Isogonic variants resistant to alkylating agents have been isolated from the human lymphoblast cell line TK6. The cell lines may be divided into four classes on the basis of resistance to N-methyl-N' -nitro-N-nitrosoguanidine (MNNG). The sensitive TK6 parental line shows a 37% survival after 45-min exposure to 0.04 \( \mu \text{M} \) MNNG; the three classes of more resistant mutants show a 37% survival after 45-min exposure to 2 \( \mu \text{M} \) (MT lines), 6 \( \mu \text{M} \) (MT lines), and \( \geq 10 \mu \text{M} \) (MX line) MNNG. A representative MF line, MF1, is resistant to both killing and mutation by MNNG or N-methyl-N-nitrosourea. An MT clone, MT1, is highly resistant to killing but hypermutable by MNNG. The MT1 line, like the parental TK6, does not remove 6\( \text{MeG} \) from the DNA. Our data are consistent with the hypothesis that the MT1 line possesses a nonexcision pathway of defense against killing by alkylating agents. Rather than preventing alkylation of DNA or removing alkylated adducts, the MT1 cells appear to be tolerant of the adducts that are not removed from the DNA.

Whereas a large body of literature has accumulated on the toxic and mutagenic actions of simple alkylating agents (Gichner and Veleminska, 1982), there is still no clear understanding of the mechanisms of the phenomena. In order to study the mechanisms of cell defense against alkylating agents, we have begun to employ the techniques of biochemical genetics: the analysis of somatic cell mutants that are resistant to alkylating agents. By this analysis, we hoped to find mutants that would be similar to the parental line except for a difference in a single gene product involved in the repair of alkylated DNA. If found, such mutants could help us to identify the gene and gene products involved in the damage of DNA by alkylating agents or in the subsequent DNA repair. This paper describes the isolation and characterization of somatic cell mutants that are resistant to MNNG.\(^1\) ICR-191, a potent and moderately toxic mutagen for human cells (DeLuca et al., 1977; Slapikoff et al., 1980), was used for the generation of the mutants. This mutagen induces only frameshift mutations in bacterial cells (Calos and Miller, 1981) and apparently causes frameshift mutations in Chinese hamster ovary cells (Gupta and Siminovitch, 1980) and TK6 human lymphoblasts (Furth, 1979). Such mutations should result in the inactivation of derived single gene products. Thus ICR-191-induced mutants with different phenotypic properties should have a high probability of representing mutations in different genetic loci.

The TK6 line was used as the parental line for generation of mutants. TK6 cells are sensitive to both toxic and mutagenic effects of alkylating agents and are deficient in removal of the 6\( \text{MeG} \) DNA adduct (Sklar and Strauss, 1981). These properties of TK6 cells were considered advantageous for the study of the cytotoxic effects of alkylating agents.

**EXPERIMENTAL PROCEDURES AND RESULTS\(^2\)**

MNNG-resistant Clonal Derivatives of a TK6 Cell Culture—MNNG-resistant clonal derivatives were selected from populations of TK6 cells treated with ICR-191 and from nontreated TK6 cell populations (Fig. 5). ICR-191-treated populations of TK6 cells contained about 2.5 times more survivors of treatment with 0.34 \( \mu \text{M} \) MNNG and 50 times more survivors of treatment with 6.5 \( \mu \text{M} \) MNNG in comparison to the control cultures (Fig. 5). It is unlikely that this increase in surviving fraction in ICR-191-treated populations was due to enrichment of pre-existing MNNG-resistant variants during treatment with ICR-191 since no correlation has been found among several human lymphoblast cell lines (including a TK6 cell line) between ICR-191 toxicity and MNNG toxicity (Slapikoff et al., 1980). The majority of cells in the ICR-191-treated population of the TK6 cell line is resistant to the same sensitivity to the toxic and mutagenic action of MNNG as nontreated TK6 cells. The shape of the survival curve suggests that cells in the ICR-191-treated cultures can be broadly divided into three categories: the first is as sensitive as TK6 cells to MNNG (99.9% of the population), the second is resistant to 0.34 \( \mu \text{M} \) MNNG (0.1% of the population), and the third is resistant to 6.8 \( \mu \text{M} \) MNNG (5 \( \times 10^{-4} \) of the population).

\(^1\) Portions of this paper (including "Experimental Procedures," part of "Results," Figs. 1–4, Table 1, and additional references) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 960 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-3651, cite the authors, and include a check or money order for $3.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
FIG. 5. ICR-191-induced and spontaneous MNNG-resistant variants of TK6 cells. The toxic action of MNNG (45-min exposure) toward the TK6 cell populations is represented on an expanded scale compared with Fig. 1. ▶, TK6 cells; ○, ICR-191-treated TK6 cells. Survival fractions were determined by measurements of clone-forming ability. Vertical bars represent 95% confidence limits.

TABLE II

Clone-forming ability of human lymphoblasts

<table>
<thead>
<tr>
<th>Cells</th>
<th>In the absence of feeder cells</th>
<th>In the presence of feeder cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>TK6</td>
<td>0.36 ± 0.07 (n = 7)</td>
<td>0.75 ± 0.12 (n = 7)</td>
</tr>
<tr>
<td>MT1</td>
<td>1.4 ± 0.3 × 10^{-2} (n = 2)</td>
<td>0.20 ± 0.08 (n = 6)</td>
</tr>
<tr>
<td>MT2</td>
<td>6 ± 3 × 10^{-3} (n = 2)</td>
<td>6 ± 2 × 10^{-3} (n = 2)</td>
</tr>
<tr>
<td>MT4</td>
<td>3 × 10^{-3}, 3 × 10^{-3} (n = 2)</td>
<td>6 ± 1 × 10^{-3} (n = 2)</td>
</tr>
<tr>
<td>MT5</td>
<td>6 × 10^{-3}, 9 × 10^{-4} (n = 2)</td>
<td>3 ± 1 × 10^{-4} (n = 2)</td>
</tr>
<tr>
<td>MX</td>
<td>1.8 ± 0.4 × 10^{-3} (n = 2)</td>
<td>8 ± 4 × 10^{-3} (n = 2)</td>
</tr>
</tbody>
</table>

A population). Twenty-five colonies of the second category were isolated and designated as MF and MT lines, respectively. These lines were established and then continuously grown in suspension cultures without feeder cells. Later, one of the MT lines was designated as MX because it was significantly more refractory to MNNG toxicity than were the other MT lines. In addition, the MF1 line was established from a survivor cell after a treatment of TK6 cells with MNU that left a surviving fraction of about 0.07%. This surviving fraction was similar to the surviving fraction that was left in the TK6 cell cultures as MT cells increased in the presence of feeder cells (Table II). At high cell densities, clone-forming abilities were essentially unaffected by the number of cells per well or by the presence of feeder cells. Therefore, frequencies of appearance of MNNG-resistant mutants could be estimated. Using data shown in Table II, we estimated the clone-forming ability at high cell densities for TK6 as about 0.8 and for clones surviving treatment with 6.8 μM MNNG as 0.1. Using these data and Fig. 5, we estimated that the spontaneous fraction of MNNG-resistant variants selected with 6.8 μM MNNG was about 4 × 10^{-3}, whereas the ICR-191-induced mutant fraction selected under similar conditions was about 1 × 10^{-8}.

Toxic and Mutagenic Action of Alkylating Agents toward MNNG-resistant Variants—Several clonal isolates were characterized with regard to their resistance to the toxic effect produced by MNNG or MNU. The cell lines formed four categories according to their sensitivity to MNNG: sensitive (TK6 line), resistant (MF2, MF3, MF4, MF5, and MF13 lines), highly resistant (MT1, MT2, MT4, and MT5 lines), and "superresistant" (MX line) (Fig. 6). All these MT lines selected with MNNG were found to be resistant to MNNG as well as to MNNG. The resistance of MT lines to MNNG was inherited and remained uniform over the 4 months that representative MT lines were grown in the absence of alkylating agents. However, two sublines of the MF phenotype that were grown for 4 months steadily lost their resistance to MNNG as a population.

MF1 and MT1 lines had different toxic and mutagenic responses to alkylating agents. Whereas MF1 cells were resistant to both toxic and mutagenic effects (Fig. 7a), MT1 cells were highly resistant to alkylating-induced toxicity but somewhat hypersensitive to mutagenic effects (Fig. 7b).

The levels of DNA alkylation in MF1 cells after exposure to [3H]MNU and in MT1 after exposure to [3H]MNNG were similar to those in the sensitive TK6 cells, as shown by measurements of radioactivity in purified DNA from exposed cells. This result indicates that MF1 and MT1 cells did not...
Somatic Cell Mutants Resistant to Alkylating Agents

possess a mode of defense that would prevent DNA alkylation.

We found that TK6 cells and MT1, MT2, MT4, and MT5 cells contained equal levels of intracellular acid-soluble thiols; whereas in MX cells, the thiol content was 2-fold lower. This thiol deficiency might contribute to the superresistance of the MX line since it is known that thiols activate the alkylating ability of MNNG and that reduced intracellular levels of acid-soluble thiols enhance the resistance of cells to MNNG (Schulz and McCalla, 1968; Lawley and Thatcher, 1970; Sedgwick and Robins, 1980). However, MX cells could possess an additional mode of resistance to alkylation since these cells are also resistant to MNU, and MNU has been reported to be equally toxic in the presence and absence of extracellular thiols (Wheeler and Bowdon, 1972).

The MT1 line possessed the phenotypic combination of hyposensitivity to toxicity and hypersensitivity to mutation by MNNG, and we characterized this line further.

MT1 Line Is a Mutator—The rate of accumulation of spontaneous mutation in the MT1 line was about $3 \times 10^{-6}$ events per cell per generation in both *hgprt* and *tk* loci (Fig. 8, upper), about 10-15-fold higher than the rate in the parental TK6 line (Fig. 8, lower). Preliminary data indicated that the MT2 line is also a mutator.

**DNA Adduct Formation and Removal by Cells**—Formation and removal of the six adducts 7MeG, O'MeG, 3MeG, 1MeA, 3MeA, and 7MeA in MT1 cells exposed to MNNG were analyzed by HPLC cationic exchange and reverse-phase separation. We observed no differences in DNA adduct formation analyzed by HPLC cationic exchange and reverse-phase separation. We observed no differences in DNA adduct formation.

**FIG. 7.** a, upper: toxic action of 24-h MNU exposure in TK6 (O) and MT1 (■) lines. Surviving fractions were determined by extrapolation of growth curves of the treated cell cultures. Lower, mutagenic action of 24-h MNU exposure in TK6 (O) and MT1 (■) lines. Vertical bars are 95% confidence limits. b, upper: MNNG-induced (45-min exposure) mutagenesis in TK6 (O) and MT1 (■) cells. Closed symbols represent the 6-thioguanine-resistant fraction in the *hgprt* cell populations, and open symbols represent the 6-thioguanine-resistant fraction in the *hgprt* cell populations.

**FIG. 8.** Upper, spontaneous mutant fraction in *hgprt* and *tk* loci in MT1 cells during 24 days of exponential growth. Each point represents the mean ± S.E. of four independently growing cultures. Lower, spontaneous mutant fraction in *hgprt* and *tk* loci in TK6 cells during 24 days of exponential growth. Each point represents the mean ± S.E. of four independently growing cultures.

**FIG. 9.** Removal of alkylated bases from DNA of TK6 (a) and MT1 (b) cells exposed to [3H]MNNG. Cells were exposed to 1.0 μM MNNG for 45 min at 37 °C, washed, and incubated in fresh medium at 37 °C. HPLC separation and calculations were done as outlined under “Experimental Procedures.” Open and closed symbols represent two independent experiments. a, bottom: amount of DNA relative to initial amount in the TK6 cell population exposed to 1.0 μM MNNG for 45 min at 37 °C as a function of post-treatment time.
in TK6 cells (Fig. 9a) and MT1 cells (Fig. 9b), either immediately after treatment or as a function of time thereafter. Only 3MeA was found to be removed from the DNA as a function of time after treatment. Other adducts, 7MeG, 7MeA, 3MeG, and O'MeG, were removed slowly if at all between the end of MNNG treatment and the time of the last point studied 10 h later. During this 10-h period, the number of TK6 cells continued to increase as did total amount of DNA (Fig. 9a, lower). MT1 cells behaved similarly. Therefore, the apparent decrease in adducts per unit of DNA is accounted for by a 40% increase (see below) in the amount of DNA per cell. That the O'MeG adduct was persistent in the DNA of TK6 cells is consistent with the findings of Sklar and Strauss (1981), who studied the TK6 line under our earlier denomination as H2BT. No lMeA was detected, either because it did not appear in any significant amount after MNNG treatment or because it had been removed from the DNA.

The amounts of alkylated adducts per cell as well as per toxic and mutagenic event were estimated (Table III) by interpolating the data of Figs. 1 and 4. According to our data, accumulation of about 1000 O'MeG molecules per TK6 cell DNA was coincident with a single lethal hit as derived from the survival curve. Such calculations reveal only broad possibilities. It could be that the lethal target for O'MeG, for instance, is only 0.1% of the cellular DNA or that any O'MeG molecule in the DNA could be lethal but has 0.1% probability of being so.

**Cell Cycle Progression after Exposure to MNNG—MT1 and TK6 cells were exposed to equally toxic concentrations of MNNG (killing 90% of the cells), 10 and 0.14 μM, respectively; and cell cycle progression was analyzed by flow cytometry (Fig. 10). The division of MT1 cells was immediately arrested after the treatment so that cells did not divide even once (the number of cells did not increase within at least 4 days after treatment with MNNG); in contrast, TK6 cells underwent a single cell division and were arrested at or immediately after their second S phase after treatment with MNNG. The behavior of TK6 cells is similar to that of HeLa cells reported by Plant and Roberts (1971). Cell cycle distributions in normal exponentially growing populations of TK6 and MT1 cells were similar.

Analysis of clone-forming abilities as a function of time after treatment indicated that the toxic response of TK6 cells was “all or nothing,” i.e. a fraction of the cells died immediately while the remaining cells continued to grow at a normal rate. On the other hand, MT1 cells seemed to be “injured” following MNNG treatment since they continued to grow but at slower rates. These slow-growing MT1 cell “survivors” completely lost clone-forming ability for 1 week or more after treatment even in the presence of feeder cells.

**DNA Synthesis after Exposure of Cells to MNNG—Rates of DNA replication by TK6 and MT1 cells after exposure to 6.8 μM MNNG were compared by measuring levels of [3H]dThd incorporation into DNA (30-min pulse). This concentration of MNNG is superlethal for TK6 cells but is almost nontoxic for MT1 cells. Following exposure to 6.8 μM MNNG, DNA replication essentially ceased within 4 h in TK6 cells but was reduced only 20% in MT1 cells (Fig. 11a). However, 8 min after exposure, no inhibition of [3H]dThd incorporation could be detected. Since within 2 days after treatment of TK6 with 6.8 μM MNNG the fraction of S phase cells in the population increased (data not shown), the inhibition of DNA replication in the exposed cells apparently was not sufficiently stringent to represent a block of cell entry into S phase.

**Hydroxyurea-insensitive DNA Synthesis—DNA excision repair synthesis (unscheduled DNA synthesis) appears to be
classes of MNNG resistance—The parental TK6 cell line and its clonal derivatives, the MF, MT, and MX lines, form four distinct classes of resistance to MNNG: (a) TK6 parental cell line, which is sensitive to both toxic and mutagenic activity of alkylating agents and is deficient in ability to remove the O'MeG DNA adduct; (b) MF lines, which are about 50-fold more resistant to toxicity and, in the case of the MF1 line, resistant to mutagenicity of alkylating agents; (c) MT lines (MT1, MT2, MT4, and MT5), which are about 1500 times more resistant to MNNG-induced toxicity than is the TK6 line, including the representative MT1 line, which is hypersensitive to MNNG-induced mutation; and (d) MX line, which is superresistant to MNNG, possibly due to the decreased level of intracellular acid-soluble thiols.

Nature of MF and MT Clonal Derivatives—Although the TK6 human lymphoblastoid cell line originated from a single cell, phenotypic variants arise at a slow but steady rate during proliferation of TK6 populations. Accumulation of spontaneous forward mutations in the tk and hgpRT loci takes place at the rate of about 1-2 × 10⁻⁷ cell⁻¹ generation⁻¹ (Fig. 8, lower). Our data also indicate accumulation of spontaneous mutation-like events in other loci, which results in variants resistant to the toxic effects of MNNG.

In principle, MNNG-resistant variants might result from a stable genetic change (mutation or broad chromosomal aberration) or from gene amplification or a gene activation/inactivation. Properties of MT variants indicate mutational origin (Siminovich, 1976; Wright et al., 1980). (a) Frequencies of appearance of variants in the wild-type cell populations were dramatically increased by a mutagen. (b) Phenotypic expression of resistance to the selective agent was stable over a period of at least 4 months of exponential growth in the absence of the agent. (c) These frequencies were low, which is characteristic of somatic mutations. The origin of MF lines is not at all clear; recombined populations of MF cells steadily accumulated cells that had sensitivities similar to the parental TK6 line.

DNA Adducts—As noted in Table III, our analysis accounts for only 50–60% of the methyl groups associated with the whole DNA preparation. Reactions forming phosphotriesters, pyrimidine adducts, and reactions with molecules associated with the DNA preparation are not taken into account. Our observations with purine adducts in MNNG-treated human cells accord reasonably with the observations of Beranek et al. (1980) with MNU-treated salmon sperm DNA. With regard to 7MeG, 3MeG, O'MeG, and 7MeA, our observations indicate no significant removal after treatment with 1 μM MNNG. However, 1 μM MNNG is extremely toxic to TK6 cells, although it does permit some 50% of the cells to proceed through a single mitosis in the 24 h following treatment. It is possible that specific kinds of DNA repair are inhibited at this concentration, which is near the edge of our ability to perform radiochemical analysis. The removal of 3MeA was, however, rapid and accounted for 75% of the initial 3MeA adducts, with an apparent half-life of 1 h or less. In principle, any or all of the adducts detected could be premutational or prelethal lesions since all except 1MeA were present at initiation of exponential growth in the absence of the agent.

Of particular interest was the observation of the wide variation in mutability and sensitivity of killing by MNNG between the TK6 and MT1 lines, both O'MeG methyltransferase-deficient. We discuss this finding in terms of previous studies of O'MeG as a mutagenic and below toxic DNA adduct.

Role of O'MeG DNA Adduct—First, the O'MeG DNA adduct and its analogue, O'-ethylguanine, appear to be principal mutagenic lesions when present in DNA of various organisms
(Samson and Cairns, 1977; Schendel and Robins, 1978; Newbold et al., 1980; Sklar and Strauss, 1980). This adduct is believed to mispair with thymine (Loveless, 1969; Coulondre and Miller, 1977; Saffhill and Abbott, 1979; Meuth, 1981), which is consistent with the apparent GC → AT specificity of MNNG mutagenesis (Krieg, 1963; Prakash and Sherman, 1973; Coulondre and Miller, 1977). Similar specificity of mutagenesis was found for ethyl methanesulfonate (Prakash and Sherman, 1973; Coulondre and Miller, 1977), which also is known to produce large amounts of O-$^3$-ethylguanine (Pegg, 1977). In contrast, methyl methanesulfonate and dimethyl sulfate, which produce low levels of O-$^3$MeG (Pegg, 1977), have no such specificity of mutagenesis (Prakash and Sherman, 1973); and predictably, the size of intracellular pools of dCTP and dTTP has much less influence on methyl methanesulfonate mutagenesis than on MNNG mutagenesis (Meuth, 1981).

Mutagenesis of bacteriophage T7 was induced by incorporation of O-$^3$MeG (from O-$^3$Me-dGTP) during in vitro DNA synthesis (Dodson et al., 1982). Recently, the O-$^3$MeG DNA adduct was directly demonstrated to cause mutations in E. coli (Loechler et al., 1984).

Second, circumstantial evidence has been used to link the O-$^3$MeG DNA adduct to the cytotoxicity of MNNG and of other S$_2$1 alkylating agents. (a) A correlation was found between toxicity of alkylating agents and their ability to methylate or ethylate the O-$^3$-position of guanine in DNA (Peterson and Peterson, 1982). (b) The resistance of cells to the toxicity of alkylating agents has heretofore been generally correlated with both their resistance to mutagenic effects (Jegg et al., 1977; O'Neill and Hsie, 1977; Samson and Cairns, 1977; Sedgwick and Robins, 1980) as well as their capacity (constitutive or inducible) to remove O-$^3$MeG from DNA (Samson and Cairns, 1977; Day et al., 1980; Sedgwick and Robins, 1980; Shihol and Becker, 1981; Sklar and Strauss, 1981). Accordingly, in sharp contrast to sensitive cells, resistant cells were shown to possess or be able to induce large amounts of O-$^3$MeG methyltransferase, the acceptor protein that specifically removed alkyl groups from the O-$^3$-position of alkylated guanine (Lindahl et al., 1982; Mitra et al., 1982; Waldstein et al., 1982; Yarosh et al., 1983; Domaradzki et al., 1984).

The O-$^3$MeG adduct was not found to block DNA replication since the alkylated DNA template containing O-$^3$MeG adducts allows by-pass of replication in cell-free systems (Abbott and Saffhill, 1979; Lockhart et al., 1982) and in intact cells (Abanobi et al., 1980). Our data also indicate that the lethal alkylation of DNA after incubation of cells in 0.14 $\mu$M MNNG still permitted the first round of replication. A human cell line (HeLa) was reported to behave similarly after treatment with MNU (Plant and Roberts, 1971).

Of particular value in thinking about these results may be the evidence that O-$^3$MeG-T mismatch formation is responsible for the toxicity of the O-$^3$MeG DNA adduct. The frequency of O-$^3$MeG-T formation during DNA replication in a cell-free system is increased by elevated dTTP concentrations and decreased by elevated dCTP concentrations (Abbott and Saffhill, 1979). This fact implies that intracellular pool sizes of dTTP and dCTP may influence the frequency of formation of the mismatch in cells treated with alkylating agents. Therefore, if toxicity of MNNG is induced by the formation of the O-$^3$MeG-T pair, then preventing formation of this pair would protect cells from toxic effects after MNNG treatment. Indeed, in several independent studies, elevated dCTP levels protected cells from the toxicity of alkylating agents, whereas elevated dTTP levels sensitized cells (Peterson et al., 1978; Brennaud et al., 1981; Meuth, 1981; Peterson and Peterson, 1982). A possible mechanism of toxicity of O-$^3$MeG-T mismatches may be through the operation of a DNA mismatch repair system, which has been found in bacteria as well as mammalian cells (Glickman, 1982; Hare and Taylor, 1985). These bacterial and mammalian repair systems seem to remove the "wrong" base specifically from the daughter strand of DNA. Since O-$^3$MeG is in the parental strand during replication, the DNA mismatch repair system might be inefficient in correcting O-$^3$MeG-T mismatches and would remove thymine from the daughter strand, leaving O-$^3$MeG in the parental strand of DNA (Karran and Marinus, 1982). Subsequent repair synthesis would again introduce the mismatch, so that the repeated removal of thymine and following events could lead to a continuous secondary lesion in DNA, such as a gap. Data of Fujimura (1975) are consistent with the formation of such DNA breaks. He reported that pretreatment with MNU increased the number of single-strand breaks only in the newly synthesized part of DNA of mouse L-cells.

Recently, Doutriaux et al. (1986) have reported that λ phage DNA with mismatches is cleaved at mismatch-proficient DNA and that growth of λ in mismatch-deficient bacteria increases survival of transfecte λ. An analogous situation may arise in human cell's action on its own mismatched DNA. In any case, the passage of highly alkylated DNA through S phase as in TK6 cells without attendant removal of any adduct measured save 2MeA implies that daughter strands contain many mismatches. It is quite possible that unremoved O-$^3$MeG adducts represent both prelethal and premutagenic lesions independent of our knowledge of the role of other adducts.

MT1 Cells Differ in Cell Cycle Progression—Since MT1 is a clonal derivative of TK6 isolated after a frameshift mutagen exposure that would yield a mutant fraction of 3 x 10$^{-2}$ for an X-linked marker such as hgpR (data not shown), we make the assumption that all of the aspects of the new phenotype arise from a single frameshift mutation in a single gene but not necessarily the same gene for each member of the MT group. Table IV summarizes 10 phenotypic characteristics for the two cell lines, with seven of the characteristics showing significant differences between the parent and MT1 clonal derivative.

From Table IV, it is clear that for the five DNA adducts measured after MNNG treatment, there is no difference between TK6 and MT1 lines with regard to initial amounts or rates of subsequent removal (see also Fig. 9). Furthermore, both cell types show the same amount and time of appearance of hydroxyurea-insensitive [3H]thymidine uptake (see also Fig. 11), which is used here as a gross estimate of DNA excision/polymerization repair activity. At the level of cell survival, it is clear that TK6 cells are more sensitive to MNNG than are MT1 cells by a factor of 1500 (see also Figs. 1 and 6). The spontaneous mutation rate of MT1 is 45 times greater than that of TK6, but the rate of MNNG-induced mutation of MT1 is only 2 times greater than that of TK6 (see also Figs. 7 and 8). Immediately after toxic MNNG treatment, TK6 cells continue cell cycle progression, eventually accumulating in the S or G2 phase of the next division cycle. The doubling time of survivors immediately after treatment is similar to that of untreated control cells. A quite different behavior follows MNNG treatment of MT1 cells. Toxic MNNG treatment arrests these cells prior to S phase, and eventually the population as a whole takes on a cell cycle distribution similar to untreated cells. However, doubling times of survivors are more than twice those of untreated cells for some days after treatment. Finally, there is the curious and repeated observation that colony-forming efficiency of
DNA adducts would be expected. However, this mechanism adducts might be related to a deficiency in a mismatch repair of MT1 cell line. 

TBoxions favor this hypothesis. (a) One would expect the mismatch repair systems to act would have a higher probability of survival. Initially lethal and potentially mutagenic DNA lesions, then the cell that stopped DNA synthesis and allowed those constitu-
tively defective systems to be a mutator, as was reported in bacteria (Glickman and Radman, 1980); and according to match repair-deficient line to be a mutator, as was reported et al. (1982) isolated a human lymphoblast line that possessed the O'MeG-removing system but was still more sensitive to MNNG killing than were other similar lines. However, this mechanism alone does not explain the high spontaneous rate of mutation of MT1 cell line.

MT1 Cell Line May Be Mismatch Repair-deficient—The resistance of the MT1 cell line to MNNG via tolerance of the adducts might be related to a deficiency in a mismatch repair system. Alkylated base mismatches would form but would not lead to formation of prelethal DNA lesions. Several observations favor this hypothesis. (a) One would expect the mismatch repair-deficient line to be a mutator, as was reported in bacteria (Glickman and Radman, 1980); and according to our data, MT1 is a mutator. (b) This model predicts that this mechanism of resistance of the MT1 cell line should not prevent mutation of the cells by MNNG since the principal mutagenic O'MeG DNA adduct is not removed and its mutagenic replication is not prevented. Indeed, our data (Fig. 7) show that MT1 is not resistant to MNNG-induced mutation. (c) Also, if the model is correct and MNNG-induced lethal lesions do not appear in the DNA of resistant cells, MNNG toxicity in the MT1 resistant cells must be due to some other mechanism. There is some indirect indication that the mechanisms of MNNG-induced toxicity in TK6 and MT1 cells are different. First, exposure of TK6 and MT1 cells to equally toxic MNNG concentrations allows TK6 cells to undergo one division before the cells die, whereas MT1 cells are arrested immediately. Second, whereas in the TK6 cell populations exposed to MNNG, survivors continue to grow at a normal rate with some cells killed and some cells unharmed, MT1 survivors grow at a slower rate with all cells injured. Third, following superlethal MNNG exposure, TK6 cells eventually accumulate toxic MNNG concentrations allows TK6 cells to undergo one division before the cells die, whereas MT1 cells are arrested immediately. Therefore, the apparent average half-life of 17 h for O'MeG, 3MeG, 7MeG, and 7MeA is accounted for by the increase in total DNA rather than by adduct removal, i.e. it appears that none of these adducts is removed during the period of observation.

**TABLE IV**

Phenotypic characteristics of MNNG-sensitive and MNNG-resistant cell lines

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>TK6</th>
<th>MT1</th>
</tr>
</thead>
<tbody>
<tr>
<td>(D_{57}) (MNNG), 45 min</td>
<td>0.04 (\mu M)</td>
<td>6 (\mu M)</td>
</tr>
<tr>
<td>Spontaneous mutation rate (6TG(^6))</td>
<td>$1.3 \times 10^{-7}$ mutations/day</td>
<td>$5.8 \times 10^{-6}$ mutations/day</td>
</tr>
<tr>
<td>MNNG-induced mutation (6TG(^6), 45 min)</td>
<td>$5.7 \times 10^{-4}$ mutations/cell (\times 10^{-6}) MNNG</td>
<td>$1.25 \times 10^{-4}$ mutations/cell (\times 10^{-6}) MNNG</td>
</tr>
<tr>
<td>Absolute clone-forming ability (low density, 10(^7) feeder cells)</td>
<td>0.36 ± 0.07</td>
<td>0.014 ± 0.003</td>
</tr>
<tr>
<td>Initial survivor doubling time after MNNG treatment</td>
<td>18 h (unchanged from untreated cells)</td>
<td>≥36 h (increased from 20 h in untreated cells)</td>
</tr>
<tr>
<td>Initial formation of adducts after 8 (\mu M) MNNG, 45-min treatment</td>
<td>adducts/cell (\times 10^{-4})</td>
<td>adducts/cell (\times 10^{-4})</td>
</tr>
<tr>
<td>O'MeG</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>3MeG</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>3MeG</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>7MeG</td>
<td>125</td>
<td>125</td>
</tr>
<tr>
<td>7MeA</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Half-life of adduct(^a)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O'MeG</td>
<td>20 h</td>
<td>20 h</td>
</tr>
<tr>
<td>3MeG</td>
<td>19 h</td>
<td>17 h</td>
</tr>
<tr>
<td>3MeG</td>
<td>≤1 h</td>
<td>≤1 h</td>
</tr>
<tr>
<td>7MeG</td>
<td>~17 h</td>
<td>~17 h</td>
</tr>
<tr>
<td>7MeA</td>
<td>~13 h</td>
<td>~13 h</td>
</tr>
<tr>
<td>Initial cell cycle progression effect (one generation time, lethal dose)</td>
<td>No effect after treatment with 0.14 (\mu M) MNNG, 45 min</td>
<td>Marked decrease in S phase fraction after 10 (\mu M) MNNG, 45 min</td>
</tr>
<tr>
<td>Cell cycle block (three generations)</td>
<td>Marked S and G2 + mitosis accumulation</td>
<td>Return to ~normal distribution</td>
</tr>
<tr>
<td>Hydroxyurea-insensitive ([^{35}S])thymidine uptake into DNA</td>
<td>7-fold over background (1-h post-treatment)</td>
<td>7-fold over background (1-h post-treatment)</td>
</tr>
</tbody>
</table>

\(^a\)DNA increased with a doubling time of 18.4 h in population after treatment. Therefore, the apparent average half-life of 17 h for O'MeG, 3MeG, 7MeG, and 7MeA is accounted for by the increase in total DNA rather than by adduct removal, i.e. it appears that none of these adducts is removed during the period of observation.
clones. The parental and the transformant cell lines were equally sensitive to MNNG, which led the authors to believe that O'MeG adduct might not be toxic for these cells. However, the parental Chinese hamster ovary cell line was extremely resistant to MNNG (about 100-fold compared with TK6 and other Mex- cell lines). The large doses of MNNG that were required to kill these cells might, according to our data (Fig. 4), induce formation of 2 x 10^4 to 1 x 10^5 O'MeG DNA adducts per cell, which would exceed the capacity of the repair system (10^4 molecules of O'MeG methyltransferase per cell).

Thus, these data are merely consistent with the suggestion that O'MeG adduct is not toxic, but do not prove it. The hypothesis that O'MeG adduct is not toxic would not explain the correlation between the presence of this adduct in cell DNA and cytotoxic effects induced by S,1 alkylating agents as found in numerous studies. On the contrary, the hypothesis put forward in this paper satisfactorily explains the experimental data concerning O'MeG adduct toxicity: O'MeG adduct is toxic due to the formation of O'MeG-T pairs and the subsequent functioning of the DNA mismatch repair system. A deficiency in this system may cause the cells to tolerate this base pair. Studies of the DNA mismatch repair system that has been recently discovered in mammalian cells (Hare and Taylor, 1985) may provide a basis for further testing of this hypothesis.

Acknowledgments—We thank Dr. D. A. Kaden for permission to use her preparation of ICR-191-treated populations of TK6 cells. B. Brunengraber and J. McDowell provided technical assistance for part of the work.

REFERENCES


Fujisawa, Y. (1975) Cancer Res. 35, 2780-2789


Additional references are found on p. 12471.

Continued on next page.
Somatic Cell Mutants Resistant to Alkylation Agents

**Supplemental Material**

**ISOLATION AND CHARACTERIZATION OF HUMAN CELL MUTANTS DIFFERING IN SUSCEPTIBILITY TO KILLING AND MUTATION BY METHYL-NITROSOUREA AND N-NITRO-NITROSOQUATERNARY AMINES**

By Victor S. Goldman, Robert A. Galva, Jr., and William S. Thrilly

**EXPERIMENTAL PROCEDURES**

**Chemicals**

MNU, 5,6-dimethyl-2-pyrimidinonic acid, 1-methylden, 1-methylglyzer, 7-amino-3-methylcholamidopterin, N-trifluoracetic acid, HCl, ampicillin, and tryptophan were purchased from Bio-Rad Laboratories, INC. (Mortlake, Hounslow, Middlesex, England). Hydroxyurea (B.D. Hjorf, Inc., Arlington, VA), dimethyl sulfoxide, 1 molar perchloric acid, and sodium citrate (0.015 ml) were purchased from Sigma Chemical Co. (St. Louis, MO). 100X RNAase solution was purchased from Roche (Nutley, NJ). 200X Tris-phosphate buffer solution (9.5 molar Tris and 0.5 molar NaCl) was purchased from Miles Laboratories (Indianapolis, IN). 1% Polyethylene glycol (PEG-4000) (supplied by Mallincrodt Baker, Inc., Phillipsburg, NJ) was used to precipitate DNA. Other chemicals and solvents were analytical or HPLC grade.

**Media**

Cells were grown in RPMI 1640 medium (Flow Laboratories, McLean, VA, or GIBCO, Grand Island, NY) supplemented with 5% or 10X horse serum (Faw Labs or GIBCO). The horse serum was heat-treated before use to eliminate a factor that degraded calf thymus and calf thylus DNA. In the presence of calf and in corresponding control plates, cells were plated in RPMI-1640 medium supplemented with 10% heat-treated (3, 50°C) horse serum. Cell medium was RPMI-1640 medium supplemented with 10% or 10X horse serum and 2% HNO3.

**Cells and Cell Culture Maintenance**

The human lymphoblast line 766 is a clone derivative of the WI-38 line, which was isolated from a male donor with hereditary spherocytosis (Ley et al., 1969).

**Cell Synthesis**

Cells were continuously maintained in a humidified atmosphere containing 10% CO2 and were daily subcultured to 1 x 106 cells/ml (cycling time was 16-37 days). Cell counts were based on a Coulter Counter (Coulter Electronics, Hialeah, FL).

**Alkylating Agents**

In order to observe colony formation from single cells, cultures were plated in tissue culture grade multiwell plates (Nalge Division of Labomed, Random, CA) containing 0.2-0.4 ml flat plates per well. The lymphoblasts wereformed mononuclear colonies of about 2 x 10Scells after two weeks of incubation at 37°C in a humidified atmosphere containing 10% CO2. Cell cultures were collected for bacterial, yeast, or mycoplasma infections and found to be free of these organisms.

**Cell Cycle Analysis by Flow Cytometry**

Quantitative analysis of cell cycle distribution in asynchronous lymphoblast populations was performed on the Flow Cytometer System 580 (Ortho Diagnostic Systems, Westwood, MA). The analysis is based on differences in DNA content in cells that are in different stages of growth. DNA was stained with propidium iodide (PI) and analyzed by using a Dolop II flow cytometer to detect changes in PI fluorescence as a result of DNA content.

**Isolation of Mutants Resistant to Alkylation Agents**

The isolation of mutants resistant to alkylation agents was performed using the WI-38 cell line. A TK6 cell population, which was labeled with 14C-labeled thymidine at 10 mCi/ml followed by incubation with 1 mCi/ml, was exposed to MNU dissolved in dimethyl sulfoxide OP at a concentration of 10-4 M. Cells were treated and untreated as a control and incubated in fresh medium for 10 min or 60 min. Cultures were then washed with fresh medium and subcultured in the presence of 18 mCi/ml of 3H-thymidine. After 24 hr of incubation, cultures were incubated for 3 hours in fresh medium containing 100 μg/ml of acridine orange, which was used to stain the cell nuclei. The cells were then washed with fresh medium and incubated for 24 hours in fresh medium containing 100 μg/ml of acridine orange and 10 μg/ml of ethidium bromide. The cultures were then harvested and spread onto slides for microscopic examination.

**RESULTS**

**Determination of DNA Alkylation by High Performance Liquid Chromatography**

DNA was isolated from cells that were treated with MNU and analyzed by high performance liquid chromatography (HPLC). The DNA was isolated from cells that were treated with MNU and analyzed by high performance liquid chromatography (HPLC) using an Acquity UPLC system (Waters Associates, Milford, MA), with fluorescence detection at 254 nm. A 100-μl aliquot of cell culture DNA was treated with 1 M MMS for 1 hr at 37°C. The mixture was then centrifuged and the supernatant was removed and diluted to 1 ml with 0.1 M sodium acetate, pH 6.7. Then proteinase K (30 μg) was added to the mixture, and the mixture was incubated at 4°C. DNA was precipitated with 2 M ammonium acetate and 2 M isopropanol, and the DNA was pelleted by centrifugation at 1500 x g for 1 hr. The DNA pellet was washed with 80% ethanol and 70% ethanol, and the DNA was resuspended in 2 μl of water.

**Measurement of ODe**

DNA was purified by phenol-chloroform extraction followed by ethanol precipitation. The DNA was then resuspended in water and analyzed by electrophoresis in an agarose gel. The DNA was analyzed by gel electrophoresis and quantitated by densitometry. The amount of DNA in the sample was determined by measuring the absorbance at 260 nm. The concentration of DNA was calculated using the absorbance at 260 nm. The concentration of DNA was calculated using the absorbance at 260 nm. The concentration of DNA was calculated using the absorbance at 260 nm. The concentration of DNA was calculated using the absorbance at 260 nm. The concentration of DNA was calculated using the absorbance at 260 nm.
Cells were plated in two plates (152 wells) per plate in 0.2 ml medium per well in nonselective (3 cells/well) or selective (10 cells/well) conditions. Selective medium contained 4 µg/ml of 6-TG. The plates were incubated at 37°C in a humidified incubator with 5% CO₂ for 13.15 days until colonies formed, after which colonies were counted for determination of mutant fractions. Each experiment consisted of treating two independent lymphoblast cultures on the same day for each concentration of mutagen used. Two separate experiments were performed. Toxicity of alkylating agents did not affect our estimates of the mutant fraction, since the cells were allowed to recover from the toxic effects of the mutagenic treatment before plating for determination of mutant fraction. Calculations of surviving fraction and mutant fraction were performed by application of Poisson statistics, as described by Fath et al., 1981.

RESULTS

Toxic and Mutagenic Action of MNNG and MNU toward TK6 Cells

The toxic and mutagenic effects of MNNG and MNU towards TK6 cells are shown in Figs. 1 and 2. In these experiments, the TK6 cells appear to be 100% to 1000 times more sensitive to the lethal effects of MNNG than many other cell lines such as human lymphoblast and fibroblast lines, HeLa variants, and Chinese hamster cells (Baker et al., 1979; Day et al., 1980; Samson and Schwartz, 1980; Sklar and Strauss, 1980; Peterson and Peterson, 1980).

FIG. 1. Toxic and mutagenic action of MNNG (48-hr exposure) toward TK6 cells. Fractions of 157°C to 17°C were determined from the appearance of TK6-resistant colonies. Each point represents the mean from two independent experiments. Vertical bars are standard errors of the means. A x 50 cells/well were exposed to MNNG in RPMI-1640 medium supplemented with 5% horse serum at 37°C. Surviving colonies were determined from back-extrapolation of growth curves of the treated cultures.

FIG. 2. Toxic and mutagenic action of MNU (24-hr exposure) toward TK6 cells. Fractions of 15°C to 17°C were determined from the appearance of TK6-resistant colonies. Each point represents the mean from two independent experiments. Vertical bars are standard errors of the means. Cells were exposed to MNNG in RPMI-1640 medium supplemented with 10% horse serum at 37°C. Surviving colonies were determined from back-extrapolation of growth curves of the treated cultures.

DNA Alkylation Adducts

We used HPLC separation to quantitatively measure the levels of several methylated DNA adducts which might be induced by MNNG. These were 2-methylguanine, 3-methylguanine, 1-methyladenine, 3-methyladenine, 7-methylguanine, and 7-methyladenine. Fig. 3 represents a typical chromatogram for MNNG and MNU. The peaks were separated and monitored at 254 nm from 15°C to 17°C. The purity of each peak was determined by thin-layer chromatography and by reversed-phase high-performance liquid chromatography. The peaks were individually applied to a reverse-phase separation column. The retention times for methylphosphonate standards in the reverse phase column were 7 min for 7MeA, 12 min for 7MeG, 18.5 min for 3MeA, and 18 min for 3MeG. All peaks of radioactivity coeluted with the same standards of methylated purines in both separations. Peaks 4 and 6 were extracted exclusively with 1N HCl, and no detectable amounts of DNA were found.

In control experiments, the stability of methylated and nonmethylated purines to the depurination procedure was analyzed. For this purpose, a methylphosphate or a purine at a concentration of about 1 x 10⁻¹⁸ M was treated as described in the procedure for DNA depurination and then applied to the cationic exchange HPLC column. The peak corresponding to the standards was isolated, and the concentration was measured spectrophotometrically and compared with the starting solutions. Two independent experiments were performed. The recovery was measured (mean ± S.E.M.): 92 ± 2% for 6, 94 ± 2% for 6m, 94 ± 3% for 7MeA, 94 ± 1% for 7MeG, 88 ± 3% for 7MeA, 92 ± 5% for 7MeA, 88 ± 1% for 7MeA, and 68 ± 0% for 7MeA.

REFERENCES FOR REPRINT SECTION


References


