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Genetics

Mutagen-induced resistance to mycophenolic acid in hamster cells can be associated with increased inosine 5′-phosphate dehydrogenase activity

(gene regulation/Chinese hamster V79 cells/*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine)

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ABSTRACT **Cell variants resistant to the cytotoxic effect of mycophenolic acid, an inhibitor of IMP dehydrogenase (IMP:NAD$^+$ oxidoreductase, EC 1.2.1.14), were selected by a one-step procedure from Chinese hamster V79 cells. The frequency of these variants was increased in a dose-dependent manner after treatment with the mutagen *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine and after an expression time of 8 days. The degree of resistance in five of the six isolated cell variants was associated with a comparable increase in the specific activity of IMP dehydrogenase, which was 3- to 6-fold higher than that of the parent V79 cells. The IMP dehydrogenase activity from both the variants and the V79 cells had a similar affinity for the substrate IMP with a K_m of about 20 μM and a similar response to mycophenolic acid with a K_i of 12–16 nM. It is suggested that cell variants with an altered regulation of IMP dehydrogenase activity may be helpful in studying the control of nucleic acid biosynthesis, cell growth, and carcinogenesis. Mycophenolic acid resistance also may be useful as a marker in short-term assays for the identification of potential chemical carcinogens.**

Mutations in regulatory genes, including those that control cell growth, may be the initial step involved in malignant cell transformation (1–13). Cell replication is regulated in part by the activity of the nucleic acid biosynthetic enzymes. IMP dehydrogenase (IMP:NAD$^+$ oxidoreductase, EC 1.2.1.14) is one of these enzymes, and its activity is enhanced in malignant cells (14). A correlation can be observed between the activity of this enzyme and the growth rate of various cell types (14). It therefore would be of interest to determine whether a carcinogen/mutagen can induce a hereditary change in the control of this enzyme. This can be achieved by inducing and selecting cell variants that are resistant to the cytotoxic effect of a specific inhibitor of the enzyme essential for cell growth. Such a resistance can result either from alterations in the structural genes that code for the enzyme, as in the case of hypoxanthine phosphoribosyltransferase (HPRT) (15), or from overproduction of the target enzyme either by regulatory gene alteration or by gene amplification (16, 17). Mycophenolic acid (MPA) (18) is useful for such studies because it is a potent inhibitor of IMP dehydrogenase (19) and is highly cytotoxic to various mammalian cell types (19–21).

In the present studies, by means of the chemical mutagen/carcinogen *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) we induced in Chinese hamster V79 cells a series of cell variants with a stable heritable change in the regulation of IMP dehydrogenase activity. These cells, which are resistant to MPA, are characterized by increased IMP dehydrogenase activity. Furthermore, the degree of resistance to the cytotoxic effect of MPA is associated with a comparable increase in the level of enzyme activity. These results indicate that at least some chemical carcinogens/mutagens can induce hereditary changes in the control of enzymes involved in the regulation of growth of mammalian cells.

MATERIALS AND METHODS

Chemicals. β-NAD, GMP, IMP, xanthine 5′-phosphate (XMP), DL-dithiothreitol, protamine sulfate, and MNNG were purchased from Sigma. Formic acid (98–100%) was obtained from MCB Chemicals (Norwood, OH). MPA was a gift from R. L. Davis (Lilly Laboratories, Indianapolis, IN), and [8-^{14}C]IMP (specific activity, 61 mCi/mmol; 1 Ci = 3.7×10^{10} becquerels) was purchased from Amersham. MPA was initially dissolved in 0.15 M $NaHCO_3$ and then diluted further in distilled water and culture medium.

Cell Culture. Chinese hamster V79 cells, derived from a subclone of V79-4, were kindly supplied by E. H. Y. Chu (University of Michigan, Ann Arbor, MI), and the various MPA-resistant cell variants were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and Fungizone (0.25 μg/ml) (GIBCO) at 37 ± 0.5°C. The cultures were incubated in a humidified incubator supplied with a constant amount of 10% CO_2 in air.

Selection of MPA-Resistant Cells. To test for MPA resistance, we treated 4×10^6 2-day-old exponentially growing V79 cells cultured in 100-mm Petri dishes with MNNG. After 3 hr of treatment, both the control and the MNNG-treated cells were dissociated with trypsin/EDTA solution and seeded at 10^5 cells per 100-mm Petri dish. Unless otherwise noted, the cells were dissociated 6 days later and reseeded at 200 cells per 60-mm dish in 5 ml of medium for the determination of cloning efficiency and at 2×10^4 cells per 60-mm dish in 4 ml of medium for the determination of the number of MPA-resistant cells. Two days later, MPA was added in 1 ml of medium to give a final concentration of 1 μg/ml. Thus, an expression time of 8 days was used for the selection of MPA resistance. Cloning efficiency was determined by counting the number of Giemsa-stained colonies of six to eight dishes per point at 7–8 days after cell seeding; the number of MPA-resistant cell variants was determined by counting Giemsa-stained colonies in 40 Petri dishes per point at 18–21 days after cell seeding. The frequency of the drug-resistant colonies was calculated per 10^5 colony-forming cells based on the cloning efficiency and the number of cells seeded for mutant selection.

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Abbreviations: HPRT, hypoxanthine phosphoribosyltransferase; MPA, mycophenolic acid; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; XMP, xanthine 5′-phosphate.

In addition, after a 6-day expression time, we incubated a sample of control and MNNG-treated (0.5 μg/ml) cells with MPA at 0, 0.1, and 0.3 μg/ml. Both the control and MNNG-treated cells had a similar growth rate, yielding, after 5 days of growth, means (± SD) of 5.0 ± 0.3, 1.4 ± 0.2, and 0.2 ± 0.05 × 10^6 cells per Petri dish for MPA at 0, 0.1, and 0.3 μg/ml, respectively. These results indicate that control and MNNG-treated cells exhibit not only a similiar growth rate but also a similiar susceptibility to the cytotoxic effect of MPA.

IMP Dehydrogenase Assay. Dehydrogenase activity was measured by determining the conversion of [^{14}C]IMP into XMP. For the isolation of this enzyme, 30 dishes of exponentially growing cultures containing about 2 × 10^8 cells were used. The cells were rinsed, trypsinized, resuspended in 15 ml of 15 mM potassium phosphate buffer (pH 7.4), and sonicated three times at 15 sec each (Sonicator, Heat System/Ultrasonics, Plainview, NY). The particulate material was removed by centrifugation at 20,000 × *g* for 15 min. The remaining supernatant was further centrifuged at 3°C in a SW 50.1 rotor at 100,000 × *g* for 90 min. To eliminate 5′-nucleotidase activity, we treated the supernatant with 2% (wt/vol) protamine sulfate (1 ml for each 300 mg of protein) for 10 min at 4°C and centrifuged at 20,000 × *g* for 15 min. The protein fraction containing IMP dehydrogenase was precipitated from the remaining supernatant by $(NH_4)_2SO_4$ at concentrations between 30% and 40% of saturation. The pellet was resuspended in 3 ml of 15 mM potassium phosphate, pH 7.4/1 mM EDTA/1 mM dithiothreitol; it was then dialyzed against 1 liter of the above solution for 4 hr. This dialyzed cellular extract was stored at −70°C and served as a source of enzyme.

The enzyme assay was a modification of that of Leyva *et al.* (22). The 150-μl reaction mixture contained 0.075 μCi of [8-^{14}C]IMP, 0.15 μmol of IMP, 0.0625 μmol of β-NAD, 10 μmol of KCl, 0.1 μmol of EDTA, 0.05 μmol of dithiothreitol, 5 μmol of potassium phosphate, and 1–10 μg of the cellular extract; it was adjusted to pH 7.4. The enzyme activity was linear with increasing cellular extract from 1 to 20 μg of protein and with time up to 2 hr. The reaction mixture was incubated for 1 hr at 37°C; the reaction was then terminated by the addition of an equal volume of cold 95% ethanol. The precipitated proteins were removed by centrifugation in a microcentrifuge (12,000 × *g*) for 1 min, and a 40-μl aliquot of the supernatant, to which unlabeled IMP, XMP, inosine, and xanthosine were added, was spotted on Whatman DE81 paper and subjected to descending chromatography for 6 hr in 0.2 M formic acid (brought to pH 5 with ammonium hydroxide). Under these experimental conditions, R_F values were 0.48 for IMP and 0.15 for XMP. Nucleotidase activity was determined by a descending chromatogram in 0.2 M formic acid (brought to pH 3.5). In this case, the R_F values were 0.64 for inosine, 0.57 for xanthosine, 0.50 for IMP, and 0.39 for XMP. In the crude extract, up to 25% of IMP and XMP was dephosphorylated whereas no such activity could be detected after treatment of the extract with protamine and ammonium sulfate. The spots were located with UV light, cut out, and assayed at 60% efficiency in a Tri-Carb liquid scintillation counter. Protein was measured by the Lowry method (23) with bovine serum albumin as a standard.

RESULTS

Induction of MPA-Resistant Cells in Chinese Hamster V79 Cells. Cells resistant to the cytotoxic effect of the IMP dehydrogenase inhibitor MPA were selected by a one-step procedure either without mutagenesis or after treatment with MNNG. Optimal induction of resistance to MPA at 1 μg/ml, a highly cytotoxic dose, was obtained after allowing an expression time of about 8 days (Fig. 1) and seeding a low number of cells (2 × 10^4 cells per 60-mm petri dish) prior to addition of the selecting agent MPA (Fig. 2). The part of the curve showing a decrease in the frequency of MPA-resistant cells as a function of the number of cells seeded presumably results from the production of cytotoxic metabolites from MPA (24), which can be transferred from susceptible to resistant cells because of metabolic cooperation (25, 26). Using an 8-day expression time and the appropriate cell density prior to selection of the cell variants, we found that the frequency of MPA-resistant cells was induced by MNNG in a dose-dependent manner (Fig. 1). At 1 μg/ml, the highest MNNG dose tested, the frequency of MPA-resistant cells was increased by about 6-fold over the spontaneous frequency. These results indicate that, under the appropriate culture conditions, MNNG can induce MPA resistance in the V79 cells.

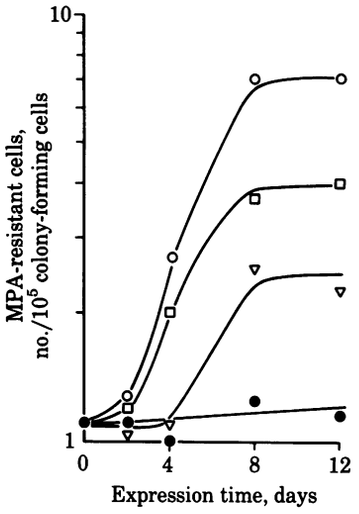

FIG. 1. Induction of MPA resistance in V79 cells at various expression times. MPA was added at 2, 4, 8, and 12 days after the initial treatment with MNNG. ●, Control; MNNG dosages: ▽, 0.25 μg/ml; □, 0.5 μg/ml; ○, 1.0 μg/ml.

To test the hereditability and stability of the MPA-resistant phenotype, we isolated 11 resistant colonies from the MNNG-treated cultures and 1 resistant colony from the control. After the cells were cultured for 4 weeks, they were tested for their chromosomal number and susceptibility to the cytotoxic effect

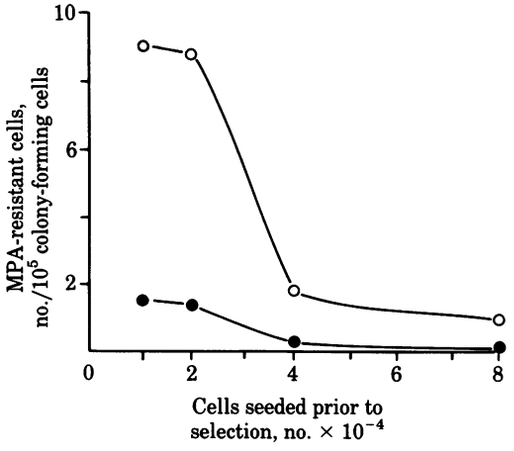

FIG. 2. Induction of MPA resistance by MNNG in V79 cells seeded at various cell densities prior to selection with MPA. Selection of the MPA variants was performed after an expression time of 8 days. ●, Control; ○, treated with MNNG at 1 μg/ml.

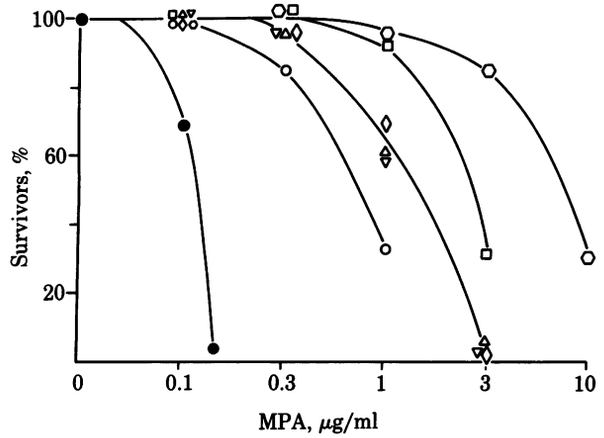

FIG. 3. Resistance of the V79 and cell variants to the cytotoxic effect of MPA. Cells were seeded at 200 cells per 60-mm Petri dish in 4 ml of medium. MPA was added to the cultures 18 hr after cell seeding, and the cloning efficiencies were determined 8 days later. The cloning efficiency of the untreated cells varied from 58% to 94% but was considered as 100% for comparative determinations of the susceptibility to the cytotoxic effect of MPA. ●, V79; ○, clone no. 1; □, no. 2; ◇, no. 3; △, no. 4; ▽, no. 5; ○, no. 6.

of MPA and 6-thioguanine. Cells from 11 of the 12 MPA-resistant colonies had a near-diploid chromosomal karyotype; the 12th, from an MNNG-treated culture, exhibited a near-tetraploid karyotype. Cells from all 12 colonies were more resistant than V79 cells to the cytotoxic effect of MPA but not to that of 6-thioguanine. This was determined by their ability to form colonies in the presence of MPA at 0.1–10 μg/ml and 6-thioguanine at 0.5–5.0 μg/ml. Results on the susceptibility of six of these diploid cell clones to the cytotoxic effect of MPA are presented in Fig. 3. Cells from three of these clones (clones 1, 2, and 3) were retested 4 weeks later and were found to maintain a similar resistance to the cytotoxic effect of MPA and to exhibit growth rates that were similiar to the growth rate of the parent V79 cells.

IMP Dehydrogenase and Resistance to MPA. To determine whether the resistance to MPA was associated with IMP dehydrogenase activity, extracts of the V79 cells and of six of the MPA-resistant clones were analyzed for IMP dehydrogenase activity (Table 1). In five of the six clones, the specific activity of IMP dehydrogenase was higher than that of the V79 cells and the degree of resistance in these five clones was associated with a comparable enhancement in IMP dehydrogenase activity. These activities were 3- to 6-fold higher than the specific activity of the V79 cell extract. The extract from clone 6 had a specific activity similar to that of the V79 cell extract. To determine whether the IMP dehydrogenase activities from V79 and those from the variant clones differed in their K_m for IMP and in their K_i for MPA, we determined these constants in the extracts of the V79 cells and in two of the most resistant clones, numbers 1 and 2, as well as in number 6 which exhibited IMP dehydrogenase activity similar to that of V79 cells. All four extracts exhibited a similar range in their K_m for IMP and in their K_i for MPA. IMP dehydrogenase activity from V79 and clone 1 cells also did not differ in the K_m for β-NAD, 29 μM. MPA caused an uncompetitive inhibition of the enzyme activity (Fig. 4) with similar K_i values that ranged from 12 to 20 nM (Table 1).

Table 1. IMP dehydrogenase activity in V79 cells and in MPA-resistant clones

Clone	Specific activity,* μmol/mg/hr	K_m (IMP), μM	K_i (MPA), nM
V79	0.2 ± 0.03	22	16
1	1.2 ± 0.04	19	12
2	0.9 ± 0.06	20	16
3	0.6 ± 0.03	—	—
4	0.6 ± 0.04	—	—
5	0.6 ± 0.05	—	—
6	0.2 ± 0.04	13	20

* Results are the mean ± SD of four experiments.

To determine whether the difference in the specific activities of the IMP dehydrogenases was associated with the presence of inhibitors or enzyme activators, we analyzed a combined sample of the V79 (20 μg protein) and clone 1 (5 μg protein) extracts. The combined extracts yielded a rate of 7.4 nmol of XMP per hr compared to 3.1 and 4.2 nmol/hr for V79 and clone 1, respectively. These results indicate that the higher IMP dehydrogenase activity found in clone 1 was not due to the presence of an activator(s) in its extract, nor was it due to an inhibitor present in the extract from V79 cells. Clone 6 presumably represents a cell variant with a poor permeability for MPA or with an increased level of β-glucuronidase that inactivates the cy-

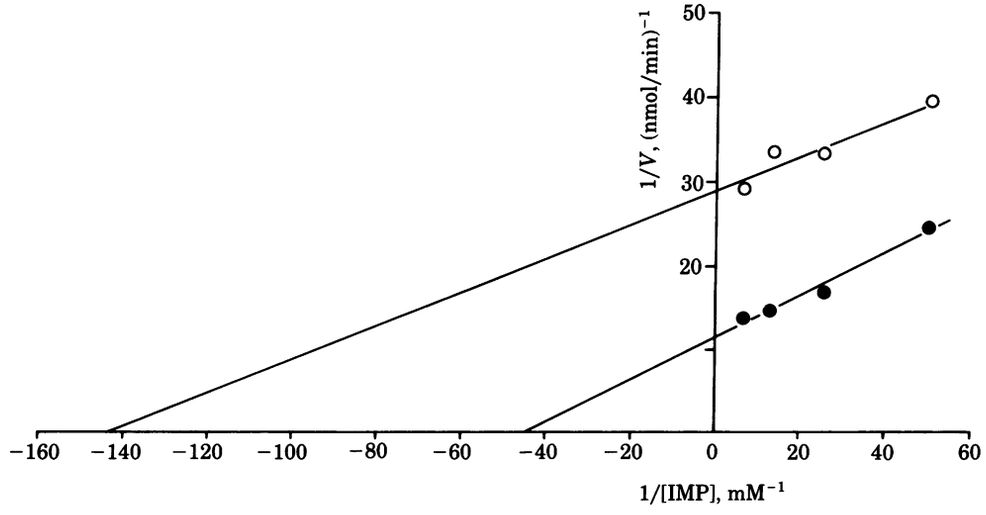

FIG. 4. Inhibition of IMP dehydrogenase by MPA. The enzyme (20 μg) was prepared from V79 cells. Similar kinetics were obtained with enzyme preparations from clones 1, 2, and 6 (Table 1). Each point represents the mean of three determinations. Enzyme activities in the absence of MPA (○) and presence of 0.03 μM MPA (●) are plotted.

totoxic effect of MPA (27) or with an altered yet unidentified enzyme. We presumably can rule out alterations in the structure of HPRT because the cells from this clone were as susceptible as V79 cells to the cytotoxic effect of 6-thioguanine, an HPRT inhibitor.

DISCUSSION

IMP dehydrogenase, which catalyzes the conversion of IMP into XMP, is a key enzyme in the control of nucleic acid biosynthesis, cell growth, and presumably malignancy (14). Cell variants with altered regulation of IMP dehydrogenase can be useful in studying these events as well as in control of gene expression. In the present studies we isolated a series of such variants which are characterized by an enhanced IMP dehydrogenase activity. The increased activity of this enzyme could result from gene amplification (16, 17) or from alterations in transcriptional (28) and posttranscriptional controls (29). At later stages this could include alterations in translational level (29), mRNA stabilization (30), blocking of enzyme turnover (31), or alteration in the structural gene, yielding a more efficient enzyme. However, we have some pertinent data that are in disagreement with the possibility of an alteration in enzyme structure. Comparisons of the K_m for IMP and β-NAD as well as the K_i for MPA failed to exhibit any significant differences between the enzyme in the wild-type V79 cells and in the variant cells. These results suggest that the variant cells have hereditary alterations in the regulation of IMP dehydrogenase. Such cell variants should therefore be useful in studying gene expression, control of nucleic acid biosynthesis, cell growth, and carcinogenesis.

Two important features of these cell variants are that their frequency can be increased after treatment with the mutagen/carcinogen MNNG and that they do not express abnormal chromosomal numbers. Furthermore, induction of MPA resistance by MNNG required an expression time of about 8 days. Similiar expression times are characteristic of other genetic markers including 6-thioguanine resistance, which is associated with structural changes in HPRT (15, 26). Expression times in mutagenesis experiments are required for fixation of the DNA damage by the mutagen as well as for selection against the constitutive level of the wild-type protein (in our case, perhaps a regulatory factor) and its mRNA to a negligible level, presumably by normal turnover in the cell (15).

Based on these studies we suggest that mutagens/carcinogens such as MNNG can induce "mutations" in regulatory elements that may affect genes associated with the control of cell growth and perhaps malignancy. Such "mutations" may thus be appropriate markers in short-term mutagenesis assays for the identification of potential chemical carcinogens (5, 8, 13).

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