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TEMPORARY CULTURE IN ISOLEUCINE-FREE MEDIUM ENHANCES TRANSFORMATION OF 10T½ CELLS BY N-METHYL-N'-NITRO-N-NITROSOGUANIDINE (MNNG)

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SUMMARY: Arrest of proliferation of C3H 10T½ cells in isoleucine-free medium sensitizes them to the transforming action of MNNG when they are exposed to this chemical after release. Exposure of 10T½ cells to MNNG for 30 min causes three to four times the rate of transformation in these cells when they are recycling following arrest of proliferation by isoleucine deficiency than does similar treatment of the same cells when they are recycling after arrest of proliferation by growth to confluence. Rates of transformation differ even though the magnitude of binding of MNNG to DNA is similar in cells recycling after suppression of proliferation by either method.

Cells of the line 10T½ (clone 8), derived from embryos of C3H mice, have been used extensively to investigate chemical carcinogenesis in vitro (1, 2). Untreated cells have a low transformation frequency, but transformation can be induced by exposing them to a variety of carcinogenic chemicals (2). Recent studies have demonstrated that 10T½ cells whose proliferation is synchronized are differentially sensitive to induction of neoplastic change, depending on the point during the cell cycle at which they are exposed to carcinogen (3-5). Two popular methods to establish cycle synchrony are growth of the cell population to confluence and growth in a tissue culture medium lacking the amino acid isoleucine (3-5). Both techniques arrest cell proliferation in the G_1 phase of the cell cycle, and following release from growth arrest virtually all of the cells enter S phase during a period of 6 to 8 hr (3-5). In this study we demonstrate that 10T½ cells recycling after arrest of growth by culture in isoleucine-free medium respond differently to the directly active alkylating agent MNNG than do similar cells recycling after arrest of growth by culture to confluence in complete medium. Although DNA of 10T½ cells synchronized by either method is equally alkylated by

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exposure to MNNG, the rate of toxicity and transformation in cells cultured in isoleucine-free medium is greatly enhanced.

METHODS

C3H 10T½ (clone 8) cells, a gift of Dr. John B. Little (Harvard University, Boston, MA), were maintained in Eagle's basal medium (BME) (GIBCO, Grand Island, NY) supplemented with 10% heat inactivated fetal bovine serum and gentamicin (Schering Corporation, Kenilworth, NJ) (50 µg/ml), according to the methods delineated by Reznikoff et al (1, 2). Cells used for this study had been cultured in vitro for less than 20 transfer generations and met all basal cytologic and growth criteria established by Reznikoff et al (1, 2).

Proliferation was arrested by growing cells for 48 hr in isoleucine-free BME supplemented with dialyzed serum (prepared in our laboratory) or by growing them to confluence in the same medium containing isoleucine by previously detailed techniques (6). Under both situations of growth arrest, cell proliferation as assessed by measuring the rate of DNA synthesis, was reduced to less than 0.6% of that occurring in logarithmically growing populations. Cells were harvested by exposure for 5 min to Hank's balanced salt solution lacking Ca^{++} and Mg^{++} (BSS) and containing 0.1% trypsin and 0.2% EDTA. Arrested cells were replated in complete medium at concentrations of 1 to 5 x 10^4 cells/100 mm dish for transformation assays or at 500 to 1000 cells/100 mm dish for toxicity assays. At the time of treatment (3 to 8 hr after replating), medium was removed and cultures were rinsed with BSS. Five to 10 replicate plates were exposed for 30 min to MNNG (Aldrich Chemical Co., Milwaukee, WI) at concentrations that had been determined previously to reduce colony forming efficiency by about 20%, 50% or 90%. MNNG was dissolved in spectrophotometric grade acetone (Aldrich Chemical Co.), and added directly to cultures in 5 ml BSS at maximal final concentrations of 0.5% acetone. Controls were treated with 0.5% acetone only which was not toxic for treated cells.

After 30 min exposure to MNNG, cultures were washed with BSS and refed with complete medium, and thereafter they were refed with complete medium twice each week. Toxicity was assessed by determining the relative colony forming efficiency of treated, as compared to control, cells 10 to 14 days after treatment. The basal colony forming efficiency for 10T½ cells in complete medium was 28.5 ± 4.6% (M. ± S.D., N = 9). Isoleucine deficiency reduced the basal colony forming efficiency by about 18%. Transformation rates were evaluated by tabulating type III foci in confluent cultures that were fixed and stained 5 wks after treatment (2). Type III foci were quantified by relating the number of cells plated (corrected for basal CFE) and the number of cells surviving treatment, assuming that each focus derived from a single transformed cell.

To evaluate binding of MNNG to DNA, 10T½ cells were plated at 1 x 10^6 cells/100 mm dish and 96 hr later released from postconfluence growth arrest by trypsinization and replating at 1 x 10^6 cells/100 mm dish. S phase was entered synchronously 20 hr after replating. Cells synchronized by growth for 48 hr in isoleucine-free medium after plating at 1 x 10^6 cells/100 mm plate entered S phase synchronously 10 hr after release by trypsinization and replating at 1 x 10^6 cells/100 mm plate in complete BME media. Cells synchronized by either procedure were exposed to MNNG as they entered S phase. The medium was removed, cells were rinsed with BSS, and incubated for 30 min in 5 ml of BSS to which was added ^{14}C-MNNG (370 µCi/mg) in 25 µl of DMSO. The cells then were rinsed free of MNNG with BSS, harvested by scraping, and resuspended in a salt solution containing 10mM EDTA, 2% Sarkosyl, 10mM Tris-HCl, pH 8.0, Proteinase K (1.5 µg/ml). This mixture was incubated at 37°C for 30 min, to allow deproteinization, and CsCl in amount to give a refractive index of 1.4000 was added directly to the hydrolysate. DNA was purified by banding at equilibrium in centrifugally generated gradients following a 66 hr centrifugation at 35,000 rpm in a type 40

Table 1

Rates of Toxicity and Transformation of Isoleucine-Deficient and -Sufficient Cells After Treatment with MNNG

Method to Arrest Growth	Conc. MNNG (µg/ml)	Colony Forming Efficiency (% of untreated control value)	Cells Transformed/ 10^6 treated	10^6 surviving	10^6 treated/ µg MNNG	10^6 surviving/ µg MNNG
Isoleucine Deficiency	0.45	82.8± 3.7	77.6± 7.1	93.7± 6.7	172.5±10.4	208.3± 12.0
	0.85	55.0± 2.9	166.7±27.9	307.3± 53.8	211.7±36.5	392.1± 72.6
	1.80	11.7± 2.1	153.9±27.5	1534.5±278.5	97.3±17.6	957.4±120.6
Growth to Confluence (Isoleucine-Sufficient)	1.50	81.8±10.3	67.5± 5.4	82.5± 9.2	45.0± 6.6	55.0± 11.4
	2.50	58.7± 6.2	139.4±28.2	287.2± 54.7	55.8±15.8	95.0± 34.2
	5.00	7.1± 3.2	90.8±18.2	1277.6±299.0	18.2± 3.4	255.5± 46.2

rotor at 20°. Following dialysis of the gradient fractions, specific activities of binding to ^{14}C-MNNG to DNA were determined by quantitation of A_{257} and liquid scintillation counting.

RESULTS

Table 1 shows that the rates of both cytotoxicity and neoplastic transformation in 10T½ cells treated with MNNG for 30 min are increased in cells recovering from growth arrest produced by isoleucine deficiency. Following a period of isoleucine deficiency, 10T½ cells require only about one-third as much MNNG as do postconfluent cells to comparably reduce colony-forming efficiencies. When normalized for the quantity of MNNG used, cells surviving treatment with MNNG after isoleucine deficiency transform at nearly four times the rate of surviving postconfluent cells. However, the extent of alkylation of nuclear DNA of 10T½ cells is not altered by deficiency of this amino acid (Figure 1). Alkylation of DNA of 10T½ cells exposed to concentrations of MNNG from 0.2 to 4.5 μg/ml culture medium was linearly related to dose, irrespective of whether cells were isoleucine-deficient or not. Cells recycling after isoleucine deficiency bound 5.5 ± 0.41 (M. ± S.D.) DPM/μg DNA/μg MNNG, while cells recycling following postconfluence arrest bound 4.5 ± 0.70 DPM/μg DNA/μg MNNG. These values do not differ significantly.

DISCUSSION

The mechanism(s) by which a period of isoleucine-deficiency augments the neoplastic response of 10T½ cells to MNNG is not established. We have previously noted the increased toxicity of MNNG for 10T½ cells recovering from isoleucine deficiency (6). Within 6 hr after culture in an isoleucine-free medium, 10T½ cells become maximally sensitive to the cytotoxic effects of MNNG and they retain this heightened responsiveness for at least one cell cycle after they are returned to an isoleucine-supplemented medium (6). Since MNNG is spontaneously activated, cellular metabolic activation is not involved. Equivalent levels of alkylation in both types of cells suggest that the permeability of the cell membrane to MNNG is not altered and that neither the avidity of binding

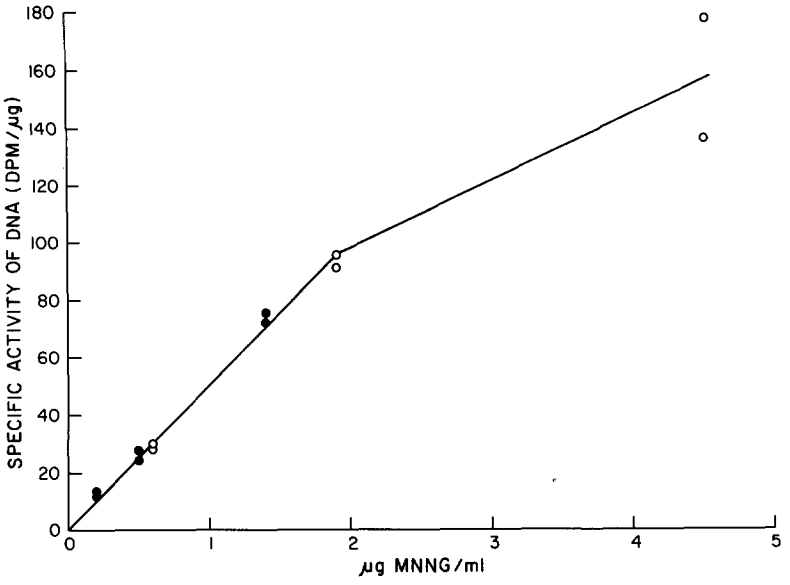

Fig. 1. Binding of MNNG to DNA in cells recycling following arrest of proliferation by growth in isoleucine-free medium (●) or by growth to confluence (O).

of MNNG to DNA nor the rate of catabolism of this chemical are affected by the presence or absence of isoleucine. Since the magnitude of binding of MNNG to DNA is similar in 10T½ cells cycling after growth arrest by either isoleucine deficiency or confluence, it is possible that differential rates of removal of alkylation products from DNA or differential abilities to bypass alkylated sites during DNA replication may explain the observed differences in toxicity and transformation. In other studies, we have found that alkylation of DNA drastically slows the rate of DNA replication in postconfluent cells, whereas a prior period of isolucine-deficiency allows alkylated DNA to be replicated with little delay (6). These effects are similar to those of caffeine on some rodent cells following treatment with methylating agents (8). Inhibition of postreplication or bypass repair by caffeine in sensitive cells may be due to an alteration of repair patterns associated with DNA synthesis (9).

A previous report by Bertram and Heidelberger did not describe an increased rate of MNNG-induced transformation in 10T½ cells synchronized by isoleucine

deficiency, possibly because they used only one concentration of MNNG that killed more than 99% of treated cells (3). At this extreme level of cell killing, calculation of specific rates of transformation (transformants/10^6 surviving cells) is made inaccurate by the paucity of proliferating survivors. However, inspection of the data in their paper (Charts 7 and 10 in Reference 3) indicates that the peak rate of transformation in postisoleucine-deficient cells exposed to 4 μg MNNG/ml was about 9% of survivors, compared to about 3 to 4% of survivors in postconfluent cells exposed to the same concentration of MNNG. These results agree with our findings reported here. In contrast, Jones and his coworkers have demonstrated that an episode of isoleucine deficiency does not sensitize 10T½ cells to the cytotoxic or transforming effects of the halogenated pyrimidine nucleoside, flurodeoxyuridine (FudR) (4), or to 1-β-arabinofuranosylcytosine (ara-CTP) (5). This apparent discrepancy may be explained by production of different types of cellular damage or activation of separate repair mechanisms by these agents.

It is important that investigators who utilize cultured 10T½ cells to evaluate toxicity and carcinogenicity in vitro be aware that presence or absence of isoleucine and perhaps other essential amino acids in the culture medium may profoundly affect responses of these cells to some chemicals. Other types of cells may respond differently to isoleucine deficiency; we have previously shown that the extreme toxicity of MNNG for CHO cells is unaffected by prior culture in the presence or absence of isoleucine (6), possibly because these cells appear to lack the protective mechanism affected by isoleucine in 10T½ cells. Since the culture of 10T½ cells in an isoleucine-free medium rapidly enhances their toxic and neoplastic responses to MNNG, detailed study of this system may help clarify the mechanisms by which cells repair damage produced by alkylating chemicals.

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