Oncogenic Transformation and Mutation of C3H/10T½ Clone 8 Mouse Embryo Fibroblasts by Alkylating Agents

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Oncogenic Transformation and Mutation of C3H/10T½ Clone 8 Mouse Embryo Fibroblasts by Alkylating Agents[1]

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ABSTRACT

Two-hr treatments with *N*-methyl- and *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidines and ethyl methanesulfonate induced ouabain-resistant mutants in C3H/10T½ cells. The alkylnitronitrosoguanidines gave linear dose-response curves and were more potent mutagens than were ethyl methanesulfonate and methyl methanesulfonate. These differences in potency were largely due to differences in the half-lives of the alkylating agents in culture medium. Differences in mutation frequencies at equitoxic concentrations of the alkylating agents are considered to reflect differences in the chemical mechanisms of alkylation and mutagenesis by the compounds. However, the frequencies of mutations produced at equitoxic concentrations were not uniformly associated with the nucleophilic selectivities of the compounds as expressed by their published Swain-Scott substrate constants. Whether or not followed by repeated replating, the yield of oncogenically transformed foci of asynchronous cells after treatment with the alkylating agents was so low that we could not obtain dose-response curves, and the yield may not be significant. By contrast, in previous experiments with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidines and polycyclic hydrocarbons in Syrian hamster embryo fibroblasts and with ultraviolet light and polycyclic hydrocarbons in C3H/10T½ cells, transformation occurred to an equal or greater extent than mutation measured in the same cells.

INTRODUCTION

The C3H/10T½ Cl[3] 8 line of mouse embryo fibroblasts can be oncogenically transformed with a variety of carcinogenic chemicals and radiations (13, 14). In these cells, mutagenesis to ouabain resistance (4, 17), inhibition of DNA synthesis, cytotoxicity, and alkali-labile DNA damage (26, 27) produced by a variety of mutagens can also now be measured. The use of such measurements in Chinese hamster cells has been put forward as a means of early detection of environmental carcinogens (26). However, the C3H/10T½ cell line offers the additional advantage of a tier of screening tests, ranging from the very rapid one of inhibition of DNA synthesis to the longer term but doubtless more relevant assays of oncogenic transformation and its initiation and promotion. These tests can be used to provide in the same cells a systematic and quantitative analysis of the genotoxic potential of environmental chemicals.

Using 4 alkylating agents of different oncogenic potencies, we have delineated previously (27) the associations among some of the above properties in Chinese hamster V79 cells and also in the C3H/10T½ cells, but we were unable to measure mutation to 8-azaguanine resistance in the hypotetraploid C3H/10T½ cells. We have now measured mutations to ouabain resistance produced in C3H/10T½ cells by the alkylating agents used in the previous studies. We describe here the associations of these measurements with assays of mutagenesis in V79 cells and assays of cytotoxicity, alkali-labile DNA damage, and inhibition of DNA synthesis in V79 and C3H/10T½ cells that we reported previously (26) and with the chemical reactivities of the compounds. We also report data from our experiments on the oncogenic transformation of C3H/10T½ cells by the above alkylating agents.

MATERIALS AND METHODS

Chemicals and Cell Culture Media. The sources of MNNG, ENNG, MMS, and EMS, of the C3H/10T½ Cl 8 and V79 cells, and of the media and plasticware used for culturing them have been described in detail previously (27). Ouabain was obtained from Sigma Chemical Co., St. Louis, Mo.

Reaction Rates of Alkylating Agents. Half-lives of the alkylating agents were determined in Dulbecco's medium without serum, because it has been shown that the 10% serum concentration does not affect the half-lives of MNNG and MMS (20, 31) but can be expected to interfere with the NBP assay (8, 11). Half-lives of the alkylating agents were determined at 37° for starting concentrations of 1.0 mM MMS, 1.3 mM EMS, 5 mM MNNG, or 5 mM ENNG using a modification of a published method (11, 30). At various time intervals, 0.2 ml of the reaction mixtures was added to 1.0 ml of ethylene glycol:glycerol:water (2:2:1) containing 0.8% (w/v) NBP and 40 mM Tris-HCl (pH 7.0). The NBP reactions were carried out at 95° for 40 min in the case of MMS and for 3 hr for EMS, MNNG, and ENNG. After cooling to 4°, the NBP chromophore(s) was extracted by the addition of 1.0 ml of acetone, 2.5 ml of ethyl acetate, and 1.0 ml of 0.25 N sodium hydroxide, followed by centrifugation at 1300 × *g* for 45 sec. The absorbance at 540 nm of the ethyl acetate phase was measured using a Beckman Model 25 spectrophotometer 3 min after adding alkali.

Treatment of C3H/10T½ Cells with Alkylating Agents. Beginning 24 hr after plating, C3H/10T½ cells were treated for 2 hr with the alkylating agents, acetone solutions of which were added directly to the complete serum-supplemented medium in the dishes in which the cells were growing. Control cultures were treated with the same volume of acetone (final concentration, 0.5% in culture medium) as that used as a solvent for the alkylating agents. These treatment conditions are identical to those used in our studies of the effects of these alkylating agents on DNA damage, DNA synthesis, and cytotoxicity (26, 27).

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[3] The abbreviations used are: Cl, clone; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; ENNG, *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine; MMS, methyl methanesulfonate; EMS, ethyl methanesulfonate; NBP, 4-(4'-nitrobenzyl)pyridine; *oua*^{r}, ouabain-resistant; LD, dose lethal to percentage of cells indicated by subscript number; *AG*^{r}, 8-azaguanine-resistant.

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Cytotoxicity and Mutation to Ouabain Resistance. For one measurement of mutagenesis (17, 18), 10^5 cells were plated in each of 5 to 10 100-mm Petri dishes. After 24 hr, the cells were treated with alkylating agents for 2 hr, rinsed twice in 0.9% NaCl solution buffered with 0.01 M sodium phosphate (pH 7.2) and then incubated in fresh medium, not containing alkylating agents, for 2 or 5 days. The cells were then trypsinized (17), pooled, counted, and replated to score for induced *oua*^{r} mutants according to the methods in Ref. 17. Mutation frequencies were calculated using the Poisson method (18). Each data point is the mean of at least 2 measurements. Cytotoxicities of the alkylating agents were determined by colony counting as described previously (27).

Transformation Assays. For measurements of transformation, either 2,000 (22, 27, 28) or 10,000 cells were plated in each of 10 to 100 60-mm dishes. With 2,000 cells, the cells were not replated after treatment. With 10,000 cells, the cells were replated at 2.5×10^4 cells/dish at 8 days after treatment. The medium was changed immediately after treatment at 5 and 10 days after treatment and at weekly intervals once the cells had attained confluence. At 5 weeks after the cells had attained confluence, transformation was scored by counting the number of dishes containing type 3 foci of piled-up cells (28). Surviving fractions for all the transformation assays were determined by the methods for measuring cytotoxicity with 200 cells/dish (27). Plating efficiencies for the replating procedure were also measured, with 200 cells replated per dish.

Calculations. The concentration-time integral exposures (25) to alkylating agents that the cells received during the 2-hr treatment period were calculated from the following formula:

$$\text{Integral exposure} = C(_0\int^t e^{-kt}dt)$$

where C is the concentration (μM) of the alkylating agent; k is the pseudo-first-order rate constant calculated from the half-life of the compound in Dulbecco's medium at 37°; and t is the treatment time in hr.

The results of the mutation experiments are expressed in 3 ways: mutation frequency (mutants/10^5 survivors), for the purpose of graphing the raw data; mutagenic potency defined as 1/integral exposure (μM × hr) required to induce one mutant per 10^5 survivors, for comparison with chemical reactivity; and mutation frequency/D_0, where D_0 is the integral exposure that reduces the surviving fraction by 1/e on the exponential portion of the survival curve.

RESULTS

Mutagenicity Assays in C3H/10T½ Cl 8 Cells. Expression times of 2 days, as in previous experiments (17), or of 5 days, so as to allow mutant expression in C3H/10T½ cultures treated with growth-inhibiting concentrations of alkylating agents, produced dose responses that were not significantly different on linear regression analysis (Chart 1). This similarity of the mutational response at the 2 expression times was found with MNNG, ENNG, and EMS at all concentrations tested.

Treatment of the C3H/10T½ cells with LD_{20} to LD_{90} concentrations (27) of the alkylating agents ENNG, MNNG, and EMS induced *oua*^{r} mutants at frequencies in the range of 1 to 30 mutants/10^5 survivors (Chart 1), but no *oua*^{r} mutants were induced with LD_{20} to LD_{90} concentrations of MMS. Since MMS is a weak inducer of *AG*^{r} mutants in Chinese hamster cells (1, 23, 27), it has been suggested that this compound is relatively ineffective in producing point mutations of the type that yields *oua*^{r} mutants (1). The molar concentration of EMS needed to induce 2 *oua*^{r} mutants/10^5 survivors was ~3 orders of magnitude higher than the concentrations of MNNG and ENNG that induced the same frequency of mutants. Moreover, MNNG was 5 times more mutagenic than was ENNG (Chart 1). These results with MNNG, ENNG, and EMS are in close agreement with their relative potencies for producing *AG*^{r} mutations in Chinese hamster cells (27) and alkali-labile DNA damage, inhibition of DNA synthesis, and cytotoxicity in Chinese hamster and C3H/10T½ Cl 8 cells (26, 27).

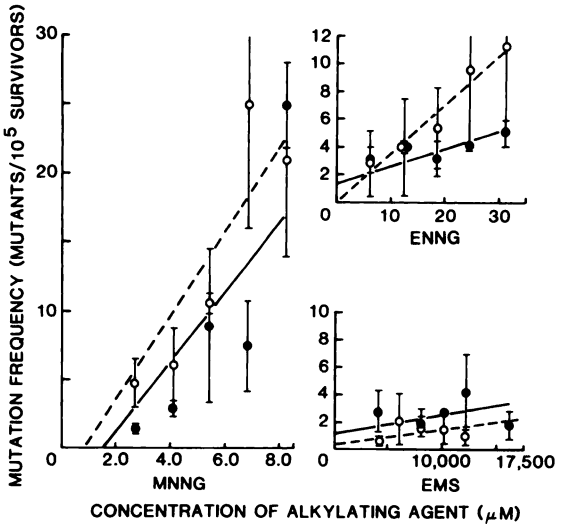

Chart 1. Induction by alkylating agents of *oua*^{r} mutants in C3H/10T½ cells after a 2-day (●) or 5-day (○) expression time. *Bars*, S.E. of 2 or more independent experiments. In 80 control dishes, no *oua*^{r} mutants were observed.

The concentration-time integral exposures to the 4 alkylating agents that produced equal cytotoxicities and those that produced equal mutation frequencies were roughly proportional to the half-lives of the compounds in Dulbecco's medium at 37° (Table 1), indicating an association between biological activity and chemical reactivity of the compounds. However, the integral exposures did not correlate with the published Swain-Scott constants (Table 1), showing that the differences in exposures were not uniformly associated with differences in nucleophilic selectivity (19, 24, 25) of the compounds. Therefore, we consider that the differences in exposures result largely from differences in the concentrations of alkylating agents that penetrate to and react with intracellular targets for cytotoxicity and mutation during the 2-hr treatment periods.

At equitoxic concentrations MNNG produced about 3 times as many *oua*^{r} mutants in C3H/10T½ cells as did ENNG and about 20 times as many as did EMS. MMS was the least mutagenic of the compounds at equitoxic concentrations in C3H/10T½ and in V79 cells. Moreover, the mutation frequencies produced by equitoxic concentrations of the alkylating agents did not correlate with the Swain-Scott constants nor with the half-lives of the compounds in Dulbecco's medium (Table 1).

Transformation Assays. The experimental protocol, in which 1000 to 2000 cells plated per 60-mm dish are treated with the test agent for 24 hr, allowed to grow to confluence, and scored for transformation 4 to 6 weeks later without replating, is considered optimal for measurement of transformation by poly-

Table 1

Chemical reactivities and biological effects of monofunctional alkylating agents in C3H/10T½ and V79 cells

	Swain-Scott constants, *s* (24, 25)	$t_{1/2}$ in medium[a] (hr)	D_0[b] (μM × hr)[c] in V79 cells (26)	D_0 (μM × hr) in C3H/10T½ cells (26)	Mutagenic potency^{-1} (μM × hr/mutant × 10^5 survivors) *AG*^{r} mutants in V79 cells (27)	*oua*^{r} mutants in C3H/10T½ cells (Chart 1)	Mutants × 10^{-5} survivors/D_0 in V79 cells (27)	in C3H/10T½ cells
MMS	0.83	4.1	270	401	48.6	>1,400	5.5	<0.30
EMS	0.64	26	9,800	15,000	635	14,000	15.4	1.1
MNNG	0.42	1.1	7.6	7.0	0.1	0.4	76.9	18.6
ENNG	0.26	2.1	12.7	28	0.6	4.1	21	6.8

[a] Dulbecco's medium without serum at 37°.
[b] D_0, dose that reduces surviving fraction by $1/e$.
[c] Integral exposure.

Table 2

Oncogenic transformation of asynchronous C3H/10T½ Cl 8 cells with alkylating agents

Two thousand cells/60-mm dish were treated with no replating.

Compound	Dose (μM)	Time (hr)	Surviving fraction	Dishes scored	Dishes with type 3 foci
Acetone	(0.5%)	2–24	1	125	0
Benzo(*a*)pyrene	4	24	1	61	41
3-Methylcholanthrene	4	24	1	74	41
Phenanthrene	4	24	1	70	1
Anthracene	4	24	1	76	0
MNNG	3.4	24	0.50	72	2
MNNG	3.4	2	0.50	36	0
MMS	136–454	2	0.63–0.20	58	0
ENNG	12.4	2	0.50	11	0
EMS	1000	24	0.83	66	0
EMS	6045	2	0.50	9	0

cyclic hydrocarbons (22, 28, 29). With benzo(*a*)pyrene and 3-methylcholanthrene, this protocol gave the expected high yield of type 3 transformed foci (Table 2), which have been shown to be the most tumorigenic type of foci produced in these cells (28, 29). Control experiments, in which the cells were treated with acetone, gave no foci of any kind in 125 dishes, and the noncarcinogen, anthracene, produced no type 3 foci in 76 dishes. The noncarcinogen, phenanthrene, produced one type 3 focus in 70 dishes (Table 2). We have not expressed the transformation data as transformation frequencies (transformants/survivor), because such "transformation frequencies" vary with cell density and are therefore ambiguous (9).

Using the above protocol, in experiments in asynchronous C3H/10T½ cells done over the period, 1975 to 1979, with concurrent negative (acetone, anthracene, phenanthrene) and positive (benzo(*a*)pyrene, 3-methylcholanthrene) controls, the LD_{50} level of MNNG produced 2 type 3 foci in 72 dishes. This is only twice as many type 3 foci as that produced by 4 μM phenanthrene, which is a noncarcinogen and also produced no cytotoxicity in C3H/10T½ cells. Neither ENNG, EMS, nor MMS produced any type 3 transformed foci (Table 2). Moreover, Table 3 shows that, in experiments where 12.5 to 25 times as many cells were exposed to MNNG and replated once or several times prior to being allowed to grow to confluence, the frequency of dishes containing transformed foci was no greater than that obtained in the nonreplating protocol and was not dose dependent.

Table 3

Oncogenic transformation of asynchronous C3H/10T½ Cl 8 cells with MNNG

Cells (10^4)/60-mm dish were treated and replated (2.5×10^4 cells/60-mm dish) at 8 days after treatment.

Dose (μM)	Time (hr)	Surviving fraction	PE[a] of replated cells (%)	Dishes treated	Dishes scored	Dishes with type 3 foci
0	2	1	27	12	45	0
1.36	2	0.74	31	8	29	1
2.72	2	0.58	29	12	41	0
3.40[b]	2	0.50	26	8	100	0
4.08	2	0.38	22	12	29	1
5.44	2	0.29	24	12	29	1
6.80	2	0.18	20	8	22	0

[a] PE, plating efficiency. The root mean square of the S.E.'s of PE is 2.6%.
[b] In this set of experiments, cultures were replated (5×10^4 cells/60-mm dish) at weekly intervals for 4 weeks before being allowed to grow to confluence. These cells underwent 56 doublings prior to attaining confluence.

DISCUSSION

Chan and Little (4) and Landolph and Heidelberger (17) have shown that ouabain resistance can be used as a criterion for measuring mutagenesis in C3H/10T½ Cl 8 cells. There is evidence that the *oua*^{r} colonies produced in these experiments result from mutation, presumably at the locus for the ouabain binding site of the Na-K-ATPase in the C3H/10T½ cells (4, 18).

Linear dose-response curves have been obtained with UV light, ionizing radiation, and chemical mutagens, using several mutational markers including *oua* resistance, in a variety of mammalian cells including C3H/10T½ Cl 8 cells (4, 6, 7, 10, 15–17, 23, 27). However, to obtain strictly linear dose-response curves for mutation in mammalian cells, it is often necessary to measure the mutant frequencies at the optimal expression time, which can vary with the toxicity of the mutagen

treatment (1, 4). Linear dose-response curves for induction of *oua*^{r} mutants can readily be obtained using a single expression time of 48 hr. Longer expression times, particularly after treatment with alkylating agents, can reduce the yield of *oua*^{r} mutants (5, 17). Chart 1 shows statistically indistinguishable dose-dependent increases in *oua*^{r} mutant frequencies after expression times of 2 and 5 days with 3 alkylating agents. Moreover, the highest correlation coefficients (0.91 and 0.98) were obtained with MNNG and ENNG using the 2-day expression time, which is consistent with other reports that found a 2-day expression time adequate for measuring *oua*^{r} mutants (5, 17). EMS and MMS were less mutagenic than were ENNG and MNNG in C3H/10T½ cells and in V79 cells (Table 1).

In large part, the differences in the D_0 concentrations and mutagenic potencies of the compounds may be ascribed to differences in the concentrations of alkylating agents that penetrate to and react with critical cellular targets during the 2-hr treatment period. This period is much shorter than the half-lives of the alkyl alkane sulfonates, which were least mutagenic in our studies (Table 1). However, at equitoxic concentrations, the 4 alkylating agents gave different frequencies of *AG*^{r} mutants in V79 cells and of *oua*^{r} mutants in C3H/10T½ cells (Table 1), which correspond to different extents of alkali-labile DNA damage produced under identical treatment conditions in the same cells (27). By contrast, DNA synthesis was inhibited to the same extent by equitoxic concentrations of the compounds (26). These results are in agreement with our previous findings in V79 cells, which led us to the interpretation that the alkali-labile lesions and mutations result largely from oxygen alkylation of the DNA, while cytotoxicity and the inhibition of DNA synthesis can result from other kinds of lesions (26, 27).

Mutation frequencies and the extent of oxygen alkylation of the DNA by methylating agents in mammalian cells are consistent with chemical theories of alkylation mutagenesis (19, 24, 25). According to these theories, ENNG, which has the lowest reported Swain-Scott constant of the 4 compounds used in our study (24, 25), should react least selectively with nucleophiles (19, 25) and therefore should produce more oxygen alkylation and higher mutation frequencies than do the other compounds at equitoxic concentrations (19, 24, 25). However, ENNG produced a lower mutation frequency than did MNNG at equitoxic concentrations (Table 1). Thus, in C3H/10T½ and V79 cells, the mutation frequencies induced by equitoxic concentrations of MNNG, ENNG, MMS, and EMS are not uniformly associated with nucleophilic selectivity expressed as Swain-Scott substrate constants. A similar conclusion can be drawn from studies of mutations produced by monofunctional alkylating agents in *Arabidopsis thaliana* (33), other mammalian cells (6, 7, 10, 15), *Drosophila* (34), barley seeds (25), and bacteria (32).

Although MNNG, ENNG, and EMS evidently produced mutagenic lesions in C3H/10T½ cells, we show that EMS, MMS, ENNG, and MNNG did not yield sufficient transformants in asynchronous cells to establish dose-response curves (Tables 2 and 3). This is in marked contrast with the much higher frequencies of oncogenic transformants relative to mutants found with polycyclic hydrocarbons and UV light in these cells (4, 17), with MNNG and polycyclic hydrocarbons in mouse M2 fibroblasts (21), and with polycyclic hydrocarbons in Syrian hamster embryo fibroblasts (2, 16). However, oncogenic transformation with MNNG occurs readily in synchronized C3H/10T½ cells (3, 12). Thus, in asynchronous C3H/10T½ cells containing mutagenic lesions produced by the potent carcinogens MNNG and ENNG, either additional genetic events are necessary for transformation to occur, or expression of the transformed phenotype can be suppressed.

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