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Biochemistry

Possible depletion of a DNA repair enzyme in human lymphoma cells by subversive repair

(*O*^{6}-methylguanine/suicide repair/methylating agents/Mex phenotype)

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ABSTRACT **Mex$^+$ human lymphoma cell lines contain *O*^{6}-methylguanine-DNA methyltransferase, a DNA repair enzyme that undergoes suicide inactivation on interaction with its substrate. The cells are therefore competent to remove the alkylation lesion *O*^{6}-methylguanine from their DNA. However, several repair-deficient lymphoma cell lines (Mex$^-$) are also known. It is shown here that Mex$^+$ cells can be converted temporarily to a Mex$^-$ phenotype by growth in nontoxic concentrations of free *O*^{6}-methylguanine. The depletion of methyltransferase activity is not a result of *O*^{6}-methylguanine incorporation into DNA and subsequent demethylation by the enzyme. It is proposed that *O*^{6}-methylguanine is mistakenly incorporated into tRNA molecules by means of a post-transcriptional ribosyl transfer reaction. The demethylation of such bases in tRNA has been demonstrated by using bacterial and human DNA repair enzymes. The existence of such a subversive repair of a methylated base in tRNA raises the possibility of competition between DNA and RNA for cellular DNA repair enzymes. Furthermore, it is proposed that the known aberrant methylation of tRNA in certain transformed cells, together with subversive tRNA repair, could account for the Mex$^-$ phenotype.**

To counter the effects of cytotoxic and mutagenic methylating agents, cellular repair mechanisms recognize and remove most types of methylated bases from DNA. This removal is commonly initiated by DNA glycosylases that excise the methylated residue as a free purine or pyrimidine base (1, 2). In contrast, DNA is freed of the important lesion *O*^{6}-methylguanine (*O*^{6}MeGua) by *O*^{6}MeGua-DNA methyltransferase (*O*^{6}MeGua MeTase), which abstracts the methyl group from the *O*^{6} position of guanine and transfers it to one of its own cysteine residues (3). The reaction mechanism is similar in bacteria and mammalian cells (4, 5) and is unusual in being stoichiometric; each MeTase molecule can repair only a single *O*^{6}MeGua residue.

Since uncorrected *O*^{6}MeGua residues in DNA are mutagenic, the distribution of MeTase activity among different organs and cell types has been the focus of considerable recent attention. The *O*^{6}MeGua MeTase is present in varying levels in different cells, and there is a reasonable inverse correlation in rat tissues between MeTase activity and the site of tumor induction by methylating carcinogens (6). Moreover, although all untransformed human cells so far examined contain active MeTase, malignant transformation may result in cell lines that lack measurable activity (7, 8). Such cell lines, designated Mex$^-$ (or Mer$^-$), are defective in the removal of *O*^{6}MeGua from DNA *in vivo* (8, 9). Cells of the Mex$^-$ phenotype exhibit an increased sensitivity to killing by methylating agents (10).

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It has been suggested that an important feature of damage reversal by methyl group transfer is that it avoids the generation of free *O*^{6}MeGua (11). Pathways exist to scavenge purines for reincorporation into DNA and RNA. Since DNA polymerases will accept *O*^{6}Me-dGTP and incorporate the methylated nucleotide in place of dAMP into DNA *in vitro* (12), the potential exists *in vivo* for the incorporation of free *O*^{6}MeGua into DNA, where its ability to code ambiguously may represent a mutagenic threat to the cell.

This study was undertaken to investigate whether free *O*^{6}MeGua could be incorporated into DNA and interfere with the cellular capacity to repair alkylation damage. It was found that incorporation into DNA does not occur to any appreciable extent and that the incorporation of the base into tRNA may be a critical event.

MATERIALS AND METHODS

Cell Culture. Raji TK$^+$ and Raji TK$^-$ cell lines were obtained from G. Klein, Stockholm. Cells were maintained in RPMI 1640 medium (GIBCO) supplemented with 10% fetal calf serum. Routine checks confirmed that cultures were free of mycoplasma contamination. Cells exhibiting spontaneous resistance to 6-thioguanine were selected by the method of Penman *et al.* (13).

Radiochemicals. *N*-[^{3}H]Methyl-*N*-nitrosourea (MNU) (1 Ci/mmol; 1 Ci = 37 GBq) was obtained from New England Nuclear. [*methyl*-^{3}H]Thymidine (25 Ci/mmol), [^{3}H]-hypoxanthine (3.6 Ci/mmol), and [^{3}H]adenine (22 Ci/mmol) were obtained from Amersham. *Micrococcus luteus* DNA containing *O*^{6}[^{3}H]MeGua was prepared as described (4). Purified yeast tRNA (a gift from T. Lindahl) was methylated in an analogous fashion, although $MgCl_2$ (5 mM) was included to maintain the tRNA in its native conformation. The specific activity of this methylated tRNA was 2600 cpm/μg of RNA. It contained <1% DNA.

Chemicals. Reagent enzymes were purchased from Sigma unless otherwise stated. Nonmethylated purines and their deoxy- and ribonucleosides were obtained from Sigma. *O*^{6}MeGua was prepared from 6-chloroguanine as described by Demple *et al.* (14). Radioactively labeled *O*^{6}[^{3}H]MeGua was prepared from acid hydrolysates (0.1 M HCl, 70°C, 30 min) of [^{3}H]MNU-treated DNA. *O*^{6}-Methylguanosine (*O*^{6}MeGuo) was prepared by the action of MNU (a gift of P. Swann) on guanosine. Guanosine (1 mg) in 1 ml of 0.2 M sodium cacodylate, pH 7.0/1 mM EDTA was treated with MNU (0.4 M) for 18 hr at 20°C. Preparative chromatography on Whatman 3MM paper in butanol/ethanol/water, 80:10:25 (vol/vol), yielded two UV-absorbing bands and one band (R_f = 0.45) that exhibited a light blue fluorescence. The fluores-

Abbreviations: *O*^{6}MeGua, *O*^{6}-methylguanine; *O*^{6}MeGuo, *O*^{6}-methylguanosine; MNU, *N*-methyl-*N*-nitrosourea; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; *O*^{6}MeGua MeTase, *O*^{6}MeGua-DNA methyltransferase.

cent material was eluted from the paper into water and lyophilized.

Analysis and Purification of O^6MeGua and O^6MeGuo. The methylated base and nucleoside were further purified by preparative HPLC before use. Unlabeled O^6MeGua and O^6[^{3}H]MeGua were purified by chromatography on a Varian micropak MCH-10 reverse-phase column with a Varian 5000 liquid chromatograph. Samples were eluted isocratically with 25% aqueous methanol at a flow rate of 1 ml/min. Both the UV absorbance (A_{254}) and the fluorescence of the column eluate were monitored. Fluorescence was measured by passage through the flow cell of a Perkin-Elmer LS-3 fluorescence spectrometer. An excitation wavelength of 295 nm was used and emission was monitored at 395 nm. This has the advantage of substantially reducing the fluorescence of adenine and its derivatives while affecting O^6MeGua and its derivatives only slightly. Fractions containing the fluorescent O^6MeGua were pooled and lyophilized.

The purity of O^6MeGua was checked by ion-exchange chromatography on a Whatman Partisil 10/25 SCX column, developed with a gradient of ammonium formate in 6% aqueous methanol. It contained a single UV-absorbing peak that coincided with the fluorescent material at the previously determined position of elution of O^6MeGua (14) and contained <1% contamination with guanine or 6-chloroguanine.

O^6MeGuo was also purified by reverse-phase chromatography on MCH-10. The eluted O^6MeGuo exhibited a single fluorescent and UV-absorbant peak following lyophilization and rechromatography under the same conditions. The coincident peaks of absorbance and fluorescence were completely abolished by prior incubation with adenosine deaminase, which is known to demethylate O^6MeGuo (15).

Analysis of nucleoside digests of tRNA was performed by reverse-phase chromatography on MCH-10 developed isocratically with 20% aqueous methanol. The elution times of relevant reference compounds are guanine, 6 min; O^6MeGuo, 16 min; O^6MeGua, 30 min.

Enzyme Assays. Preparation of lymphoma cell extracts for assay of O^6MeGua MeTase was carried out as follows. Generally, 1–3 × 10^7 cells in exponential growth were harvested from 40 ml of medium by low-speed centrifugation, washed twice by resuspension in phosphate-buffered saline, and suspended in 150–400 μl of extraction buffer (50 mM Tris·HCl, pH 7.8/10 mM EDTA/10 mM dithiothreitol/0.3 M KCl). Cell suspensions were disrupted by sonication at 0°C. Crude extract were clarified by centrifugation at 4°C and used to assay O^6MeGua MeTase as described by Harris *et al.* (16).

Homogeneous *Escherichia coli* MeTase, fraction VI (ref. 14), was assayed as described (14) except that a final volume of 500 μl was employed. MeTase activity on MNU-treated tRNA was determined in an analogous fashion. The assay volumes and buffers were the same except 5 mM $MgCl_2$ replaced 1 mM EDTA in the reaction mixture. Each assay contained 4–8 μg of methylated tRNA (1–2 × 10^4 cpm) and following incubation, usually for 10 min at 37°C, RNA was digested to ethanol-soluble oligonucleotides by pancreatic RNase. Protein was removed by precipitation with ethanol and the supernatants containing the oligonucleotides were evaporated to dryness, dissolved in 50 mM Tris·HCl, pH 8.0/10 mM $MgCl_2$, and further digested to nucleosides by the successive addition of pancreatic RNase, phophodiesterase, and bacterial alkaline phosphatase (heated for 5 min at 95°C to destroy contaminating adenosine deaminase activity). The digests were analyzed by reverse-phase HPLC as described above. The precipitated protein was redissolved and successively digested with proteinase K followed by aminopeptidase M. The protein hydrolysate was supplemented with authentic *S*-methylcysteine and analyzed by descending chromatography on Whatman 3MM paper as described (3).

Hypoxanthine phosphoribosyltransferase activities were determined in Raji TK$^+$ cells and cells from the cloned 6-thioguanine-resistant lines selected. The conversion of [^{3}H]hypoxanthine to [^{3}H]IMP by crude cell extracts was determined by the method of Olsen and Milman (17). Cell extracts of the 6-thioguanine-resistant sublines contained <1% of the hypoxanthine phosphoribosyltransferase activity of the parental Raji TK$^+$ line. Adenine phosphoribosyltransferase activities were determined in extracts of Raji TK$^+$ cells by the method of Jones and Sargent (18). O^6[^{3}H]MeGua could not replace [^{3}H]adenine in this reaction and O^6MeGMP was formed at <1% of the rate of AMP formation.

RESULTS

Mex$^+$ and Mex$^-$ Human Lymphoma Cells. The Burkitt lymphoma cell line Raji can efficiently remove O^6MeGua from DNA after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (9). Extracts of these cells contain active O^6MeGua MeTase (16). Thus, Raji cells are of the Mex$^+$ phenotype. However, a subline of Raji, lacking thymidine kinase (TK$^-$), derived from the original Raji (TK$^+$) cells is Mex$^-$ (I. Teo, personal communication). Thus, Raji TK$^-$ cells are more sensitive than the parental cells to killing by MNU and MNNG and no O^6MeGua MeTase activity is detectable in crude cell extracts. Extracts of Raji TK$^+$ cells contained 0.5 ± 0.05 enzyme unit/mg of protein; Raji TK$^-$ extracts contained <0.05 unit/mg of protein (Fig. 1).

Since the Raji TK$^+$ and Raji TK$^-$ lines share a common origin, they were used to investigate the effects of free O^6MeGua on Mex$^+$ and Mex$^-$ cells.

Effect of Free O^6MeGua on Cellular O^6MeGua MeTase Levels. Continuous growth in the presence of O^6MeGua at concentrations of <0.3 mM was nontoxic to both Raji TK$^+$ and TK$^-$ cells. However, exposure to such nontoxic concentrations of O^6MeGua reduced the level of O^6MeGua MeTase activity in Raji TK$^+$ cells. Fig. 2 shows that cells grown for 16 hr in medium containing up to 0.1 mM O^6MeGua were depleted of the MeTase in a concentration-dependent manner, whereas extracts of cells grown in the presence of equivalent concentrations of guanine or adenine contained normal levels of enzyme activity. Extracts of Raji TK$^+$ cells grown with >0.1 mM O^6MeGua contained no detectable O^6-MeGua MeTase activity. This reduction in activity was not due to enzyme inhibition by residual free O^6MeGua present in the cell extracts, as separate experiments confirmed that

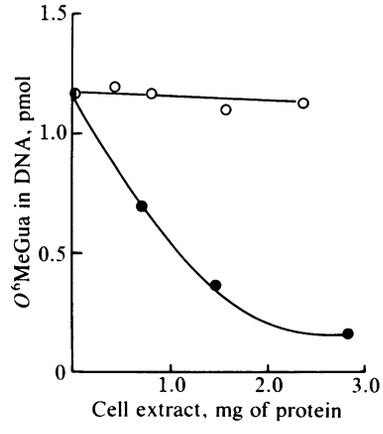

FIG. 1. O^6MeGua MeTase activity in extracts of Raji TK$^+$ (Mex$^+$) and TK$^-$ (Mex$^-$) cells. *M. luteus* DNA containing O^6[^{3}H]MeGua residues was incubated with extracts of either Raji TK$^+$ (●) or Raji TK$^-$ cells (○) for 20 min at 37°C and the amount of O^6MeGua remaining in the DNA was determined.

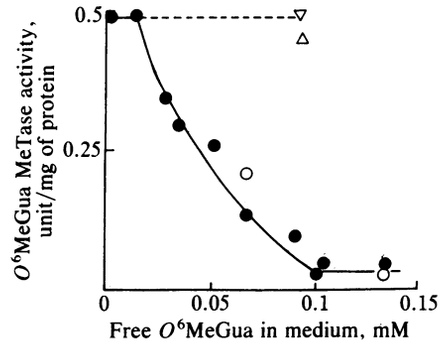

FIG. 2. Depletion of O^6MeGua MeTase activity by growth in the presence of free O^6MeGua. Exponentially growing Raji TK$^+$ cells were exposed to O^6MeGua in complete growth medium at the concentrations shown (circles). Following 16 hr of growth, the cells were harvested and washed, and O^6MeGua MeTase activity was determined in cell extracts. ●, Raji TK$^+$ cells; ○, 6-thioguanine-resistant subline of Raji TK$^+$ cells; ▽, activity in extracts of cells grown in the presence of 0.1 mM adenine; △, activity in extracts of cells grown in the presence of 0.1 mM guanine.

the free base does not interfere with enzyme activity when present in the standard assay (data not shown).

Biochemical Basis of MeTase Depletion. Since the only substrates for the O^6MeGua MeTases thus far reported are O^6MeGua and O^4-methylthymine in DNA (or polydeoxynucleotides), the possibility that the inactivation of MeTase occurred as a consequence of O^6MeGua incorporation into DNA was tested. First, DNA was isolated from Raji TK$^+$ cells grown in the presence of 0.3 mM O^6MeGua. Following acid hydrolysis of the DNA, the released purines were analyzed by reverse-phase HPLC. The column eluate was monitored fluorimetrically. O^6MeGua exhibits marked fluorescence at 395 nm and can be separated from other fluorescent material (principally adenine) present in acid hydrolysates of DNA. This permits the ready detection of as little as 2 pmol of O^6MeGua and therefore approaches the sensitivity of methods using the radioactively labeled base. Less than 2 pmol of O^6MeGua was present in hydrolysates of 500 μg of DNA obtained from 2×10^8 Raji TK$^+$ cells. In addition, no O^6MeGua (<2 pmol) was recovered in DNA hydrolysates from Raji TK$^-$ cells grown in the presence of 0.1–0.5 mM O^6MeGua. Thus, following growth in the presence of O^6MeGua concentrations up to five times those required to abolish MeTase activity in the Mex$^+$ Raji TK$^+$ cells, each cell contained on average $<10^4$ O^6MeGua residues in its DNA.

In a different approach, two spontaneous mutants of Raji TK$^+$ cells resistant to 6-thioguanine were tested for inactivation of O^6MeGua MeTase by growth in the presence of different concentrations of free O^6MeGua. The concentrations of the base needed to inactivate the enzyme activity in both of these TK$^+$ 6-thioguanine-resistant cells were the same as those required by the TK$^+$ parent line (Fig. 2). Since these 6-thioguanine-resistant sublines lack one salvage pathway for purines and O^6MeGua is not a substrate for adenine phosphoribosyltransferase (see *Materials and Methods*), O^6-MeGua is not being incorporated into DNA by means of these pathways under conditions that result in MeTase inactivation.

To confirm that the MeTase depletion in Raji TK$^+$ cells was independent of active DNA synthesis, cells in which DNA replication had been inhibited by >98% compared to control (as measured by [^{3}H]thymidine incorporation) by preincubation for 30 min with 1 mM hydroxyurea and 1 μM 5-fluorodeoxyuridine were exposed to free O^6MeGua. To avoid toxic effects of the DNA synthesis inhibitors, the incubation period with O^6MeGua was reduced to 5 hr at 37°C. Under these conditions a concentration of 0.15 mM O^6MeGua depleted the MeTase of TK$^+$ cells equally in both the presence and absence of DNA synthesis inhibitors (data not shown). Taken together, the absence of detectable O^6MeGua in DNA hydrolysates and the lack of dependence of the enzyme inactivation on either the purine salvage pathways or DNA synthesis suggest that the observed inactivation of the MeTase is not due to O^6MeGua incorporation into DNA and, subsequently, demethylation by this suicide enzyme.

Since free O^6MeGua, its ribo- or deoxyribonucleoside (19), or O^6MeGua residues in DNA did not seem to account for the enzyme inactivation, the possibility that O^6MeGua might be incorporated into RNA to provide a substrate for the MeTase was considered. Incorporation of the nucleotide during transcription seemed unlikely because this route should be dependent on functional pathways of purine salvage. Transfer RNA, however, undergoes numerous post-transcriptional modifications, of which two known examples utilize a free purine base as substrate.

The possibility that tRNA containing O^6MeGua would be a substrate for O^6MeGua MeTase was tested by using the homogeneous *E. coli* enzyme of Demple *et al.* (14) and [^{3}H]MNU-treated tRNA. Following incubation with [^{3}H]-MNU-modified yeast tRNA, the MeTase was digested to amino acids and the amount of *S*-methylcysteine was determined (3). From yeast tRNA that contained 24 pmol of [^{3}H]methylated bases, ≈0.5 pmol of *S*-methylcysteine was generated following incubation with an excess of MeTase (Fig. 3 *Upper*). The reaction was complete in 10 min at 37°C. More than 90% of the radioactivity initially present as O^6MeGua was transferred to cysteine residues. Digestion to nucleosides followed by HPLC analysis confirmed that a stoichiometric disappearance of O^6[^{3}H]MeGuo from the [^{3}H]MNU-treated tRNA had occurred during incubation with MeTase (Fig. 3 *Lower*). It is concluded that tRNA containing O^6MeGua residues is a substrate for the O^6MeGua MeTase.

The corresponding human O^6MeGua MeTase is not available in highly purified form. Nevertheless, crude extracts of Raji TK$^+$ cells were able to catalyze the disappearance of O^6[^{3}H]MeGuo from [^{3}H]MNU-treated tRNA (Fig. 4). The likelihood that this removal was effected by the O^6MeGua MeTase was supported by the observation that extracts of Raji TK$^-$ cells, which contained normal amounts of another DNA repair enzyme, 3-methyladenine-DNA glycosylase, were unable to carry out the reaction (Fig. 5).

DISCUSSION

The finding that the O^6MeGua in tRNA can be recognized and demethylated by the homogeneous O^6MeGua MeTase of *E. coli* or the analogous enzyme in extracts of human cells together with the observation that depletion of MeTase is not due to O^6MeGua incorporation into cellular DNA (see also ref. 20) suggest that tRNA metabolism could be involved in the reversible conversion to the Mex$^-$ phenotype demonstrated here. The first position of the anticodon of several tRNA species from many organisms may contain a modified nucleoside (21). For example, queuine or hypoxanthine can be inserted into specific sites of certain tRNA species in ribosyl transfer reactions that displace free guanine from the precursor tRNA (22, 23). It appears that ribosyl transfer reactions are a preferred method of generating specifically modified bases in preformed tRNA molecules. It seems possible that O^6MeGua is being incorporated into tRNA by such an enzyme. Although O^6MeGua is not a substrate for purified mammalian queuine ribosyl transfer enzyme (ref. 24; unpublished data), it is likely that other ribosyltransferases exist, one of which may mistakenly incorporate O^6MeGua into tRNA.

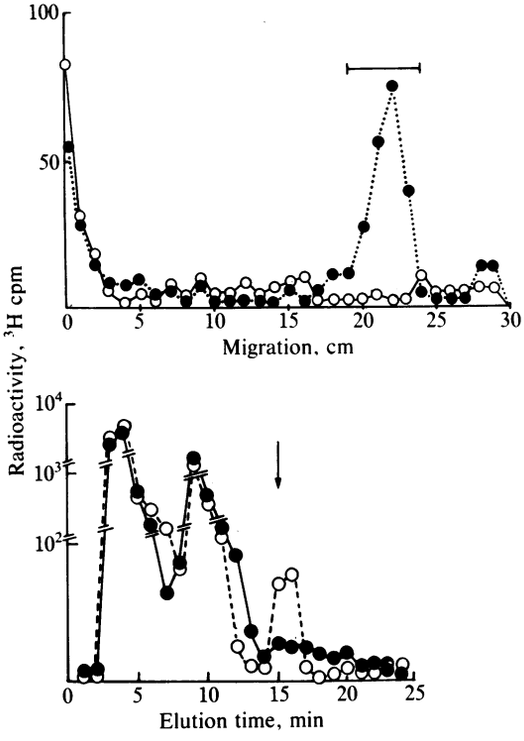

FIG. 3. Action of the homogeneous *E. coli* O^6MeGua MeTase on O^6MeGua in tRNA. [^{3}H]MNU-treated yeast tRNA (10^4 cpm) was incubated with 1 unit of purified *E. coli* MeTase for 10 min at 37°C. The tRNA was digested to nucleosides, which were analyzed by HPLC, and the MeTase was hydrolyzed to amino acids by digestion with proteinase K and aminopeptidase M. (*Upper*) Generation of *S*-methylcysteine in MeTase. Open symbols, proteolytic digests of control reaction mixtures (without added MeTase); closed symbols, digests of reaction mixtures containing MeTase. The position of migration of authentic *S*-methylcysteine is shown by the bar. (*Lower*) Disappearance of O^6MeGuo from tRNA catalyzed by MeTase. ○, Nucleoside digests of methylated tRNA incubated in the absence of MeTase; ●, digests of methylated tRNA following incubation with MeTase. The arrow indicates the elution time of authentic O^6MeGuo.

The demonstration of a potential for cellular repair of O^6MeGua in tRNA has important implications for DNA repair. Methylated tRNA may compete with DNA for the limited number of DNA repair enzyme molecules in the cell. This will be particularly serious in the case of the MeTase(s) that undergoes suicide inactivation. Cellular RNA is methylated to similar, or even greater, extents than DNA following treatment with simple methylating agents (25, 26). Inactivation of particular tRNA species is unlikely, on the grounds of target size, to be a major factor in the biological effects of methylating agents. However, the tRNA population as a whole comprises a target of similar size to genomic DNA and the partial inactivation or subversion of the DNA repair capacity by methylated tRNA molecules must now be considered as a possible contributory factor to cell killing or mutagenesis by such agents.

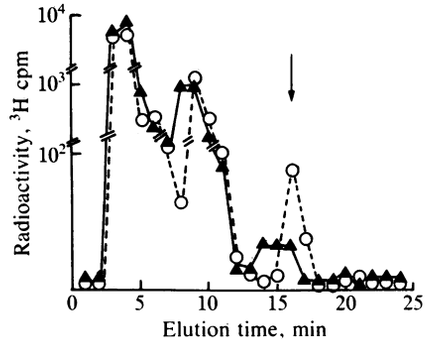

FIG. 4. Action of extracts of Raji TK$^+$ cells on O^6MeGua in methylated tRNA. Reaction conditions were the same as those in Fig. 3. Methylated tRNA was incubated without (○) or with (▲) a freshly made extract of Raji TK$^+$ cells (1.2 mg). After incubation for 10 min at 37°C, the tRNA was recovered and digested to nucleosides, which were analyzed by HPLC. The arrow marks the position of elution of O^6MeGuo.

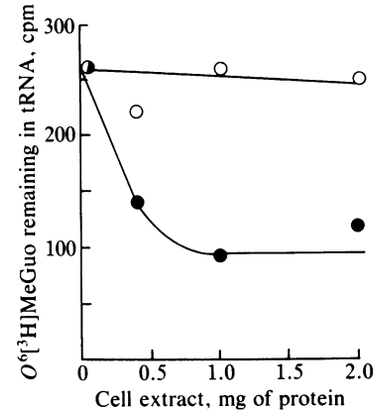

FIG. 5. Effect of extracts of Raji TK$^+$ or Raji TK$^-$ on O^6MeGua in tRNA. Freshly made extracts of Raji TK$^+$ cells or Raji TK$^-$ cells were incubated with [^{3}H]MNU-treated tRNA. The amount of O^6[^{3}H]MeGua remaining in the tRNA after incubation was determined following digestion and by HPLC analysis. ●, Raji TK$^+$; ○, Raji TK$^-$.

The ability of methylated tRNA to serve as a substrate for a DNA repair enzyme may underlie the Mex$^-$ phenotype. tRNA contains a wide variety of methylated bases (21) and all mammalian cells contain a number of specific tRNA MeTases (27). Alteration of tRNA MeTase levels is a consistent feature of the malignant state (28). This may result in the production of novel methylated bases in some tumor tRNA species (29, 30). Transformation by simian virus 40, an event that in many cases results in the transition from a Mex$^+$ to Mex$^-$ phenotype, is also associated with altered tRNA MeTase activity (31). It is proposed that in certain transformed cells, aberrant methylation results in O^6MeGuo (or possibly O^4-ribothymidine) in tRNA molecules. These methylated nucleosides are recognized and repaired by the suicidal O^6MeGua MeTase. Consequently, no active O^6MeGua MeTase molecules remain available to correct O^6MeGua in DNA—i.e., the cells are Mex$^-$. In this model, the lack of O^6MeGua MeTase in Mex$^-$ cells is an epigenetic event; expression of the O^6MeGua MeTase gene(s) is normal and the levels of active enzyme are determined by the rate of aberrant tRNA methylation.

Two lines of evidence support such a model. First, the intracellular distributions of tRNA MeTase and the repair enzymes are similar—the major fraction of both enzymes is found in the cytoplasm, although both are present in the nucleus (19, 32). Second, Ayres *et al.* (33) found no evidence for a diffusible regulator able to activate or repress the MeTase gene in hybrids between Mex$^+$ and Mex$^-$ cells. This is compatible with the full expression of both sets of O^6MeGua MeTase genes and the subsequent inactivation of a fraction of the enzyme molecules.

Treatment of Mex$^+$ cells with low concentrations of MNNG also depletes them of O^6MeGua MeTase (34). Such treatment sensitizes the cells to the toxic effects of a subsequent exposure to a variety of related alkylating agents (35). Because of the highly reactive nature of MNNG, this sensitization could be mediated by several means, including

interference with intracellular thiol levels or direct reaction with DNA repair enzymes. An advantage of depletion of O^6MeGua MeTase by growth in free O^6MeGua is that the effect should be more specific. By using this approach, it has been shown that MeTase depletion results in sensitization to the mutagenic but not the cytotoxic effects of methylating agents (36).

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