O⁶-Methylguanine Removal by Competent and Incompetent Human Lymphoblastoid Lines from the Same Male Individual

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ABSTRACT

Two cell lines, one proficient (Mex⁺) and one deficient (Mex⁻) in the ability to remove O⁶-methylguanine, have been isolated by Epstein-Barr virus-mediated transformation of a single blood sample obtained from a normal human male. Extracts of untreated cells differ in their O⁶-methylguanine transferase (methyl acceptor protein) activity. Although both lines arise from the same individual, they show great difference in their sensitivities to the toxic action of N-methyl-N'-nitro-N-nitrosoguanidine. Chromosome counts of the strains reveal a modal number of 46 for both. Neither X-inactivation nor a gross abnormality in chromosome number can be the cause of the difference between the two lines.

INTRODUCTION

The induction of mutations and the transformation of cells by methylating agents is related to the level of O⁶-MeG⁶ maintained after production of the adduct (12, 14). This level is determined in large part by the presence of an acceptor protein to which the methyl of O⁶-MeG is transferred (10). The methyl transferase activity and the acceptor protein are probably identical; the protein is inactivated on acceptance by one of its cysteines of the methyl group from O⁶-MeG (5). In Escherichia coli (16) and also in Bacillus subtilis (8), the amount of acceptor protein is controlled by an inducible system. Although the inducing signal has yet to be identified, an alkylation product is clearly involved. Since at least the O⁶-MeG acceptor and a 3-methyladenine DNA glycosylase are simultaneously induced in bacteria, it is not necessary that O⁶-MeG itself be the inducer (6, 9).

In the bacteria, the constitutive level of the acceptor protein is difficult to detect, and the increase in activity following alkylation may be 2 orders of magnitude (8). The situation in mammalian cells and in whole animals is much less clear. Treatment of animals with low doses of methylating agents can result in a 3- to 4-fold increase in the level of acceptor protein (13). Treatment of some cells with alkylating agents has also been reported to bring about an increase in the level of acceptor protein (23). However, the increase in acceptor activity in rats can be induced by substances which do not methylate but rather have some general cytotoxic effect (15). In some cases at least, it appears that increased acceptor activity is related to cell proliferation. That proliferation should play a role seems reasonable in view of the cell cycle dependence of some DNA repair activities (7).

The existence of cell types with varying capacities for O⁶-MeG provides material for a genetic analysis of the control of acceptor activity. SV40-transformed fibroblasts and tumor cells unable to reactivate MNNG-treated adenovirus have been termed Mer⁻ by Day et al. (3, 4). We have studied a series of human lymphoblastoid lines which fall into 2 major categories: Mex⁺, competent in removal of O⁶-MeG; and Mex⁻, incompetent to varying degrees (19, 20). Mer⁻ cells are phenotypically Mex⁻ (4). Mex⁺ and Mex⁻ phenotypes can be considered as "codominant," since hybrid cells containing both Mex⁺ and Mex⁻ chromosomes produce the level of acceptor protein characteristic of the Mex⁺ parent, rather than twice the level as is found for the system removing 3-methyladenine (2). It is most likely that the differences between Mex⁺ and Mex⁻ strains are epigenetic rather than genetic, since it has already been reported that Mex⁺ fibroblasts and Mex⁻ lymphoblasts (18) or Mex⁺ fibroblasts and Mex⁻ tumor cells (4) can be obtained from the same individual. Day et al. (4) have also reported that, in some cases, a cell line may be Mer⁺, whereas that same cell line after SV40 transformation may be Mer⁻. However, not all SV40-transformed lines are Mer⁻.

In this study, we show that Mex⁺ and Mex⁻ lymphoblastoid lines can be obtained from a single male individual. The normal chromosome number and the presence of a Y-chromosome in these recently developed lines permit us to eliminate the hypothesis that either X-inactivation or some sort of gross chromosome abnormality is responsible for the difference. Many of the Mex⁻ lines studied previously had a long history of cultivation and were likely to have accumulated such aberrations (2).

MATERIALS AND METHODS

Cell Culture and Analysis. We have previously described our methodology for the culture of human lymphoblastoid cells (19). We use either RPMI 1640 (KC Biologicals, Lenexa, Kans.) supplemented with 10% fetal calf serum (KC Biologicals) plus 6 μg glutamine or a "cloning medium" made with RPMI 1640 supplemented with 5% fetal calf serum, 10% horse serum (KC Biologicals), 1 mM α-ketoglutarate, and 6 μg glutamine (1). Cell counts were made in a Coulter Counter (Coulter Electronics, Hialeah, Fla.). Cells for alkylation studies and for the preparation of extracts were grown in spinner culture as described previously (19). MNNG solutions (Aldrich Chemical Co., Milwaukee, Wis.) were made in absolute alcohol and stored at −20°.

Cell Lines. Cell lines were established by the simplified procedure of Tohdes et al. (21). Three drops of donor blood were added to 2.5 ml of cloning medium containing 50 units of heparin and 0.4 ml of an Epstein-Barr virus filtrate (obtained from Dr. Elliott Kieff, The University of Chicago). The mixture was split into 20 replicate cultures and incubated for 1 month. From these 20 attempts, 2 positive cultures were obtained, Pa 1 and Pa 2. The donor was a healthy adult male not associated with the laboratory. As standards, we also used the ROT-5 (Mex⁺) and L6 (Mex⁻) lines studied previously (2).
Chromatography. Fifty ml of exponential cells were treated with vinblastine sulfate at 0.04 μg/ml for 30 min at 37°C. Cells were then centrifuged in 2 25-ml portions, and each portion was resuspended in 20 ml of 75 mM KCl and swollen for 15 min at 37°C. The cells were then collected by centrifugation, and the supernatants were aspirated. Fixation was by the stepwise addition of fresh methanol/acetic acid (3:1) with agitation. Fixed cells on slides were treated with 0.25% trypsin in Hanks’ salt base (KC Biologicals) for 60 seconds, followed by a rinse in phosphate-buffered saline [100 ml Solution A (80 g NaCl, 20 g KCl, 11.5 g Na2HPO4, and 2.0 g KH2PO4 in 1 liter H2O)], 10 ml Solution B (1.0 g MgCl2, 6 H2O in 100 ml H2O), 10 ml Solution C (1.0 g anhydrous CaCl2 in 100 ml H2O), and 88 ml H2O with Ca++ and Mg++. The slides were stained with Gurr’s Giemsa (Biomedical Specialties, Santa Monica, Calif.) for 4 min and rinsed in Gurr’s buffer (Biomedical Specialties) followed by a distilled water rinse. Cell chromosome counts were restricted to reasonably circular spreads on a clean chromosomeloose background. Y-chromosomes were identified by quinacrine staining (25).

Preparation and Analysis of Extracts. Extracts were prepared essentially as described by Waldstein et al. (24), except that 0.2 m NaCl was present during the sonication, and the extracts were dialyzed against 70 mM HEPES buffer, pH 8.0, 1 mM EDTA, 10 mM dithiothreitol, and 5% glycerol. Exponential cells were collected by centrifugation, washed with phosphate-buffered saline and with HEPES, and suspended in the above buffer with 0.2 mM NaCl for sonication. Sonication was with a Branson "sonifier" (Branson Sonic Power, Danbury, Conn.) set to deliver pulses, and sonication was continued until microscopic observation indicated that about 95% of the cells had been broken.

The DNA substrate was prepared from human lymphoblastoid cells by alkylating either with methylmethanesulfonate (1.6 Ci/mmol; New England Nuclear, Boston, Mass.) for O6-MeG studies or with dimethyl sulfate (4.1 ahnCi/mmol; New England Nuclear) for glycosylase studies. The DNA was alkylated by repeated additions of alkylating agent, followed by alcohol precipitation, solution, and retreatment, until a sufficient number of counts was obtained. Our preparations gave about 2000 dpm in O6-MeG in 5 to 10 μl of DNA solution at a concentration of 2 mg DNA per ml.

All reactions were carried out in a reaction mixture of 50 μl containing HEPES buffer, DNA (5 μl), and extract. After incubation at 37°C for 15 min, 15 μl of a mixture of 10 μl of 2 mM HCl and 5 μl of a mixture of methylated purine markers were added, and the combined solution was heated for 30 min at 70°C. The hydrolyzed lysates were then spotted on a 0.5-mm cellulose MN 300-thin-layer plate (Analtech, Inc., Newark, Del.) and chromatographed in 2-propanol:28% NH4OH:H2O (7:2:1) as described previously (19) except that the chromatography tank was vented essentially as described by Waldstein et al. (24), except that 0.2 M salt was used in our previous investigation (2) compared to no detectable removal of O-MeG from untreated cells (about 6:1) compares reasonably well to the results obtained with whole cells (Chart 1). Therefore, our extracts contained essentially all of the acceptor activity in the cells. Treatment with 0.68 μM MNNG reduces D, A, O, O'-MeG removal; •, A, •, 3-methyladenine removal. O'-MeG removal by Pa 1 and Pa 2 cell lines treated with MNNG. Cells (40 ml of about 8 x 105/ml) were treated with MNNG (760 mCi/mmol) in 0.15 mM NaCl plus 0.15 mM sodium citrate for 15 min at 37°C and then incubated in RPMI 1640 for 3 hr before lysis and preparation of the DNA as described previously (19). The DNA was treated with dilute acid to liberate purines, and the mixture was then chromatographed. We determine the amount of O6-MeG removed as a function of the amount of 7-methylguanine formed in order to eliminate the effects of differences in permeability, reactivity, or other line-specific factors (2). In order to provide reference standards, we also determined the removal of O6-MeG by the ROT-5 lymphoma strain and the L6 lymphoblastoid line (2).

It is clear from the results (Chart 1) that Pa 2 should be classified as Mex+, whereas Pa 1 has the characteristics of a Mex- strain. Pa 1 does remove about 15 to 25% of the amount of O6-MeG removed by Pa 2 or Raji, and both we (2) and others (4) have noted the appearance of intermediate Mex- strains. Since it has recently been reported that untreated Mer- strains have levels of acceptor protein equal to those of Mer+ lines, but that the ability of the 2 types of lines to regenerate acceptor protein after MNNG varies greatly with Mer strains deficient in this respect (24), we decided to determine the activity of the extracts of different lines. We also determined the activity of extracts of Raji and L6 lines, since these have been used as prototypes of Mex+ and Mex- lines, respectively.

Pa 2 yields extracts with good acceptor activity comparable to the activity found in Raji cells (Chart 2). We found activity of about 220 fmol/mg protein in extracts of the Mex+ Rot-5 strain used in our previous investigation (2) compared to no detectable activity in extracts of untreated L6 cells (<10 fmol/mg). Pa 2 extracts had activities (untreated) of about 325 fmol/mg protein as compared to about 50 fmol/mg protein for those of the Mex- Pa 1 (Chart 2). This ratio of Mex+ to Mex- activities in extracts from untreated cells (about 6:1) compares reasonably well to the results obtained with whole cells (Chart 1). Based on the number of cells lysed, we calculate a capacity of 3.4 x 10^-8 fmol of O6-MeG removed per cell by Pa 2 extracts (Chart 2) as compared to 2.9 x 10^-8 fmol/cell calculated from the whole cell data (Chart 1). Therefore, our extracts contained essentially all of the acceptor activity in the cells. Treatment with 0.68 μM MNNG reduces the activity in the cells. Treatment with 0.68 μM MNNG reduces the activity in the cells. Treatment with 0.68 μM MNNG reduces the activity in the cells.

RESULTS

Removal of O6-MeG. We first determined the ability of Pa 1 and Pa 2 cell lines to remove both 3-methyladenine and O6-MeG. Cultures were treated with MNNG (760 mCi/mmol) in 0.15 mM NaCl plus 0.15 mM sodium citrate and treated with MNNG for 15 min at 37°C. Doses of MNNG used ranged from 1.4 to 11.6 μM, and the results are expressed as a function of pmol 7-methylguanine produced/μmol of guanine (G) for reasons discussed previously (2). Treated cells were collected, resuspended in complete RPMI 1640, and incubated for 3 hr at 37°C. After incubation, the cells were harvested, and DNA was prepared and analyzed for methylated purines as described previously (19). Therefore, our extracts contained essentially all of the acceptor activity in the cells. Treatment with 0.68 μM MNNG reduces the activity in the cells. Treatment with 0.68 μM MNNG reduces the activity in the cells. Treatment with 0.68 μM MNNG reduces the activity in the cells.
the activity of acceptor protein in extracts but does not saturate the system so that there is some residual activity. We find that, under our conditions of incubation, about 10⁶ cells/ml in spinners, and with these strains, full activity in the Mex⁺ strains has not fully recovered by 24 hr as with whole cells (19). Extracts of both Pa 1 and Pa 2 strains have 3-methyladenine glycosylase activity. In our experiments, the Pa 1 strain showed about 20% more activity for this glycosylase than did the Pa 2, but this might reflect the different rates of growth of the 2 strains (7).

Loss of ability to remove O⁶-MeG is also associated with an increase in sensitivity to killing by MNNG (17). This correlation is also seen in the response of Pa 1 and Pa 2 strains to treatment with MNNG. Cells were prepared in a rapidly growing state by daily dilution with medium. The cells were then treated at the indicated concentrations of MNNG and incubated. In this experiment, the cultures were counted daily, and the cells were diluted 1:1 with medium whenever cell counts approached 4 x 10⁵. We have calculated overall (cumulative) factors of increase, and these data are shown in Chart 3. Extrapolation to the origin indicates that Pa 1 has about 37% survival at an MNNG dose of 0.01 µg/ml (0.068 µM). In contrast, it is difficult by this methodology to determine any effect of treatment of Pa 2 with MNNG (up to 0.3 µg/ml; 2 µM). The calculated survival of Pa 1, 1.8% at 0.2 µM MNNG, is close to that determined previously by microtiter plating (11) for the intermediate Mex⁻ strain, Tk⁻/⁻ (2). It is worth noting the huge differences in sensitivity to alkylating agents that can be observed in derivatives of different cells from the same blood sample.

Although the Pa 1 and Pa 2 cell lines were freshly obtained from a normal volunteer, we decided to check the chromosome number under staining conditions in which the chromosomes could be identified. The result of chromosome counts after Giemsa staining is shown in Chart 4. The great majority of the cells of both Pa 1 and Pa 2 have a chromosome number of 46. A few tetraploid cells have been observed, but the karyotype of most of these cells appears normal with easily recognizable chromosomes. In order to demonstrate that these cells do, in fact, have a normal chromosome complement, we stained chromosome spreads with quinacrine (25). Both cell lines have one Y-chromosome/cell (Fig. 1). Therefore, we conclude that Pa 1 and Pa 2 cells have a normal chromosome number with one X and one Y. Since both Pa cell lines have only the X-chromosome, we conclude that the Mex character of the lines cannot be due to differences in the activity of this chromosome, e.g., the difference between Mex⁺ and Mex⁻ cannot be due to X-inactivation. It is also clear that there is no major difference in chromosome number, which could account for the different behavior of the 2 lines with respect to the removal of O⁶-MeG.

**DISCUSSION**

Our results strengthen the argument that the difference bet-
between Mex+ and Mex− strains is epigenetic in nature (4, 18). The result of virus infection has been the production of 2 cell lines of normal chromosome number but with different O6-MeG removal characteristics. The Epstein-Barr virus transformation occurs in B-cells (21) but, since there are numerous B-cell subtypes, it is possible that the transformation event has selected 2 preformed populations, one Mex+ and the other Mex−. It is also possible that the act of transformation itself occasionally results in inactivation of the Mex+ characteristic. We suppose this to be inactivation because of the report that nontransformed human B-cells do have O6-MeG acceptor activity, although at a lower level than found in T-cells (22). Notwithstanding the normality of the chromosome counts, our results do not exclude microscopic or submicroscopic differences in chromosome structure from being associated with the changes. The finding that Mex+ and Mex− characteristics behave autonomously in crosses (2) supports the idea that some chromosome change might be responsible. For example, differences in methylation pattern or rearrangement of chromosome DNA sequences could account for our results.

That Pa 1 seems to be a stable line of intermediate acceptor level makes us suppose that the Mex characteristic is one which regulates the amount of acceptor protein although, as yet, there are no data to prove that the proteins made by Pa 1 and Pa 2 are identical. Pa 1 and Pa 2 differ not only in the O6-MeG removal activity of their cells but also in the constitutive activity of the O6-MeG acceptor protein in extracts, a result apparently different from that reported by Waldstein et al. (24). However, the Mer+ and Mer− cells used by these investigators were not of lymphoid origin as are Pa 1 and Pa 2. We do not know whether this difference in cells can account for the different results. As we have discussed previously (2), it is possible that this (hypothetical) regulatory process governs the activity of several reactions. Although lines deficient in their ability to remove O6-MeG tend to be sensitive to the killing action of MNNG, the correlation is not quantitative, which makes us suppose that reactions other than O6-MeG removal are involved in lethality in bacteria. In bacteria, a second enzyme is involved in the removal of 3-methyladenine and also of 3-methylguanine from DNA, and failure to induce this enzyme is associated with bacterial killing (6, 9). Mex− strains are capable of removing 3-methyladenine, and it is not now possible to identify the cause of lethality. It does seem possible that a chromosomally based change may alter more than one cellular process involved in producing a viable chromosome set after MNNG treatment and that the differences between Mex+ and Mex− strains lie in the structure of a regulatory element that affects these different processes.

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REFERENCES

Fig. 1. Quinacrine-stained nuclear preparation of Pa 1 and Pa 2.
\( \text{\textsuperscript{6}}\text{G}-\text{Methylguanine Removal by Competent and Incompetent Human Lymphoblastoid Lines from the Same Male Individual} \)

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