of the mitochondria. The endogenous supply of cellular energy is manifest only when the mitochondria are functioning. ATP levels rapidly disappear on anaerobiosis or upon addition of mitochondrial poisons such as cyanide (21). The data obtained in control, MGBG-treated, or EB-treated and recovered cells indicate that adenylate energy charge of the cell is a reasonable parameter for mitochondrial integrity.

Treatment of L1210 cells with MGBG or EB markedly depleted the ATP stores of the cell and lowered the energy charge. A similar finding was made previously by Pine and DiPaolo (21) who found that, under different experimental conditions and with considerably higher drug concentrations, the cellular ATP level was lowered significantly after short-term MGBG treatment. Also, it has been reported (2) that exposure of L1210 cells in vitro to MGBG inhibits respiration almost completely after several hr of treatment. On the basis of the structural resemblance of MGBG to guanidine or amidine structures, the drug would seem to belong primarily to the group of mitochondrial poisons that inhibit but do not uncouple a site of phosphorylation transfer and inhibit cellular respiration in this manner (21, 23). Pine and DiPaolo (21) found that cellular respiration was partly inhibited by the drug and that phosphorylation of isolated mitochondria was, in fact, uncoupled and could not be restored by the addition of 2,4-dinitrophenol.

The recovery studies indicate that the mitochondrial damage induced by MGBG represents a reversible sublethal lesion which the cell can overcome when placed in a drug-free environment, at least following the drug treatment used here. The morphological data suggest that this is accomplished by repair and restoration of the damaged mitochondria rather than by replacement with new organelles, as has been observed in culture cells treated with EB (27). It was not determined whether cells treated for longer times or with greater concentrations of MGBG still could recover in this manner.

Drug-treated cells examined 24 hr after being in an untreated animal display morphological damage intermediate between drug-treated and fully recovered (48-hr) cells. Moreover, the cells at this same time are uniform in morphological appearance, excluding the possibility of repopulation of the cells by drug-resistant cells. Not only are the cells capable of recovering their normal morphology, but they are also capable of maintaining their original malignancy as indicated by the leukemogenicity studies. Treatment of cells with MGBG extends the median survival of mice injected with the cells by an average of <1 day.

The damage induced by EB is apparently irreversible. Cells damaged by EB did not recover their normal ultrastructural appearance after 72 hr in the untreated mice, and the average survival time was extended by 4 days which, given the logarithmic progression of cell growth, is sufficiently long to allow for repopulation by drug-resistant cells or mitochondria (27). Thus, despite similarities between the molecular structure of MGBG and EB (8) and between their morphological effects on mitochondria, the leukemogenicity and recovery data suggest that the 2 drugs differ in their actions. This could involve differences in the molecular site of action or in the avidity of binding at the site of action.

Thus, MGBG has 3 interesting actions on cycling cell populations: it interferes with the structure and function of mitochondria; it inhibits polyamine biosynthesis; and it exerts an

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Unpublished data.
antiproliferative action. A number of studies have attempted to relate the effect of MGBG on polyamines with its antiproliferative activity, and some success has been reported in mitogen-stimulated lymphocyte systems (10, 18, 19). The recovery data reported here and findings by Pine and DiPaolo (21) suggest that, whereas the mitochondrial damage is substantial, the resultant energy depletion is not sufficient to account for growth inhibition per se. The subsequent depletion of triphosphate nucleotide pools required for mitochondrial and nuclear DNA synthesis, however, may be secondarily involved. In fact, mitochondrial damage precedes detectable growth inhibition by about 12 hr, allowing for this possibility. Consistent with this theory is the finding that MGBG inhibits the incorporation of thymidine into both mitochondrial and nuclear DNA (9). Whether drug inhibition of polyamine biosynthesis is related to these effects has not been determined.

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