ABSTRACT

Five human tumor cell lines of the Mer—phenotype sensitive to killing by the methylating agent 5-(3-methyl-1-triazeno)imidazole-4-carboxamide were sensitive to hydroxyurea (HU) compared with 15 cell lines resistant to methylating agents (Mer+ phenotype). In a study using fewer cell lines, Mer—cells were also sensitive to methotrexate but not to seven other agents including the antimetabolites 1-d-arabinofuranosylcytosine and 5-fluorouracil. Cells sensitive to HU were designated the HU—phenotype. Five autologous Mer—lines, derived in vitro by treating Mer+ lines with methylating agents, did not become resistant to HU or methotrexate (Mer+ HU—phenotype). All Mer+ lines studied had enhanced ability to reactivate methylated adenovirus. Adenovirus was inactivated by prolonged treatment with HU, but no enhanced reactivation of HU—treated virus was found in Mer+ cell lines. Cell survival after 5-(3-methyl-1-triazeno)imidazole-4-carboxamide treatment was not significantly decreased by HU, nor was replication of methylated adenovirus inhibited by HU in Mer—or Mer+ lines. Replication of untreated adenovirus was poor in Mer—cells treated with HU, indicating that sensitivity of cells to HU was associated with inhibition of DNA synthesis. These results suggest that cell sensitivity to deoxynucleotide depletion is linked, perhaps coincidentally, to the Mer—phenotype. The retention of HU and methotrexate sensitivity by cells after development of resistance to 5-(3-methyl-1-triazeno)imidazole-4-carboxamide may have therapeutic implications.

INTRODUCTION

Methylating agents react with DNA to form a variety of products including O6-MeG, a lesion often associated with mutagenesis and toxicity in mammalian cells (1-5). The methylation-sensitive Mer—phenotype is deficient in ability to reactivate methylated adenovirus (1, 6) and lacks O6-MeG transferase, a protein which acts as receptor for direct removal of the methyl group from DNA without requiring patch repair (7). However, the level of O6-MeG transferase does not always correlate with the degree of resistance to methylating agents (3, 8-10).

The antitumor agent DTIC is activated in vivo by demethylating to MTIC which rapidly decomposes and methylates DNA (11, 12). Intrinsic and developed resistances are major limitations in the treatment of melanoma with DTIC (13, 14). In previous studies using human melanoma cell lines, a Mer—line developed from a Mer+ line by treatment with MTIC (autologous Mer+ line) was found to have enhanced ability to remove O6-MeG and to reactivate MTIC-treated adenovirus in the HCR assay but was not resistant to a variety of other agents (2, 15). Here we report that Mer—cell lines and autologous Mer+ lines derived from them are sensitive to HU and MTX compared with spontaneous Mer+ melanoma lines established from the tumors of other patients (allogeneic Mer+ lines).

MATERIALS AND METHODS

The origins and general properties of the human melanoma cell lines have been described (2, 15-18). MM138-2T and HeLa-2T were derived from the Mer+ cell lines MM138 and HeLa-S3, respectively, by two treatments with MTIC (0.3 mM). The human melanoma line SK-MEL-13 (19) was obtained from Dr. C. W. Beattie, University of Illinois. Cells were cultured in Roswell Park Memorial Institute Tissue Culture Medium 1640 (Commonwealth Serum Laboratories, Melbourne, Australia) containing 10% fetal calf serum, penicillin (100 IU/ml), streptomycin (100 ug/ml), and 3 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. Assays for Mycoplasma by the uridine/uracil ratio method (2) and by culture on agar were routinely conducted and found to be negative.

Unless otherwise stated, cell survival was determined by thymidine incorporation in colonies 5 to 7 days after treatment. This method correlates with visual counting of colonies using a variety of agents and enables survival to be determined in cell lines where colonies are too diffuse to be readily counted (17, 18, 20). Duplicate cultures (2 x 10^3 cells/16-mm well) were seeded in 1 ml of medium 24 h before treatment. Five to 7 days after treatment was initiated the medium was replaced with medium containing (methyl-3H)thymidine (1 #Ci/ml, 40 Ci/mmol; Radiochemical Centre, Amersham, United Kingdom). After 2 to 4 h at 37°C the cells were washed with Tris buffer (0.1 M NaCl/10 mM Tris/2 mM EDTA, pH 6.8), detached with trypsin solution [trypsin (0.2 mg/ml)/0.1 M NaCl/8 mM Na2HPO4/2.7 mM KCI/1.5 mM KH2PO4/7.5 mM EDTA, pH 7.2], filtered onto glass fiber discs (Whatman GF/A), and lysed and washed exhaustively with water prior to liquid scintillation counting. To avoid thymidine pool size effects when using HU, the medium was changed 24 h before labeling. Survival curves were plotted (log percentage of control cpm versus dose) for determination of D50, D60, and D90. In the absence of a shoulder, D50 = D90. MTIC was synthesized as previously described (21) and handled under minimum illumination. MTIC and MNNG were dissolved in DMSO and ethanol, respectively (100 #l/mg), before dilution with medium at 0°C and further immediate dilution into cultures.

MTX toxicity could not be assayed reproducibly using [3H]thymidine incorporation and was therefore determined by the colony-counting method (17). Cells (1000-60-mm plate seeded 24 h previously) were treated with various doses of drug. The cultures were maintained until colonies in the control plates had more than 50 cells (7 to 20 days), fixed with methanol, and then stained with Giemsa, and the colonies (>50 cells) were counted. To obtain the single cell response, the surviving fraction required correction for the contribution of 2 to 4 cell clusters present at the time of treatment (22).

Methylating agents were obtained from Dr. A. J. Bellet, John Curtin School of Medical Research, Canberra, Australia, and shown to be Mycoplasma free by agar culture. The ability of cells to reactivate drug-damaged adenovirus (HCR) was assayed by detection of viral antigens 2 days after infection. Successive 10-fold dilutions of virus were used to infect duplicate cultures in microtiter plates (5 x 10^3 cells/6-mm well) seeded 24 h previously. After 2 h the cells were washed twice with phosphate-buffered saline (pH 7.4) and fixed with methanol. For detecting viral antigen, cultures were incubated for 1 h with human plasma (25 #l of 1/15 dilution) containing adenovirus antibody, followed by peroxidase-labeled Protein A (25 #l of 5 #g/ml; Sigma); each step was preceded by 2 washes with phosphate buffer. After washing with Tris buffer (10
RESULTS

Cell Survival. As reported previously (2,15) the Mer~ human melanoma cloned line MM253cl was highly sensitive to killing by MTIC compared with the autologous Mer* line MM253-3D derived by in vitro treatment of MM253 with MTIC (Fig. 1A); a similar difference in survival was found using MNNG (Fig. 1B). MM253cl had the same drug sensitivities as the uncloned MM253 parent line (results not shown). Other human tumor lines available in this laboratory exhibited MTIC and MNNG survival curves typical of either the Mer~ (Do < 0.10 mm) or Mer* (D0 > 0.1 mm) phenotype (Fig. 1; Table 1).

The sensitivity of the autologous Mer* MM253-3D line to HU was similar to that of the Mer~ parent (Fig. 2A). Four other autologous Mer* cell lines, derived from Mer~ lines by treatment with various methylating agents, also showed little increase in HU resistance compared with the increase in MTIC resistance (Table 1; Fig. 3). Allogeneic Mer* lines, however, were significantly more resistant to HU compared with Mer~ cells (Fig. 2A; Table 1). In a comparison of 20 allogeneic Mer~ and Mer* lines, the correlation appeared to be linear up to a D0 for HU of 0.3 mM (r = 0.91 for melanoma and 0.71 for all lines), above which little further increase in HU resistance was found; the overall correlation coefficient was 0.62 (Fig. 3A). Of the allogeneic Mer* cells, MHB fibroblasts, MM200, and MM384 showed the poorest correlation between resistance to HU and MTIC. MM418 showed the poorest correlation between resistance to MTIC and MTIC-resistance (Table 1; Fig. 3). When plotted using a logarithmic scale for MTIC survival, the overall correlation was improved (r = 0.78), and a clear separation of cells (Fig. 2A; Table 1). In a comparison of 20 allogeneic Mer~ lines, the correlation appeared to be linear up to a D0 for MTIC of >1 mM and was therefore not included in Fig. 3. When plotted using a logarithmic scale for MTIC survival, the overall correlation was improved (r = 0.78), and a clear separation of cells (Fig. 2B; Table 1).

Sensitivity to a variety of other agents was compared in several lines. Mer~ cells were found to be highly resistant to MTX compared with Mer~ or autologous Mer* cells (Fig. 2B, Table 1), the difference being greater than that found using HU. Two Mer~ lines (MM253cl and HeLa) variably displayed a plateau on the dose-response curves with MTX (Fig. 2B). It should be noted that supratoxic levels of MTX allowed many colonies of 10 to 40 Mer~ cells to form, but no further proliferation occurred when the cultures were replated or maintained in situ for several weeks. Thus although care was taken to score only large colonies (>50 cells), the survival of Mer~ lines at high MTX doses may have been underestimated if more than 5 doublings occurred in some colonies before growth ceased.

Fig. 1. Sensitivity of human tumor cell lines to methylating agents. A, MTIC. O, MM253cl (Mer~); C, HeLa (Mer~); , MM96L (Mer~); , MM253-3D (Mer~); A, MM418 (Mer~). B, MNNG. Symbols as in A. Points are means of duplicates.

Fig. 2. Sensitivity of human cells to HU and MTX. A, HU. O, MM253cl (Mer~); C, HeLa (Mer~); , MM96L (Mer~); , MM253-3D (Mer~); A, MM418 (Mer~). B, MTX. Symbols as in A. Points are means of duplicates.
MTIC AND HU CROSS-SENSITIVITY

Fig. 3. Correlation of the cell $D_0$ for HU with the cell $D_0$ for MTIC. $A$, linear scale for MTIC dose. $\bullet$, Mer* cell lines; $\Box$, Mer* human fibroblasts (MHB); $O$, Mer cell lines; $\Delta$, autologous Mer* cell lines. $B$, logarithmic scale for MTIC dose. Symbols as in $A$. Points are means of quadruplicates.

Table 2 Toxicity of various agents in Mer* (MM96L), Mer' (MM253cl), and autologous Mer* (MM253-3D) melanoma cell lines

<table>
<thead>
<tr>
<th>Agent</th>
<th>MM96L</th>
<th>MM253cl</th>
<th>MM253-3D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colcemid (µM)</td>
<td>0.063</td>
<td>0.63</td>
<td>0.41</td>
</tr>
<tr>
<td>Dopa (µM)</td>
<td>8</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>Deoxyadenosine (µM)</td>
<td>51</td>
<td>1200</td>
<td>2900</td>
</tr>
<tr>
<td>1 β-D-Arabinofuranosylcytosine (µM)</td>
<td>0.002</td>
<td>0.021</td>
<td>0.021</td>
</tr>
<tr>
<td>5-Fluorouracil (µM)</td>
<td>2.3</td>
<td>2.3</td>
<td>2.1</td>
</tr>
<tr>
<td>UV (J m⁻²)</td>
<td>1.5</td>
<td>3.8</td>
<td>2.3</td>
</tr>
<tr>
<td>γ-Rays (rads)</td>
<td>110</td>
<td>300</td>
<td>190</td>
</tr>
</tbody>
</table>

Fig. 4. HCR of drug-treated adenovirus compared in various cell lines. $A$, virus treated with MTIC. $O$, MM253cl (Mer*); $□$, HeLa (Mer); $●$, MM96L (Mer*); $●$, MM253-3D (Mer*). $B$, virus treated with HU. Symbols as in $A$. Points are means of quadruplicates.

MTIC treatment of Mer~ cells, however, either immediately after (Fig. 4A) or 24 h before infection (results not shown), had little effect on virus replication.

Effect of Combined Treatments on Cell and Virus Survival. A nontoxic level of 3-aminobenzamide (2.5 mM) enhanced MTIC toxicity in the autologous Mer* line MM253-3D and in the Mer* MM96L line, whereas replication of MTIC-treated adenovirus was not significantly different in 3-aminobenzamide-treated cells compared with control (results not shown). Since the MTIC breakdown product AIC may inhibit purine synthesis (25), synergism between AIC and MNNG, a methylating agent similar in action to MTIC, was investigated. Over the concentration range of AIC generated by MTIC breakdown in the cell survival experiments, AIC alone was not toxic and did not affect the toxicity of MNNG.

Radical scavengers have previously been shown to decrease killing by HU in mouse cells (26, 27), implicating a radical mechanism in HU cytotoxicity including possibly DNA damage. To test whether this occurred in human melanoma cell lines, DMSO (2%) and catalase (2000 IU/ml) were included in HU cell survival assays. In the cell lines tested (MM418 and MM253cl), these chemicals had no effect on the toxicity of HU.

Extensive treatment time and dose-response experiments were carried out to determine whether combinations of MTIC and HU were synergistically toxic. No consistent effects other than additive levels of toxicity were found in any of the Mer phenotypes. This was also found to be the case for HCR of
MTIC-treated adenovirus in HU-treated cells (results not shown), in contrast to the moderate level of synergism between MNNG and HU reported in Mer− human fibroblasts (28).

DISCUSSION

This study found a strong degree of cross-sensitivity in Mer− cells between two classes of apparently unrelated drug: methylating agents and inhibitors of deoxynucleotide synthesis. For convenience in discussion, sensitivity to HU is tentatively designated the Hu+ phenotype. MTX, like HU, appears to be selective against Mer− cells, but a definitive study of MTX toxicity in a large number of lines may require the use of an exceptionally rigorous criterion for cell survival. Methylation-like genome damage by HU, acting either directly or indirectly through generation of an alkylating agent from components of the cells or medium, was ruled out by the lack of enhanced HCR of HU-treated virus in Mer+ cells. That adenovirus was inactivated at all by HU indicates some form of DNA or protein damage, possibly through HU autoxidation, but this aspect was not investigated further. DNA synthesis as judged by the effect on viral capacity was strongly inhibited by HU in Mer+ cells, suggesting that HU acts in its commonly accepted role as an enzyme inhibitor rather than as a genotoxic agent. HU and MTX deplete deoxynucleotide pools by inhibition of ribonucleotide reductase and dihydrofolate reductase, respectively (29–32). Mer− cells therefore appear to be sensitive to a type of deoxynucleotide deprivation specific to these drugs. No sensitivity was found to other agents which affect deoxynucleotide pools in various ways: deoxyadenosine (32) 1-β-d-arabinofuranosylcytosine (33); and 5-fluorouracil (34).

None of the present evidence provides a functional link between sensitivity to MTIC and Hu or MTX. The semilogarithmic relationship between the toxicities of MTIC and HU may result from varying expression of structurally linked Mer+ and Hu+ genes, modulated in individual cell lines by other factors such as growth rate. The HCR assays using MTIC-treated virus reinforced previous conclusions (1, 4, 5, 15) that the Mer− defect operates primarily at the level of DNA repair; and the lack of significant inhibition of viral capacity in MTIC-treated Mer− cells suggested that MTIC has no antimetabolite or other marked epigenetic effects on DNA synthesis. A proportion of Mer− cell lines has been reported to be sensitive to deoxynucleotide deprivation specific to these drugs. No sensitivity was found to other agents which affect deoxynucleotide pools in various ways: deoxyadenosine (32) 1-β-d-arabinofuranosylcytosine (33); and 5-fluorouracil (34).

Tumor becomes resistant in vivo, it may be feasible to use HU or MTX. Previous lack of success in treating melanoma with the latter agents may have been due to the relatively low proportion of patients (10 to 15%) from whom a favorable response to DTIC could be expected (13, 14).

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REFERENCES

Cross-Sensitivity of Methylating Agents, Hydroxyurea, and Methotrexate in Human Tumor Cells of the Mer™ Phenotype

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