Comparison of DNA Damage by Methylmelamines and Formaldehyde

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ABSTRACT—The cytotoxicity and DNA-damaging activity of S9-activated hexamethylmelamine (HMM) and pentamethylmelamine (PMM) were compared with suspected active metabolites in mouse leukemia L1210 cells. The presence of semicarbazide hydrochloride did not alter the cytotoxicity of S9-activated HMM and PMM or that of their hydroxylated analogs monomethylolpentamethylmelamine (MPM) and trimethylotrimethylmelamine (TTM), which have been suggested as active metabolites. Following treatment of L1210 cells with high concentrations of activated HMM and PMM, there were no DNA single-strand breaks or interstrand cross-links observed by DNA alkaline elution and only a low frequency of DNA-protein cross-links. Formaldehyde (FA) at nonlethal concentrations caused far greater DNA-protein cross-linking. In contrast, the polyfunctional TTM produced both DNA-protein cross-linking and DNA interstrand cross-linking. The cytotoxicities of HMM and PMM were found unlikely to be related to extracellular or intracellular release of FA.—JNCI 1981; 67:217–221.

MATERIALS AND METHODS

The methylmelamines and their derivatives were obtained from the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. HMM and MPM were dissolved in dimethyl sulfoxide. PMM and TTM were dissolved in water. FA solution (Fisher Scientific Co., 37%) was diluted in sterile H2O prior to use.

Mouse leukemia L1210 cells were grown in RPMI-1630 medium with 20% fetal calf serum. One hour prior to drug treatment, log-phase cells were resuspended in fresh warm medium at a concentration of 5X10^6 cells/ml. In certain experiments as noted, this medium contained S/C (1 mg/ml) which inactivated any FA that evolved from substrate demethylation. Inhibition of cell growth was determined as follows. After drug treatment, cells were washed twice, resuspended into fresh medium at 5X10^6 cells/ml, and allowed to grow at 37°C. Cell counts were obtained at 24, 48, and 72 hours after drug treatment.

Metabolic activation of HMM and PMM was achieved with the use of reconstituted lyophilized rat liver 9,000×g supernatant supplemented with nicotinamide adenine dinucleotide phosphate and glucose 6-phosphate. The livers were obtained from animals pretreated with 60 mg phenobarbital/kg for 3 days prior to killing. Substrate concentrations were 1 mM for PMM and 0.2 mM for HMM.

DNA damage in the form of single-strand breaks and cross-links was determined by the alkaline elution technique. The theoretical basis for and technical details of DNA alkaline elution have been described elsewhere.

L1210 cells containing 14C-labeled DNA were treated with drug, washed with cold medium, and layered onto

ABBREVIATIONS USED: FA = formaldehyde; HMM = hexamethylmelamine; MPM = monomethylolpentamethylmelamine; PMM = pentamethylmelamine; S/C = semicarbazide hydrochloride; TTM = trimethylotrimethylmelamine.

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a polyvinyl chloride filter along with untreated cells containing $^3$H-labeled DNA which have been irradiated with 150 rad. These $^3$H-labeled cells serve as an internal standard. To measure single-strand breaks, we lysed cells in the presence of proteinase K and performed the elution as previously described (8). The rate of elution of the DNA from the filter is directly related to the frequency of strand breaks. For measuring DNA-protein cross-links, a similar protocol is followed, except that following drug treatment cells on ice are given 300 rad of $\gamma$-radiation and cell lysis is performed without proteinase K. Because protein adsorbs to the filter under standard eluting conditions, the presence of DNA-protein cross-links retards the rapid elution of irradiated DNA from the filter (8). The degree to which the DNA is retarded is dependent on the frequency of the DNA-protein cross-links. DNA interstrand cross-links also reduce the effects of X-ray on DNA elution. This effect is not reversed by proteinase K, thereby providing a means of studying the two independently.

RESULTS

Mouse L1210 cells were exposed to HMM and PMM, in the presence of S9, for various periods of time (text-fig. 1). As the duration of drug exposure increases from 30 to 60 minutes, there is a marked inhibition of cell growth. Cells incubated with drug and no S9 were unaffected (data not shown). Including S/C in the treatment medium had no effect on drug-induced cytotoxicity. In other experiments S/C was also included in the post-treatment incubation medium up to 4 hours after drug removal. No protection from cytotoxicity was observed (data not shown). As expected, on the basis of the work of Rutty et al. (5), MPM and TTM were directly toxic to L1210 cells without S9 activation (text-fig. 2). Again, the S/C offered no protection. Cells exposed to 125 $\mu$m FA for 1 hour (text-fig. 3) exhibited only slight loss of proliferative activity, whereas cells exposed to 300 $\mu$m FA were significantly inhibited. S/C completely protected the cells from the effect of the higher dose.

Having established conditions in which the methylmelamines and their derivatives were cytotoxic, we then examined drug-treated cells by the alkaline elution method to determine if cytotoxicity was associated with measurable forms of DNA damage. Following a 60-minute exposure to S9-activated HMM and PMM (text-fig. 4), we noted a small cross-link effect, which was reversed by lysing the cells in the presence of proteinase K, indicating that these were DNA-protein cross-links. No strand breaks or DNA interstrand cross-linking was observed. The methylols were similarly studied for their ability to damage DNA (text-fig. 5). MPM (240 $\mu$m) had little effect on DNA, but TTM (240 $\mu$m) produced a substantial cross-linking effect which was only partially reversed by proteinase K. This suggests that both DNA-protein and interstrand cross-linking is produced by this polyfunctional methylol derivative. Significantly, even a nontoxic concentration of FA (125 $\mu$m) produced extensive DNA-protein cross-linking but no strand breaks or interstrand cross-links. This is consistent with our previously published results (4).

DISCUSSION

Biotransformation of HMM and PMM results in the formation of methylol derivatives (6) and FA (3). Rutty...
et al. (3, 5) have proposed that release of FA may be the principal event leading to cytotoxicity. This proposal is based on their observation that various cell lines, including L1210 cells, were protected by S/C from the methylol derivatives MPM and TTM and from microsomally activated HMM. S/C effectively removes FA by forming semicarbazone. In contrast, Morimoto et al. (7) observed significant binding of [ring-14C]HMM and [ring-14C]PMM to the DNA of tumor cells in mice, suggesting an alternative mechanism of cytotoxicity. We sought to resolve this issue by direct examination.

TEXT-Figure 2.—Inhibition of L1210 growth after 1-hr treatment with various doses of MPM or TTM. Open symbols indicate the presence of S/C.

TEXT-Figure 3.—Inhibition of L1210 growth after a 1-hr treatment with 125 or 300 μm FA. Open symbols indicate the presence of S/C.

TEXT-Figure 4.—Alkaline elution of DNA from L1210 cells following a 1-hr treatment with 59-activated HMM (■, □) or PMM (△, △). Some cells received 300 rad (R) on ice prior to elution. Lysis was performed in the presence (closed symbols) or absence (open symbols) of proteinase K. Cells bearing [3H]DNA are given 150 rad and included in each filter as an internal standard.
of the intracellular DNA under cytotoxic conditions. In previous work, this laboratory has shown that FA produces extensive DNA-protein cross-linking in L1210 cells at nontoxic concentrations (4). Thus if the metabolism of the methylmelamines yields a toxic intracellular concentration of FA, comparable DNA-protein cross-linking should be observed. That this is not true argues strongly against a major role for FA. We considered the possibility that the methylmelamines may release FA slowly in the cell, thereby reducing or delaying the peak cross-linking effect. However, we have examined the cells by alkaline elution at various times up to 24 hours after drug removal and find no evidence for this occurring (data not shown). Since we have previously shown that repair of FA-induced DNA-protein cross-links is a slow process, occurring over many hours (4), it is also unlikely that such repair removes a significant number of cross-links during the course of our experiments. The lack of protection by S/C in our experiments is also evidence against a role for FA but must be considered weaker in the absence of documentation of uptake of S/C by the L1210 cells. Although our S/C data in L1210 cells seem in conflict with those of Rutty et al. (5), they found that S/C did not protect P815 plasmacytoma cells from the methylol derivatives. Thus cell lines may differ in this regard.

Although TTM causes interstrand cross-linking and MPM does not, the two drugs are roughly equitoxic in our experiments. This suggests that these cross-links, generally considered highly lethal, are not of primary importance to drug-induced cytotoxicity.

The fact that we observed little in the way of DNA damage in cells treated with activated HMM and PMM is surprising in view of Morimoto's data indicating substantial DNA binding by these drugs (7). Our own observation that TTM produces interstrand cross-links could also be interpreted as evidence that the methylol groups can provide a site for binding to DNA. On the basis of previous alkaline elution data with alkylating agents (9), nitrosoureas (10), radiation (11), platinum compounds (12), and bleomycin (13), it is likely that if strand breaks or interstrand cross-links were causally related to HMM cytotoxicity we would have observed them in our experiments. However, many forms of DNA damage, such as base damage and intrastrand cross-linking, are not detectable by the alkaline elution technique. We are currently studying the ability of these compounds to inhibit the transfectivity of DNA phage for further evidence of such DNA damage.

REFERENCES


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