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Relationship between O^6-alkylguanine-DNA alkyltransferase activity and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced mutation, transformation, and cytotoxicity in C3H/10T½ cells expressing exogenous alkyltransferase genes

(*O*⁶-methylguanine/*ada*/*ogt*/neoplastic transformation)

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ABSTRACT **While a great deal of evidence has directly implicated the importance of O⁶-alkylation of guanine in the mutagenicity of alkylating agents, evidence demonstrating the oncogenic potential of this lesion has been largely indirect. We have combined a well-studied *in vitro* neoplastic transformation system (using C3H/10T½ mouse cells) with a proven method of gene transfection for expressing the bacterial *O*⁶-alkylguanine-DNA alkyltransferase (AT; EC 2.1.1.63) repair genes *ada* and *ogt* to generate subclones which possess augmented repair capability toward specific DNA lesions. The products of these genes specifically and differentially repair *O*⁶-methylguanine (*O*⁶-MeGua), *O*⁴-methylthymine (*O*⁴-MeThy), and methylphosphotriesters. We show that the level of expression of either the *ada* or the *ogt* AT gene in C3H/10T½ cells directly correlates with protection against mutation to ouabain resistance by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). Subclones expressing 70 fmol of AT per 10⁶ cells exhibited a mutation frequency approximately 1/40th of that of clones expressing 15 fmol of AT per 10⁶ cells when treated with MNNG at 0.4 μg/ml. Protection against mutagenesis by MNNG at 0.8 μg/ml, however, did not exceed 12-fold even in subclones expressing greater than 100 fmol of AT per 10⁶ cells. As an MNNG dose of 0.6 μg/ml was sufficient to saturate more than 95% of the AT activity in any of the clones, the residual mutation frequency may have been caused by unrepaired *O*⁶MeGua lesions. In contrast to mutagenesis, protection against neoplastic transformation *in vitro*, in cells expressing high levels of AT, was most pronounced in cells treated with the highest dose of MNNG used (1.2 μg/ml). Low levels of transformation caused by MNNG at 0.4 and 0.8 μg/ml were not consistently inhibited in those clones. These data suggest that *O*⁶-MeGua formation is of major but not unique significance in the neoplastic transformation of C3H/10T½ cells by MNNG.**

The formation and persistence of O^6-alkylguanine (O^6-AlkGua) is strongly associated with neoplastic transformation *in vivo* and *in vitro* (1). The miscoding and mutagenic potential of this lesion has been established in *in vitro* systems using purified DNA polymerases (1, 2) as well as in prokaryotic and eukaryotic cell systems (3). Evidence that the specific mutagenic potential of O^6-AlkGua is responsible for the carcinogenicity of alkylating agents was supplied by the observation that a *ras* oncogene was frequently mutated in a manner consistent with the formation of O^6-methylguanine (O^6-MeGua) in murine mammary tumors caused by *N*-methyl-*N*-nitrosourea (4). Subsequent studies have underscored the relationship between carcinogen-specific mutations in oncogenes and tumorigenesis (5). While these data suggest that simple point mutations may be involved in the initiation of carcinogenesis, additional events are clearly necessary for complete carcinogenesis (6). The difficulties inherent in following a target cell *in vivo* from the time of carcinogen exposure to tumor formation, however, have hampered efforts to critically evaluate the relationship between mutation and carcinogenesis.

The use of *in vitro* neoplastic transformation systems offers a means of more closely characterizing the critical interactions between carcinogenic agents and target cells. The question we address in the current study is the role of the mutagenic lesion O^6-MeGua in the neoplastic transformation of C3H/10T½ (10T½) mouse cells compared with the role of this lesion in causing cytotoxicity and mutation to ouabain resistance. To dissect out the importance of O^6-MeGua in the biological effects of the methylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), we have generated a series of 10T½ subclones which express the bacterial DNA repair genes *ada* and *ogt* (7–9). The products of these genes specifically and stoichiometrically transfer the alkyl group from the O⁶ position of guanine onto one of their own cysteine residues (10, 11). Gene transfection has been used successfully to demonstrate the mutagenic, cytotoxic, and clastogenic effects of O^6-AlkGua (reviewed in ref. 1). We now report that expression of either the *ada* or the *ogt* O^6-alkylguanine-DNA alkyltransferase (AT; EC 2.1.1.63) gene in 10T½ cells protects them against MNNG-induced mutation and neoplastic transformation. The stoichiometry of protection, however, suggests that the endpoint of neoplastic transformation may be modified by MNNG-induced damage which does not influence mutation to ouabain resistance.

MATERIALS AND METHODS

Cell Culture System. 10T½ cells were maintained in Eagle's basal medium supplemented with 10% fetal calf serum as described previously (12). Standard cytotoxicity assays and mutation to ouabain resistance, using a 4-day expression time, were performed as described (13). Mutation frequency was calculated by the Poisson method (14). Neoplastic transformation (as measured by morphological transformation) was performed according to the Nesnow modification of the standard 10T½ transformation assay, which results in more efficient transformation of 10T½ cells by alkylating agents (15). This involves seeding cells at 2000 cells per 60-mm dish and treating with test agent 5 days rather than 24 hr after

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Abbreviations: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; O^6-AlkGua, O^6-alkylguanine; O^6-MeGua, O^6-methylguanine; O^4-MeThy, O^4-methylthymine; MCA, 20-methylcholanthrene; AT, O^6-alkylguanine-DNA alkyltransferase; 10T½, C3H/10T½.

seeding. Treatment time was 4 hr for MNNG (Sigma) and 24 hr for 20-methylcholanthrene (MCA) (Sigma).

Bacterial AT Genes and Transfection of 10T½ Cells. The *ada* and *ogt* AT genes used for these studies have been described previously (9, 16). The plasmid pXTO6C, which comprises the *ada* gene (the expression of which is driven by the herpes simplex virus thymidine kinase promoter) inserted into the *Bgl* II site of the retroviral vector pXT1 (17), was introduced into the retroviral packaging cell line, Ψ2, and a high-titer retroviral producer, ΨXTO6C, was isolated. Conditioned medium from confluent virus-producing cells was passed through a 0.45-μm filter and added, in the presence of Polybrene at 8 μg/ml, to a T75 flask seeded 24 hr earlier with 10^4 10T½ cells (p7). After 2 hr at 37°C, sufficient medium was added to bring the final concentration of Polybrene to 2 μg/ml, and incubation at 37°C was continued for 48 hr. After selection in G418 at 500 μg/ml and subcloning, cells were assayed for AT activity and cryopreserved in liquid nitrogen until further use.

In other experiments, the *ada* gene in the pZIP-NeoSV(X)1-based plasmid [designated pJCBO6C (16)] and the *ogt* gene, also in pZIP-NeoSV(X)1 [designated pZogtKL (L. Harris and G.P.M., unpublished data)] were transfected into 10T½ cells by lipofection (18). These vectors possess the Moloney murine leukemia virus long terminal repeat (LTR). Briefly, 2–10 μg of plasmid DNA was combined with 50 μg of Lipofectin (BRL) and added to 10T½ cells at 50% confluence in a 100-mm dish containing 5 ml of serum-free medium. After 6 hr, sufficient medium containing 20% fetal calf serum was added to bring the final concentration of serum to 10%. Sixteen hours later the medium was changed; selection and subcloning of transfected colonies were performed as described for retroviral transfection.

Qualitative differences in AT activity in *ada*- versus *ogt*-transfected cells confirmed the expression of these two bacterial genes. First, O^6-benzylguanine, which is a specific inhibitor of mammalian AT (19), only partially inhibited expression of AT activity in transfected cells (E.v.H., unpublished data). Second, we have demonstrated by the use of high-performance liquid chromatography that O^4-methylthymine (O^4-MeThy) is repaired in substrate DNA by extracts from *ogt*-transfected cells but not by extracts from *ada*-transfected or control 10T½ cells (unpublished data). This is consistent with kinetic studies comparing the substrate specificities of the purified *ada* and *ogt* gene products (20).

Determination of AT Activity. The level of AT activity in extracts of control and transfected cells was determined in cultures seeded into 150-mm dishes in parallel to mutation, transformation, and cytotoxicity experiments as described previously (21). While cells were seeded at different densities for the different biological experiments, all cells were in logarithmic-phase growth at the time of treatment and assay for AT activity. To determine the depletion of AT activity in transfected subclones, 10^5 cells were seeded per 150-mm dish and treated with MNNG for 4 hr when the cells became 80% confluent. Six hours after the end of treatment, cells were harvested and assayed for AT activity.

Determination of Cell Doubling Time. The cell doubling time was determined for three *ada*- and three *ogt*-expressing subclones. Cells from each subclone in logarithmic growth were seeded into 12 100-mm dishes (25,000 cells per dish). The cells from three dishes were trypsinized and counted individually on four successive days after seeding.

RESULTS

Transfection with AT Genes. The endogenous level of AT in 10T½ cells is roughly 20 fmol of AT per 10^6 cells, and we obtained *ada*- and *ogt*-transfected subclones expressing from 9 to 130 fmol of AT per 10^6 cells. The expression of both *ada* and *ogt* was stable over several passages of individual subclones (the coefficient of variation averaged 0.17 over five passages of four *ada*- and five *ogt*-transfected subclones with no upward or downward trend). The *ada* gene product possesses a site for accepting the methyl group from methylphosphotriesters (22) as well as a site which can repair O^6-MeGua and (to a lower extent) O^4-MeThy, while the *ogt* gene product has only an O^6-MeGua/O^4-MeThy acceptor site (20). As both the methyl-acceptor sites on *ada* can be occupied simultaneously, the total amount of O^6-MeGua and O^4-MeThy repaired per fmol of total AT activity (as measured by transfer of [^{3}H]methyl groups from both O^6-MeGua/O^4-MeThy and methylphosphotriester sites, all of which are produced by MNNG and *N*-methyl-*N*-nitrosourea) would be less than in *ogt*-expressing cells. Also, total AT activity expressed by *ada* would be expected to be depleted twice as fast as the activity expressed by *ogt*. As only ½ the number of *ada* product molecules exhibit the same total AT activity as a given number of *ogt* product molecules, ada-encoded activity would be depleted at a rate similar to half the amount of *ogt*-encoded activity. This is in fact the pattern of depletion we observe in the *ada* and *ogt* clones we have examined (Fig. 1). While *ogt*-transfected cells expressing 115 fmol of AT per 10^6 cells still had some residual AT activity after cells were treated with MNNG at 0.6 μg/ml (clone 2O2A, Fig. 1), *ada*-transfected cells expressing 121 fmol of AT per 10^6 cells were almost completely depleted after being treated with MNNG at 0.4 μg/ml (clone A3A, Fig. 1). To better compare repair activity in *ada*- and *ogt*-transfected subclones, the AT values in Tables 1 and 2 as well as Figs. 2 and 3 reflect only O^6-MeGua/O^4-MeThy repair activity (calculated by the following formula, which assumes an average endogenous activity of 20 fmol of AT per 10^6 cells: [(total AT − 20)/2] + 20.

Mutagenicity of MNNG in Transfected 10T½ Subclones. Cells expressing a high level of *ada* or *ogt* exhibited dramatically increased resistance to mutation by MNNG (Fig. 2). Even relatively small increases in AT activity conferred high levels of resistance. The correlation was further improved in *ada*-transfected cells when only O^6-MeGua repair activity (i.e., corrected for methylphosphotriester repair) was correlated with mutation frequency. The slopes of the lines obtained by power regression were 0.44 (for cells treated with MNNG at 0.4 μg/ml) and 0.73 (MNNG at 0.8 μg/ml), but the difference was not statistically significant (analysis of variance). In summary, the data indicate a strong inverse correlation between the repair of O^6-MeGua and mutagenicity.

Neoplastic Transformation of Transfected Subclones. Neoplastic transformation caused by MNNG treatment was also reduced in cells expressing high levels of either the *ada*- or

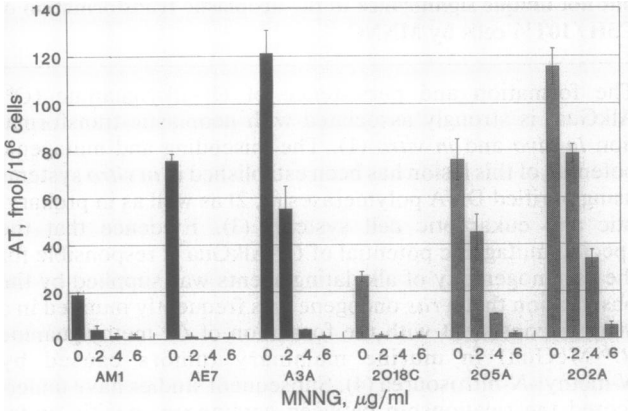

FIG. 1. Depletion of total AT activity in *ada*- (solid bars) and in *ogt*- (cross-hatched bars) transfected cells 10 hr after treatment with MNNG at 0.2, 0.4, or 0.6 μg/ml. The mean ± SD for three determinations is given for each data point.

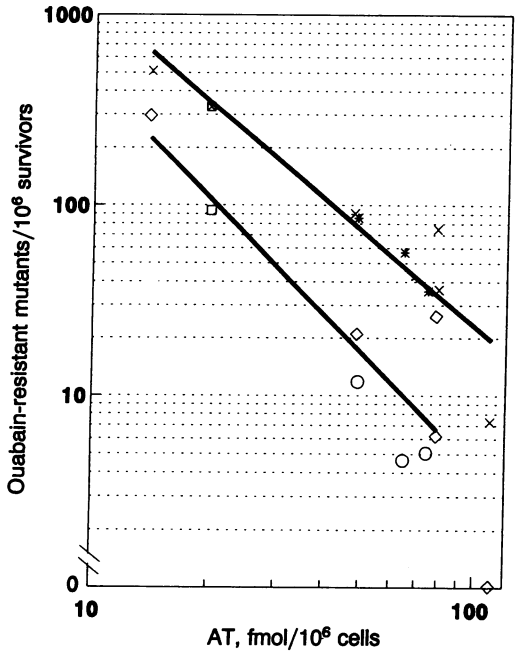

FIG. 2. Effect of AT activity on mutagenicity to ouabain resistance in *ada*-transfected cells treated with MNNG at 0.4 (○) or 0.8 (*) μg/ml, *ogt*-transfected cells treated with MNNG at 0.4 (◇) or 0.8 (×) μg/ml, and untransfected 10T½ cells treated with MNNG at 0.4 (□) or 0.8 (⊠) μg/ml. The AT activity was calculated to reflect only O^6-MeGua and O^4-MeThy repair activity (see text).

the *ogt*-encoded ATs (Tables 1 and 2, Fig. 3). In contrast to mutagenicity, however, this effect was greatest in cells treated with a high dose of MNNG (1.2 μg/ml). Surprisingly, there was significant transformation by MNNG at 0.4 μg/ml in two *ada*-transfected subclones expressing elevated levels of AT (AE7 and A6A in Table 1). While some 10T½ subclones expressing AT genes exhibited a relatively high level of spontaneous transformation (clone AE7 in Table 1 and clone 2O2A in Table 2), the protective effect afforded by increased AT expression in these subclones was comparable to protection in cells with a low level of spontaneous transformation. It was unclear why subclone A6A (54 fmol of AT per 10^6 cells) displayed higher transformation than did other subclones expressing a comparable level of AT activity. The correlation between AT activity and neoplastic transformation by MNNG at 1.2 μg/ml suggests clustering of resistant clones with high AT levels (greater than 40 fmol of AT per 10^6 cells) and sensitive clones with low AT levels (less than 40 fmol of AT per 10^6 cells) (Fig. 3). This holds whether the data are plotted as total type II and type III transformed colonies per dish (minus background) relative to transformation by MCA (Fig. 3) or as transformation frequency ($P < 0.05$ for both, Fisher exact test; figure not shown).

Cell Cycle Analysis of Transfected Subclones. The cell doubling times were consistently longer in transfected subclones (22.7 ± 1.9 hr) compared to parental 10T½ cells (17.2 ± 1.3 hr) but did not correlate with mutagenicity or neoplastic transformation by MNNG (data not shown). However, in six of the subclones for which cell doubling time was determined (AM1, 2O18C, AE7, 2O16C, AI2, and 2O5A), there was good correlation between the LD_{50} of MNNG (0.26, 0.51, 0.75, 0.80, 0.89, and 1.1 μg/ml, respectively) and cell doubling time (20.6, 21.0, 21.8, 22.9, 24.3, and 25.4 hr, respectively. Correlation between the cytotoxicity of MNNG in 10T½ cells and AT activity was poor (Tables 1 and 2).

DISCUSSION

Convincing evidence for the miscoding (2, 3) and mutagenic potential of O^6-MeGua together with the strong association between unrepaired O^6-MeGua DNA lesions and neoplastic transformation *in vitro* (23, 24) and *in vivo* (25) provide compelling but circumstantial evidence for the causative mutagenic basis of carcinogenicity by alkylating agents such as MNNG. In the 10T½ cell system, some data have suggested that transformation can occur as a single-step mutagenic event (24, 26, 27), while other data suggest that there are multiple stages in transformation and that the initial step occurs at a frequency much higher than any known mutagenic event (28, 29). Evidence implicating the importance of nonmutagenic events in the initiation of *in vitro* neoplastic transformation in 10T½ cells, as well as in other *in vitro* and *in vivo* systems, has recently been reviewed (30).

Table 1. Neoplastic transformation of 10T½ cells expressing various levels of *ada*-encoded AT

Subclone[a] Treatment	P.E.,[b] % Abs.	Rel.	No. of dishes with foci/total no. of dishes[c] Type III	Type II or III
AM1 (9)				
Control	23	100	0/18 [0]	0/18 [0]
MCA, 1 μg/ml	21	91	10/20 [17]	18/20* [38]
MNNG, 0.4 μg/ml	9.8	42	2/20 [2]	5/20§ [5]
MNNG, 0.8 μg/ml	3.4	15	2/20 [2]	9/20* [9]
MNNG, 1.2 μg/ml	0.4	1.7	3/17 [3]	13/17* [26]
AI2 (31)				
Control	33	100	0/20 [0]	2/20 [2]
MCA, 1 μg/ml	27	82	6/20 [6]	13/20* [18]
MNNG, 0.4 μg/ml	26	79	0/20 [0]	3/20 [3]
MNNG, 0.8 μg/ml	20	61	0/20 [0]	5/20 [6]
MNNG, 1.2 μg/ml	1.1	3.3	1/18 [1]	9/18‡ [15]
A6B (42)				
Control	21	100	0/32 [0]	0/32 [0]
MCA, 1 μg/ml	18	86	4/30 [2]	7/30* [9]
MNNG, 0.4 μg/ml	12	57	0/29 [0]	1/29 [1]
MNNG, 0.8 μg/ml	0.85	4.1	2/30 [2]	2/30 [2]
MNNG, 1.2 μg/ml	0.2	1.0	0/15 [0]	0/15 [2]
AE7 (45)				
Control	36	100	4/20 [11]	10/20 [20]
MCA, 1 μg/ml	33	92	19/20 [45]	20/20* [100]
MNNG, 0.4 μg/ml	28	78	10/20 [10]	17/20§ [33]
MNNG, 0.8 μg/ml	17	47	12/20 [14]	9/20† [44]
MNNG, 1.2 μg/ml	1.4	3.9	4/20 [4]	12/20 [15]
A6A (54)				
Control	25	100	1/35 [1]	3/35 [4]
MCA, 1 μg/ml	22	88	19/36 [28]	26/36* [46]
MNNG, 0.4 μg/ml	14	56	8/34 [10]	17/34* [24]
MNNG, 0.8 μg/ml	4.3	17	11/36 [13]	20/36* [34]
MNNG, 1.2 μg/ml	1.0	4.0	2/14 [2]	5/14§ [7]
A3A (61)				
Control	21	100	0/35 [0]	3/35 [3]
MCA, 1 μg/ml	18	86	17/39 [21]	34/39* [59]
MNNG, 0.4 μg/ml	10	48	1/40 [1]	6/40 [8]
MNNG, 0.8 μg/ml	2.1	10	1/39 [1]	11/39§ [12]
MNNG, 1.2 μg/ml	0.2	1.0	0/16 [0]	0/16 [0]

[a]Subclones A6A, A6B, and A3A were from the same pool of cells transfected by defective retrovirus infection; subclones AM1, AI2, and AE7 were from three separate pools of cells transfected by lipofection. The numbers in parentheses indicate the specific O^6-MeGua/O^4-MeThy repair activity in terms of fmol per 10^6 cells (see text).

[b]Cytotoxicity is reported as percent plating efficiency (P.E.), absolute (Abs.) and relative to solvent-treated controls (Rel.).

[c]Types II and III foci are discussed in ref. 12. The numbers in square brackets indicate the total number of foci. Results of statistical analysis (Fisher exact test) of number of dishes with foci divided by total number of dishes (treated vs. untreated cells): *, $P < 0.001$; †, $P < 0.005$; ‡, $P < 0.01$; §, $P < 0.05$.

Table 2. Neoplastic transformation of 10T½ cells expressing various levels of *ogt*-encoded AT

Subclone[a] Treatment	P.E.,[b] % Abs.	Rel.	No. of dishes with foci/total no. of dishes[c] Type III	Type II or III
2O18C (17)				
Control	32	100	0/18 [0]	1/18 [1]
MCA, 1 μg/ml	34	106	10/20 [16]	15/20* [25]
MNNG, 0.4 μg/ml	19	59	1/19 [1]	1/19 [1]
MNNG, 0.8 μg/ml	9.6	30	3/18 [3]	5/18 [5]
MNNG, 1.2 μg/ml	0.6	2.0	7/19 [8]	13/19* [22]
10T½ (24)				
Control	30	100	0/30 [0]	2/30 [2]
MCA, 1 μg/ml	35	116	13/20 [20]	16/20* [34]
MNNG, 0.4 μg/ml	22	73	2/37 [2]	7/37 [8]
MNNG, 0.8 μg/ml	16	53	6/38 [6]	16/38* [19]
MNNG, 1.2 μg/ml	7.5	25	13/38 [15]	30/38* [54]
2O18B (36)				
Control	22	100	0/19 [0]	1/19 [1]
MCA, 1 μg/ml	20	91	16/19 [31]	19/19* [60]
MNNG, 0.4 μg/ml	19	86	0/16 [0]	2/16 [2]
MNNG, 0.8 μg/ml	19	86	0/19 [0]	0/19 [0]
MNNG, 1.2 μg/ml	16	73	1/20 [1]	3/20 [3]
2O5A (41)				
Control	22	100	0/20 [0]	0/20 [0]
MCA, 1 μg/ml	22	100	16/19 [26]	19/19* [73]
MNNG, 0.4 μg/ml	21	95	0/19 [0]	3/19 [3]
MNNG, 0.8 μg/ml	18	82	4/20 [4]	10/20* [11]
MNNG, 1.2 μg/ml	8.7	40	1/20 [1]	6/20‡ [9]
2O16C (60)				
Control	18	100	0/20 [0]	0/20 [0]
MCA, 1 μg/ml	17	94	8/20 [8]	17/20* [21]
MNNG, 0.4 μg/ml	11	61	0/17 [0]	0/17 [0]
MNNG, 1.8 μg/ml	9.4	52	0/17 [0]	1/17 [1]
MNNG, 1.2 μg/ml	6.1	34	0/20 [0]	1/20 [1]
2O2A (112)				
Control	8.7	100	0/19 [0]	10/19 [11]
MCA, 1 μg/ml	7.2	83	10/20 [15]	19/20† [56]
MNNG, 0.4 μg/ml	6.1	70	0/20 [0]	10/20 [13]
MNNG, 0.8 μg/ml	3.2	37	0/20 [0]	14/20 [20]
MNNG, 1.2 μg/ml	6.1	16	1/15 [1]	9/15 [12]

[a]Subclones 2O18B, 2O18C, and 2O16C were from one pool of Lipofectin-transfected cells and 2O5A and 2O2A were from another. The numbers in parentheses indicate the specific O^6-MeGua/O^4-MeThy repair activity in terms of fmol per 10^6 cells (see text).
[b]Cytotoxicity reported as for Table 1.
[c]Numbers reported as for Table 1. *, $P < 0.001$; †, $P < 0.005$; ‡, $P < 0.05$.

The results of the current studies provide direct evidence for the role of O^6-MeGua in the neoplastic transformation of 10T½ cells by high doses of MNNG (Tables 1 and 2, Fig. 3). The protection of 10T½ cells against neoplastic transformation by MNNG was somewhat more pronounced in cells expressing the *ogt* gene. As has been mentioned previously, the total AT activity in *ada*-transfected subclones reflects 50% repair of O^6-MeGua plus O^4-MeThy and 50% repair of methylphosphotriesters, an activity lacking in the *ogt* gene product (8, 31). Other more subtle differences between the *ada* and *ogt* gene products are that O^4-MeThy is much more efficiently repaired by the *ogt* than by the *ada* gene product (refs. 20 and 32; also E.v.H. and G.P.M., unpublished results) and there is greater similarity between the *ogt* gene product and mammalian ATs in that neither possess a methylphosphotriester repair function (10, 33); it may be, therefore, that the *ogt* gene product is more effective at repairing lesions within the mammalian genome than is the *ada* gene product.

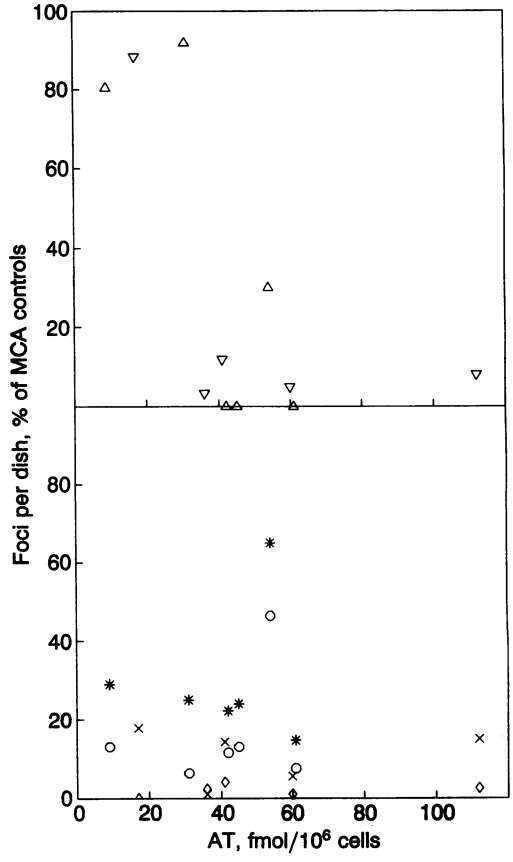

FIG. 3. Effect of AT activity on neoplastic transformation by MNNG. (*Upper*) *ada*- (△) or *ogt*- (▽) transfected cells treated with MNNG at 1.2 μg/ml. (*Lower*) *ada*-transfected cells treated with MNNG at 0.4 (○) or 0.8 (*) μg/ml and *ogt*-transfected cells treated with MNNG at 0.4 (◇) or 0.8 (×) μg/ml. The data (from Tables 1 and 2) are expressed as foci per dish minus the background, relative to the MCA-treated controls. The AT activity in *ada*-transfected cells was calculated to reflect only O^6-MeGua and O^4-MeThy repair activity (see text).

The differential protection against transformation by high versus low doses of MNNG was not observed in mutation experiments. This is comparable to the resistance observed in previous studies utilizing x-irradiation to increase endogenous AT prior to treatment with MNNG (21), suggesting that the protective effect observed in those studies was indeed the result of increased AT activity and not some other possible effect of x-rays. This consistent discrepancy between protection against mutation and against neoplastic transformation of 10T½ cells by MNNG suggests that the endpoint of neoplastic transformation is sensitive to cellular events which are not reflected in simple mutagenesis assays. In the current study, subclones of 10T½ cells expressing increased AT were obtained by gene transfection and were not exposed to genotoxic agents for selection or induction at any time prior to the experiments. In another cell transformation study, rat cells (208F) were selected with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) to obtain subclones with increased AT activity which were subsequently challenged with the alkylating carcinogen ethylnitrosourea (34). In that system, protection against neoplastic transformation more closely paralleled protection against mutation to ouabain resistance in 10T½ cells, suggesting that a simple mutagenic event is the most likely mechanism for the transformation of 208F cells by alkylating agents. It should be pointed out, however, that the alkylating agent used in that study (ethylnitrosourea) produces a higher proportion of O-alkylation (i.e., lesions with greater mutagenic potential than N-alkylation products) than

does MNNG. Also, ethylated lesions are removed less efficiently by the mammalian AT than are methylated lesions.

Gene transfection studies whereby AT-deficient cells were converted into cells expressing very high levels of AT (1, 16, 35, 36) have demonstrated a strong association between unrepaired O^6-MeGua lesions and cytotoxicity. While the LD_{50} for MNNG in the transfected subclones was indeed lowest in the subclone expressing the lowest AT activity (AM1, Table 1), the overall correlation between AT activity and MNNG-induced cytotoxicity was poor. In contrast, a strong association between protection against MNNG-induced cytotoxicity and an extended cell doubling time was observed (see *Results*). This suggests that minimal amounts of AT (less than 20 fmol of AT per 10^6 cells) are sufficient to protect against the contribution of O^6-MeGua to the overall cytotoxicity of MNNG. In cells having more than 20 fmol of AT per 10^6 cells, however, lesions other than O^6-MeGua which have a greater chance of being repaired during an extended cell cycle may play a predominant role in determining MNNG cytotoxicity.

In summary, these results suggest at least two separate mechanisms of neoplastic transformation of 10T½ cells by MNNG: (*i*) Formation of O^6-MeGua. However, the high dose of MNNG required before a protective effect of AT can be observed suggests that transforming O^6-MeGua lesions are preferentially repaired over nontransforming O^6-MeGua lesions. (*ii*) Non-O^6-MeGua-mediated damage. This damage can give rise to a low level of transformation but may be more strongly associated with cytotoxicity than is O^6-MeGua. Therefore, this type of transformation is not increased in cells treated with higher, cytotoxic, doses of MNNG.

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