An alkylation-tolerant, mutator human cell line is deficient in strand-specific mismatch repair

cell death/DNA damage/DNA repair/mutagenesis

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Communicated by Robert L. Hill, April 19, 1993

ABSTRACT The human lymphoblastoid MT1 B-cell line was previously isolated as one of a series of mutant cells able to survive the cytotoxic effects of N-methyl-N'-nitro-N-nitrosoguanidine (MNN). MT1 cells nevertheless remain sensitive to mutagenesis by MNN and display a mutator phenotype. These phenotypes have been attributed to a single genetic alteration postulated to confer a defect in strand-specific mismatch repair, a proposal that attributes the cytotoxic effect of DNA alkylation in wild-type cells to futile attempts to correct mispairs that arise during replication of alkylated template strands. Our results support this view. MNN-induced mutations in the HPRT gene of MT1 cells are almost exclusively G-C → A-T transitions, while spontaneous mutations observed in this mutant cell line are single-nucleotide insertions, transversions, and A-T → G-C transitions. In vitro assay has demonstrated that the MT1 line is in fact deficient in strand-specific correction of all eight base-base mispairs. This defect, which is manifested at or prior to the excision stage of the reaction, is due to simple deficiency of a required activity because MT1 nuclear extracts can be complemented by a partially purified HeLa fraction to restore in vitro repair. These findings substantiate the idea that strand-specific mismatch repair contributes to alkylation-induced cytotoxicity and imply that this process serves as a barrier to spontaneous transition, transversion, and insertion/deletion mutations in mammalian cells.

Isolation of N-methyl-N'-nitro-N-nitrosoguanidine (MNN)-resistant derivatives of mammalian cell lines has demonstrated that the mutagenic and cytotoxic effects of alkylation agents can be resolved by mutation (1, 2). The MT1 cell line, which is the subject of this report, was isolated from the human lymphoblastoid TK6 line after frameshift mutagenesis (1). Although the MT1 line is several hundred times more resistant than TK6 to the toxic effects of MNN, MT1 cells are slightly more sensitive to MNN mutagenesis than are cells of the parental lineage, consistent with the observation that kinetics of formation and removal of DNA adducts are similar in the two cell lines (1). Therefore, the resistance of MT1 cells to the cytotoxic action of MNN is due to their ability to tolerate alkylation adducts that lead to cell death in the parental TK6 line.

In addition to this profound tolerance for alkylation adducts, the MT1 line also differs from TK6 with respect to cell cycle progression after treatment with MNN to 10% survival (1). TK6 proceeds through a single division after MNN treatment and arrests after the second S phase—behavior similar to that observed with HeLa cells (3). In contrast, MT1 cells display an apparent block at S phase entry, but the population soon returns to a normal cell cycle distribution. Unlike TK6 survivors of MNN treatment, which proliferate at normal rates, surviving MT1 cells grow at a markedly diminished rate for several days to a week after treatment, although a proliferative rate similar to that of untreated cells is eventually achieved.

The two cell lines also differ phenotypically in the absence of alkylation agents. MT1 has a mutator phenotype, with spontaneous mutability increased about 60-fold at the HPRT locus (1). Furthermore, MT1 cells exhibit poor clone-forming ability at low cell densities, in contradistinction to the high cloning efficiency of TK6, and also display a greater serum dependence than the parental line.

MT1 was derived from TK6 by frameshift mutagenesis under conditions that yield a mutant fraction of 0.003 for the single-copy HPRT gene. Thus, phenotypic characteristics of MT1 cells were attributed to a single genetic alteration (1). Since resistance to the cytotoxic action of MNN and hypermutability had been reported as characteristics of Escherichia coli mismatch repair mutants (4–6), Goldmacher et al. (1) suggested that the MT1 phenotype is due to a defect in a strand-specific mismatch repair system analogous to the bacterial methyl-directed pathway, which is responsible for removal of biosynthetic errors from newly synthesized DNA (7). This hypothesis is based on the premise that miscoding alkylation adducts on the template strand would lead to anomalous base pairs upon replication. Provocation of mismatch correction by such lesions would result in a futile turnover of the newly synthesized strand because the offending adduct is not removed from template DNA, a process that could lead to cell death.

The use of in vitro assays for mismatch repair has demonstrated that human cells possess a strand-specific mismatch repair system that is homologous to the E. coli methyl-directed system (8–10). As in the case of the bacterial reaction (11, 12), mismatch correction by the human system can be targeted to a DNA strand by a strand break (8, 9), and both systems share a similar mismatch specificity as well as an unusual bidirectional excision mechanism (8–10, 13, 14). Using a biochemical assay, we demonstrate here that the MT1 cell line is in fact characterized by a major defect in strand-specific mismatch repair. We also show that mutations produced in MNN-treated MT1 cells are almost exclusively G → A transitions, while those occurring spontaneously comprise single-nucleotide insertions, transversions, and A → G transitions.

MATERIALS AND METHODS

Cell Lines and Nuclear Extracts. Human lymphoblastoid B-cell lines TK6 and MT1 have been described (1). These lines were grown in RPMI 1640 medium (GIBCO) supple-

Abbreviations: TGR, thiguanine resistance; MNN, N-methyl-N'-nitro-N-nitrosoguanidine.
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mented with 10% (vol/vol) horse serum (HyClone) by daily dilution after growth to a cell density of 1 x 10^6 cells per ml. Culture samples removed at the time of harvest were tested for sensitivity to killing by MNNG as described (1). HeLa S3 cells were grown, and nuclear extracts were prepared from HeLa, TK6, and MT1 lines as described by Holmes et al. (8).

**Mutational Spectra.** Hot-spot mutational spectra in the third exon of the HPRT gene were determined after en masse selection for 6-thioguanine resistance (TG^8). The frequency and nature of hot-spot mutations were determined by mutational spectrometry as described (15). Spontaneous and MNNG-induced spectra for MT1 cells were based on cultures derived from an untreated population grown from 100 cells and the same population treated with 4 μM MNNG after it had achieved a cell number of 10^6. Base pair assignments for mutations are relative to the adenine of the ATG initiation codon, which is assigned position 1, and nucleotide designations refer to the untranscribed DNA strand.

**Heteroduplexes and Mismatch Repair Reactions.** A set of homologous, 6440-base-pair circular heteroduplexes representing each of the eight possible base-base mispairs was prepared by using the collection of f1MR phages (16) as described (10). Each heteroduplex contained a mispair at position 5632 and a single-strand break in the complementary DNA strand at the Sau96I site (coordinate 5757), 125 base pairs 5' to the mismatch as viewed along the shorter path separating the two sites in the circular substrate. G-T heteroduplexes containing a site-specific incision in the complementary strand at the HindII site (coordinate 0/6440, 808 base pairs 5' to the mispair, shorter path) or in the viral strand at the gene II protein cleavage site (coordinate 5813, 181 base pairs 3' to mismatch, shorter path) have been described (10). A control G-C homoduplex with a HindII interruption in the complementary DNA strand was prepared by methods identical to those used for heteroduplex construction. Unless specified otherwise, mismatch repair was assayed in 10-μl reaction mixtures containing 24 fmol of heteroduplex and 100-120 μg of nuclear extract protein (8, 10). Incubation was at 37°C for 15 min, conditions under which product formation was limited by the rate of repair.

**RESULTS**

Spontaneous and MNNG-Induced Hot-Spot Mutations in MT1 Cells. The rate of spontaneous mutation to TG^8 was about 6 x 10^{-6} per division for MT1 cells compared with a rate of 10^{-7} per division for TK6 parental line, a mutator effect of 60-fold. Four spontaneous hot-spot point mutations were identified in exon 3 of the HPRT gene in populations of MT1 cells. Insertion of a guanine residue into the GGGGGG sequence between base pairs 207 and 212 accounted for 13% of the total TG^8 population. Hot-spot substitution mutations were observed at base pair 217 (A → T, 12% of TG^8) and at base pair 248 (A → G, 1% of TG^8), while a curious double mutation involving base pairs 223 (T → G) and 269 (A → G) accounted for 5% of the total TG^8 mutants. This mutation spectrum was distinct from that observed for MT1 populations that had been treated with 4 μM MNNG. Twenty percent of the TG^8 population isolated after MNNG treatment were due to G → A transitions occurring at base pairs 209 or 212, with mutations at each site accounting for 10% of the TG^8 population. A third G → A transition hot spot was observed at base pair 234. This mutation, which does not alter the coding properties of the exon, was a super hot spot, occurring in 1% of the treated population, irrespective of selection. Identification of an unselected mutation occurring with a frequency of 1% is without precedent in mutagen-treated mammalian cells.

During the course of this study, hot-spot mutations in HPRT exon 2 and exons 4-9 were also characterized in MT1 cells. These findings, which will be described elsewhere, were similar to those obtained with exon 3 (A.K. and W.G.T., unpublished data). Eight additional spontaneous hot spots were found of which seven were base pair substitutions, but none were G → A transitions. Eleven additional hot-spot mutations were also characterized for MNNG-treated MT1 populations. All of these were single base pair substitutions and 10 of 11 were G → A transitions.

**MT1 Cells Are Deficient in Strand-Specific Mismatch Repair.** Nuclear and whole-cell extracts derived from HeLa cells support a strand-specific repair reaction on heteroduplexes containing a single-strand break, with correction being highly biased to the incised strand (8, 9). This reaction is mismatch-provoked as judged by differing repair efficiencies for the different mismatches (8, 9) and by mismatch-dependent formation of excision tracts spanning the mismatch and the strand break that directs the reaction (10). Since aphidicolin inhibits repair of each of the eight base-base mismatches and since similar excision reactions occur on each of the representative heteroduplexes, correction of this set of substrates has been attributed to action of a single pathway (10).

We have used this set of eight heteroduplexes to evaluate mismatch repair activity of nuclear extracts derived from TK6 and MT1 cell lines. Each circular heteroduplex contained a single base-base mispair and a site-specific complementary strand break located 125 base pairs 5' to the mismatch as viewed along the shorter path between the two sites. Nuclear extract prepared from TK6 cells is as proficient in G-T heteroduplex repair as an extract derived from HeLa nuclei (Fig. 1). By contrast, G-T repair activity in nuclear extract prepared from the MT1 cell line was not significantly above background. As is evident from the mixing experiments shown in Fig. 1, this G-T repair defect is not due to presence of a diffusible inhibitor in extracts of the MT1 line. Furthermore, supplementation with cytoplasmic fractions derived from TK6 or the MT1 line did not restore repair to the MT1 nuclear fraction (not shown).

Similar results were obtained with each of the other seven heteroduplexes. As summarized in Table 1, nuclear extracts of HeLa and TK6 lines displayed similar specific repair.

![Fig. 1](image_url)
activities with each of the base–base mispairs, while extracts derived from MT1 cells were repair-deficient in each case. However, the extent of the MT1 repair deficiency depended somewhat on the nature of the mismatch. Correction of G-T, A-C, A-G, T-T, C-T, and C-C by MT1 nuclear extract was not above background, but A-A and G-G were subject to significant repair, at rates about 20% of those observed with the TK6 parent. It is not clear whether residual activity on these purine–purine mispairs is due to action of a second repair system or, alternatively, whether the lesion responsible for the MT1 phenotype confers only a partial defect on purine–purine mismatch correction mediated by the strand-specific pathway described above.

Using circular fl heteroduplexes, we have shown (10) that a site-specific strand break on either the viral or complementary DNA strand is sufficient to direct mismatch correction in HeLa nuclear extracts. Mismatch-provoked excision with either kind of heteroduplex was found to remove that portion of the incised strand spanning the shorter path between the mismatch and the strand break, thus implying a bidirectional excision mechanism (10). The heteroduplexes used in the experiments of Fig. 1 and Table 1 contained a complementary strand interruption located 5' to the mismatch as viewed along the shorter path joining the two sites. In view of the bidirectional capability of the human repair system, we have also tested MT1 nuclear extracts for their ability to support correction of the alternate heteroduplex orientation. A G-T heteroduplex with a viral strand incision 181 base pairs 3' to the mismatch (shorter path) was efficiently repaired in TK6 nuclear extract (140 fmol/mg) but was a weak substrate for correction by MT1 nuclear fractions (14 fmol/mg). The MT1 mismatch repair defect is therefore evident with both heteroduplex orientations.

**MT1 Defect Blocks Mismatch Repair Prior to Excision.** The finding that MT1 nuclear extracts are deficient in repair of both heteroduplex orientations suggested that mismatch repair in this cell line is blocked at a step prior to excision. This was confirmed by direct analysis of mismatch-provoked excision intermediates, which accumulate in repair reactions when DNA synthesis is inhibited by aphidicolin (10). These intermediates contain a single-strand region that spans the original locations of the mismatch and the strand break; directing correction, and gap endpoints can be mapped relative to a restriction endonuclease cleavage site by electrophoresis under denaturing conditions.

Fig. 2 presents an analysis of 5' termini proximal to the mismatch as a test for production of excision intermediates in HeLa, TK6, and MT1 nuclear extracts. The circular G-T heteroduplex used in these experiments contained a complementary strand incision 808 base pairs 5' to the mismatch (shorter path) with an otherwise identical G-C homoduplex used as control. Analysis of products isolated from standard repair reactions demonstrated that the complementary strand of both G-T and G-C DNAs had been largely converted to a covalently closed form in all three extracts, as evidenced by conversion of the 3.9-kilobase (kb) species to a 6.4-kb full-length strand. However, inclusion of aphidicolin in the reactions led to the mismatch-dependent accumulation of excision intermediates in HeLa and TK6 extracts, with the major species mapping about 3 kb from the site of cleavage by Bsp106 that was used to linearize repair products. These results confirm previous findings with HeLa nuclear extracts (10). Production of this class of intermediate was not observed with the G-T heteroduplex in MT1 extracts. Rather, most of the complementary strand was converted to a covalently closed species, consistent with the finding in the bacterial reaction that mismatch-dependent excision competes with ligase for the strand break that directs correction (14). We have concluded therefore that the repair defect in the MT1 line blocks repair at an early step in the reaction.

**Nuclear Extracts of MT1 Cells Are Complemented by a Partially Purified Nuclear Fraction from HeLa Cells.** Inasmuch as a diffusible inhibitor is not responsible for the MT1 repair defect, we have tested the possibility that the MT1 nuclear fraction might be deficient in an activity that is present in a "wild-type" human cell line. This would also be the simplest expectation in view of the fact that the MT1 line was produced by frameshift mutagenesis (1). This expectation was confirmed by the demonstration that MT1 nuclear extracts were complemented in vitro by a partially purified HeLa nuclear fraction obtained by chromatography on phos-

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**Table 1.** MT1 cells are deficient in repair of base-base mismatches

<table>
<thead>
<tr>
<th>Mismatch</th>
<th>Repair, fmol/mg</th>
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<tbody>
<tr>
<td></td>
<td>HeLa TK6 MT1</td>
</tr>
<tr>
<td>G-T</td>
<td>84 ± 19 84 ± 12 ± 2 ± 1</td>
</tr>
<tr>
<td>A-C</td>
<td>57 ± 9 32 ± 12 2 ± 1</td>
</tr>
<tr>
<td>A-A</td>
<td>55 ± 7 66 ± 7 9 ± 5</td>
</tr>
<tr>
<td>A-G</td>
<td>32 ± 6 17 ± 2 4 ± 2</td>
</tr>
<tr>
<td>G-G</td>
<td>89 ± 13 70 ± 17 13 ± 4</td>
</tr>
<tr>
<td>T-T</td>
<td>23 ± 8 32 ± 23 2 ± 1</td>
</tr>
<tr>
<td>C-T</td>
<td>58 ± 8 37 ± 10 4 ± 3</td>
</tr>
<tr>
<td>C-C</td>
<td>11 ± 2 11 ± 8 5 ± 5</td>
</tr>
</tbody>
</table>

Mismatch repair was determined as described in Materials and Methods by using circular heteroduplexes containing a site-specific incision at the Sau96I site in the complementary DNA strand. Each value represents the average of at least three determinations (±1 SD).

**Fig. 2.** Mismatch repair in MT1 cells is blocked prior to excision. Except for presence/absence of a mismatch at position 5632, circular G-T heteroduplex and G-C homoduplex DNAs were identical, each containing an incision in the complementary strand at the HincII site (positions 0/6440, 808 base pairs from the mismatch for the heteroduplex). DNAs were incubated with HeLa, TK6, or MT1 nuclear extracts as described, with 90 µM aphidicolin present when indicated. Products were cleaved with Bsp106 (cleavage at coordinate 2529) and subjected to electrophoresis through alkaline 1.5% agarose gels, and DNA was electrotransferred to nylon membranes as described (10). Membranes were hybridized with 5'-32P-labeled d(ATTGTTCTGGATATTACCAG) (viral strand nucleotides 5216–5235) to map excision tract 5' termini. The mapping method is illustrated on the left, with the shaded bar corresponding to the oligonucleotide probe. The mismatch and the strand break in the heteroduplex map 3.1 kb and 3.9 kb 5' to the Bsp106 cleavage site, respectively. STND, standard repair reaction; + APH, aphidicolin included in the reaction.
DISCUSSION

Since the MT1 cell line was isolated by frameshift mutagenesis under conditions that yield a very small mutation fraction for the X chromosome-linked HPRT locus, the alklylation tolerance, hypermutability, poor cloning efficiency at low cell density, and cell cycle progression phenotype characteristic of the line are likely due to a single mutation (1). Assuming this inference to be correct, our demonstration that MT1 cells are defective in mismatch repair implies that these phenotypes are due to either deficiency of a component required for this reaction or alteration of a regulatory activity that is necessary for production of the repair component lacking in the MT1 line. On the basis of similar characteristics of E. coli mismatch repair mutants, as pointed out previously (1, 2), the alklylation-tolerant and hypermutable phenotypes can be explained by a simple defect in mismatch repair. It is nevertheless premature to exclude the possibility of a regulatory defect because the low cloning efficiency and cell cycle progression phenotypes of the MT1 line cannot yet be rationalized in terms of a simple repair mutation.

Comparison of spontaneous and MNNG-induced mutational spectra in HPRT exon 3 revealed clear differences between the MNNG-sensitive TK6 parent line and the MNNG-resistant MT1 derived line. Spontaneous hot-spot mutations identified previously in the parental line include loss of an adenine at base pair 256 or 257 and the loss of G-G at base pairs 237 and 238 (17). Neither of these mutations was observed as a hot spot in MT1 cells. Furthermore, the four spontaneous hot spots described above for untreated MT1 cells were not observed as spontaneous TK6 mutations. Clearly, base pair substitutions are much more prominent in the MT1 spontaneous spectrum than in that of the parental line.

MNNG-induced spectra of the two cell lines were also distinct. The TK6 parent line shows two strong hot spots at base pairs 208 and 209, both G → A transitions occurring in a run of six guanines (base pairs 207–212) with each representing 10% of the total TG^6 population (18). The MT1 MNNG-induced spectrum also contained two G → A hot spots in this run of six guanines occurring at base pairs 208 and 212. The G → A hot spot at base pair 209 was shared by both lines, the one at base pair 208 occurred only in TK6, and the one at base pair 212 only in MT1 cells. This result was so striking that the TK6 work reported in Cariello et al. (18) was repeated in quadruplicate and confirmed. MT1 was also distinguished from TK6 by the silent G → A transition at base pair 234 that was identified as a super hot spot only in cultures of MNNG-treated MT1 cells. While differences in the spontaneous spectra of TK6 and MT1 cells can be understood in terms of the mismatch repair defect of the mutant cell line, we are unable at this point to offer plausible hypotheses to explain differences in location of MNNG-induced G → A hot spots observed in the two cell lines.

It has been argued that the cytotoxic and mutagenic potential of MNNG and other S^-1 alkylators are largely attributable to the ability of these agents to alkylate the O^-6-position of guanine (reviewed in refs. 1 and 2). The mutation spectra reported here are consistent with the notion that the primary mutagenic lesion in MNNG-treated cells is O^-6-methylguanine. While 3^-methyladenine is excised in both TK6 and MT1 lines after MNNG treatment, O^-6-methylgua-

nine, 7^-methylguanine, 1^-methyladenine, 3^-methylguanine, and 7^-methyladenine persist in the DNAs of both cell types (1). Nevertheless, MNNG-induced hot-spot mutations in MT1 and TK6 lines are almost exclusively G-C → A-T transitions—the result expected based on the mismapping properties of O^-6-methylguanine.

In contrast to the almost exclusive occurrence of G → A transitions in MNNG-treated cells, spontaneous hot-spot mutations observed in exon 3 in the hypermutable MT1 line were distributed among two A → G transitions, an A → T transition, a T → G transversion, and insertion of a single guanine residue. This spectrum of spontaneous mutations is consistent with the finding that both transition and transversion mismatches are repaired at reduced rates in the MT1 line.

Individual activities required for human strand-specific mismatch repair have not been characterized, but the mechanism of the reaction is known to be similar to that catalyzed by the E. coli methyl-directed pathway. The two systems have comparable mismatch specificities (8, 9), and both display an unusual bidirectional capability (10, 14).

In view of this apparent homology, our finding that repair in MT1 cells is blocked prior to excision suggests that the mutant line may be deficient in an activity that is functionally homologous to bacterial MutS, MutL, or DNA helicase II activities required for initiation of mismatch-provoked excision in the bacterial system (12, 14).

The properties of the MT1 cell line show that although MNNG is a potent mutagen, alklylation adducts produced by this agent are not intrinsically toxic to the mammalian cell. Rather, cytotoxicity is apparently dependent on one or more activities that function during the early steps of mismatch correction. It has been previously suggested that MNNG killing is due to futile turnover of newly synthesized DNA by the mismatch repair system when the replication fork encounters a miscoding adduct on the template strand (1, 2).
Although the nature of the MT1 repair defect is consistent with this view, our results do not prove a requirement for excision in the process of cell death because of the fact that the precise nature of the MT1 defect has not been established. Since a defect at the level of mispair recognition would block mismatch correction prior to excision, it is possible that recognition of mispairs containing an alkylated base may be sufficient to promote cell death in the absence of a hydrolytic reaction.

The demonstration that a mismatch repair activity is involved in MNNG killing indicates that cell death promoted by this agent is an active process, formally similar to programmed cell death documented in other systems (19, 20). It can be seen that this mechanism provides a developmentally complex organism with an effective means for eliminating cells that are potentially harmful because of the presence of premutagenic lesions within their genetic complement. It is worth noting in this context that our results imply, in addition to mismatched base pairs, that the human strand-specific repair system can recognize mispairs involving MNNG adducts, a property that is shared by the E. coli methyl-directed pathway (4, 5). Since the bacterial system can apparently also recognize UV-induced lesions (21), it is tempting to speculate that, in addition to its roles in rectification of DNA metabolic errors (6, 22), strand-specific mismatch repair may serve as a general sensor for genetic damage.

We acknowledge Ms. Sherry Larson and the Tissue Culture Facility of the Duke University Comprehensive Cancer Center for their assistance in culturing the cells used in this work. This work was supported by grant GM45190 from the National Institute of General Medical Sciences (to P.M.), grants from the National Institute of Environmental Health Sciences and the Department of Energy Office of Health and Environmental Research (to W.G.T.), and a predoctoral fellowship from the Ministry of Education, Republic of China (to W.-h.F.).