REPAIR SYNTHESIS AND SEDIMENTATION ANALYSIS OF DNA OF HUMAN CELLS EXPOSED TO DIMETHYLNITROSAMINE AND ACTIVATED DIMETHYLNITROSAMINE

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SUMMARY

The capacity of dimethylnitrosamine(DMN), and of DMN activated by a NADPH-fortified mouse liver microsomal preparation, to elicit DNA alterations in cultured human fibroblasts was examined. A maximum induction of DNA repair synthesis, estimated by unscheduled incorporation of tritiated thymidine, occurred following 60-minute incubation of the human cells with DMN activated by a NADPH-fortified mouse liver microsomal preparation. A low level of DNA repair activity followed exposure to DMN alone, or to DMN mixed with the microsomal preparation without NADPH or without O_2. The extent of DNA damage, estimated by velocity sedimentation of DNA through alkaline sucrose gradients, was maximum following treatment with DMN mixed with the NADPH-fortified microsomal preparation. The combined application of *in vitro* activation systems and estimation of DNA repair synthesis in cultured cells may be exploited in the detection of precarcinogens.

INTRODUCTION

DNA repair synthesis has been successfully applied to indicate DNA changes induced by physical (1) or chemical (2-5) agents in mammalian cells. It was employed to distinguish between high, weak, and non-oncogenic derivatives and isomers of 4-nitroquinoline-1-oxide and 4-nitropyridine-1-oxide (2), between the precarcinogen 2-acetylaminofluorene and its active N-hydroxy and N-acetoxy metabolites (3), and between benz(α)anthracene and its K-region epoxide or dihydrodiol (4). Thus, it has been proposed that the measurement of DNA repair synthesis in cultured mammalian cells could be developed as a screening procedure for the identification of potential chemical carcinogens with the distinct advantage of employing human cells (6).

With the utilization of human lymphocytes (5) or fibroblasts (2-4) as

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"receptor" cells, the in vitro test systems for DNA repair are severely restricted in that they are useful in detecting only those carcinogens that do not require metabolic activation. A prime requisite for the utilization of in vitro systems in screening or prescreening programs is, therefore, the development of activation procedures capable of converting precarcinogens to active metabolites which can induce DNA alterations sufficient to trigger DNA repair synthesis in human receptor cells.

In this study, we compare the level of DNA repair in cultured human fibroblasts following exposure to dimethylnitrosamine (DMN) and to DMN activated by a NADPH-fortified liver microsome preparation (7,8). DNA repair is measured as unscheduled incorporation of tritiated thymidine and as shifts in DNA sedimentation profiles (9-11).

MATERIALS AND METHODS

Human skin fibroblasts were cultured from a skin-punch biopsy which was taken from the fore-arm of a 26-year-old Caucasian woman. Cell cultures used in this study were of transfer passages 4 or 5. Monolayers of fibroblasts were maintained in Eagle's minimal essential medium (MEM), supplemented with 15% fetal calf serum and antibiotics (penicillin 200 U/ml, and streptomycin 40 µg/ml) The cells were grown on 10x35 mm glass coverslips kept in stoppered Leighton tubes.

For the activation procedure, the livers of decapitated 3 to 4 month-old male Swiss mice were pooled and homogenized in ice-cold Dulbecco's phosphate-buffered saline containing calcium and magnesium ions (PBS; pH 7.2) and sucrose (0.25 M). The tissue was homogenized by 10 to 12 up-and-down strokes of a Potter-Elvejhem Homogenizer operating at 1000 rev./min. The post-mitochondrial supernatant fraction resulting from a 9,000xg centrifugation (10 min, 4^0) was immediately used for the activation experiments following addition of co-factors and adjustment of the pH to 7.0 with 0.1 M NaOH. For the complete activation system, each Leighton tube contained microsomes from 200-300 mg liver plus 8.5 µmoles NADPH, 25 µmoles magnesium chloride, and 20 µmoles glucose-6-phos-

phate (7,8). A solution of DMN in MEM was added to the cultures in conjunction with the fortified activation system to give total reaction volumes of 1.0 ml. The contents were mixed thoroughly, flushed with O_2 gas, and left tightly stoppered without further agitation at 37^0 for the required incubation period.

For analysis of DNA repair synthesis by unscheduled incorporation of tritiated thymidine, the coverslip cell cultures were transferred into arginine-deficient medium (ADM) for 2-3 days, prior to exposure to the carcinogen, in order to block semiconservative DNA replication (12). Following carcinogen treatment, the cell cultures were rinsed 3 times with ADM and then incubated at 37^0 with ADM containing 10 µCi/ml tritiated thymidine (^{3}H-TdR, specific activity 20 Ci/mmol; New England Nuclear). The cultures were then prepared for autoradiographic analysis as previously described (12).

For analysis of DNA damage and repair by alkaline sucrose gradient centrifugation, a technique developed in the laboratory of Dr. E. Farber by R. Cox, I. Damjanov, S.E. Abanobi, and D.S.R. Sarma of the Fels Research Institute, Philadelphia, was used. Prior to exposure to the carcinogen, ^{3}H-TdR was added to the regular growth medium at a concentration of 1-2 µCi/ml for 20-24 hours. Following carcinogen treatment, the cell cultures were rinsed 4 times with cold 0.024 M EDTA/0.075 M NaCl buffer (pH 7.5), scraped into 0.5 ml of EDTA/NaCl buffer, vortexed lightly to disperse aggregates, pelletted (2 min, 1000xg), and resuspended in sufficient EDTA/NaCl buffer (100-300 µl) to give $5\text{-}10\times10^4$ cells per 100 µl. The cell suspension was added to an overlay (0.3 ml) of a lysing solution (0.3 M NaCl, 0.03 M EDTA, 0.1 M Tris-HCl buffer, and 0.5% sodium dodecyl sulfate, pH 10) on top of a linear alkaline sucrose gradient (5-20%, 5 ml) containing 0.9 M NaCl and 0.3 M NaOH, and prepared over a one ml cushion of 2.3 M sucrose. An additional aliquot of 0.1 ml lysing solution was added on top of the cells and the tube was filled with mineral oil. After a 10-minute lysis period at room temperature (25^0), the gradients were centrifuged in buckets of an SW-40 rotor on model L2-65B ultracentrifuge (Beckman Instruments Inc., Palo

Table 1.

DNA Repair Synthesis in Cultured Human Fibroblasts Following 60-Minute Exposure to DMN ($5x10^{-2}M$) and to DMN ($5x10^{-2}M$) plus Complete and Incomplete Activation Systems

TREATMENT	GRAINS/NUCLEUS
Control (No Treatment)	0.0
DMN alone	0.5
Activation System alone	0.3
DMN plus Activation System	23.2
DMN plus Activation System with N_2 flush	1.2
DMN plus Activation System without NADPH	1.0
UV (900 ergs/mm^2)	85.5
UV (900 ergs/mm^2) plus Activation System	85.7

Table 2.

Effect of DMN Concentration on the Level of DNA Repair Synthesis in Cultured Human Fibroblasts (60-minute exposure)

CONCENTRATION OF DMN (mol/l)	GRAINS/NUCLEUS	
	(a)	(b)
$5.0x10^{-4}$	3.6	0.1
$5.0x10^{-3}$	8.4	0.1
$7.5x10^{-3}$	14.2	0.2
$1.0x10^{-2}$	16.8	0.2
$2.5x10^{-2}$	17.6	0.3
$5.0x10^{-2}$	21.3	0.5
$7.5x10^{-2}$	19.3	0.6
$1.0x10^{-1}$	17.2	0.7

(a) DMN plus complete activation system
(b) DMN alone

Alto, Calif.) at 25,000 rev./min and 20^0 for 30 min. Following centrifugation, approximately 15 sequential fractions of 16 drops each were collected from the bottom of pierced tubes, and assayed for radioactivity as previously described (11): acid insoluble radioactivity was precipitated with 8-10% trichloroacetic acid (TCA), collected on membrane filters, washed with TCA, dried, and counted.

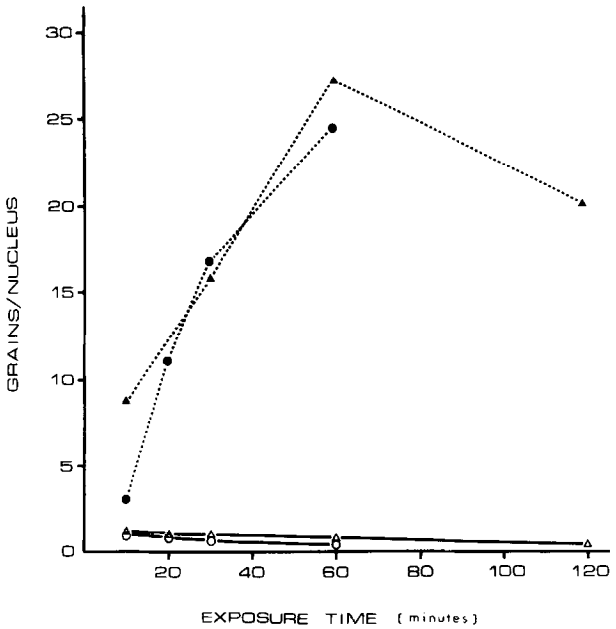

Figure 1. Effect of duration of exposure to $5x10^{-2}$M DMN alone (O—O, Δ—Δ), and to $5x10^{-2}$M DMN plus complete activation system (●···●, ▲···▲), on the level of unscheduled DNA synthesis in cultured human fibroblasts. (Autoradiographic detection of the unscheduled incorporation of ^{3}H-TdR)

RESULTS AND DISCUSSION

A relatively high level of DNA repair synthesis occurred in non-proliferating human fibroblasts following exposure to DMN incubated with a NADPH-fortified mouse liver microsomal fraction (Table 1). DMN treatment alone or treatment with DMN plus the activation mixture without NADPH or without oxygen induced a very low level of DNA repair synthesis.

The level of DNA repair synthesis increased with the time of exposure to $5x10^{-2}$M DMN plus the activation system. Exposure for 120 min appeared to result in a drop in activity (Figure 1). This raised the question of possible toxic effects of the activation mixture against the DNA repair mechanism of the cells. To obtain further information on the problem, cell cultures were exposed to 900 ergs/mm^2 UV-light (12) and subsequently incubated with the complete microsomal preparation. The cells exhibited no detectable inhibition of

unscheduled ^{3}H-TdR incorporation by exposure to the complete activation system for up to 120 min.

The effect of DMN concentration, both with and without the complete activation system, on the induction of DNA repair synthesis was examined (Table 2). A peak activity occurred at $5x10^{-2}$M DMN mixed with the complete activation system.

The application of the alkaline sucrose gradient technique revealed a shift in the sedimentation profile of ^{3}H-DNA to a region of lower molecular weight following exposure of the cultured cells to $5x10^{-2}$M DMN incubated with the complete activation system (Figure 2B). The sedimentation profiles of ^{3}H-DNA did not change from the control position following a one-hour exposure to the activation system only and to $5x10^{-2}$M DMN mixed with a heat-inactivated activation system (Figure 2A). Exposure to $5x10^{-2}$M DMN alone also produced no discernible effect (Figure 2B). These observations show that the induction of a DNA damage, as measured by velocity sedimentation in alkaline sucrose gradients, is dependent on an activation of DMN. Post-treatment incubations of the fibroblasts in the regular culture medium produced shifts of the lower molecular weight profiles to regions of higher molecular weight (Figure 2B), thus verifying that a repair of DNA damage is in fact occurring.

The results show that DMN can be converted *in vitro* by the NADPH-fortified liver microsomal activation system to a form that is capable of inducing a detectable DNA damage in cultured human cells. These DNA changes seem to lead to single-strand breaks as revealed by shifts in sedimentation profiles after centrifugation of DNA through alkaline sucrose gradients and by the unscheduled incorporation of ^{3}H-TdR. In this connection, it is of interest to note that only activated DMN induced mutations in *S. typhimurium* (8). The *in vitro* activation procedure, in combination with an estimation of DNA repair synthesis in cultured mammalian cells, may be utilized as a rapid and economic tool for the detection of chemical precarcinogens.

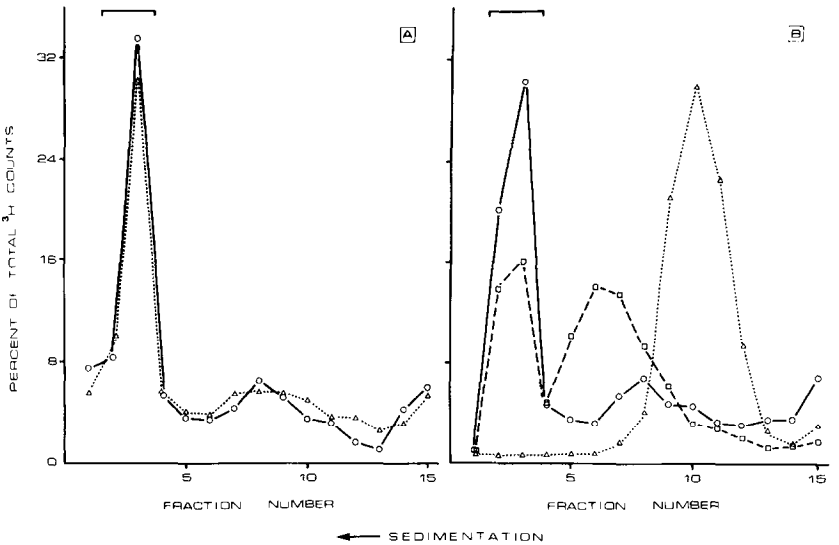

Figure 2. Alkaline sucrose gradient sedimentation profiles of ^{3}H-DNA released from cultured human fibroblasts exposed for 60 minutes to:

A) No treatment (control) (O—O); activation system only or $5x10^{-2}$M DMN plus heat-inactivated activation system (superimposable profiles) (Δ····Δ);

B) $5x10^{-2}$M DMN alone (O—O); $5x10^{-2}$M DMN plus complete activation system (Δ----Δ); $5x10^{-2}$M DMN plus complete activation system with a 4-hour post-treatment incubation in fresh medium (□—□). The horizontal bar designates the position of control DNA peaks.

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REFERENCES

1. Rasmussen, R.E., and Painter, R.B. J. Cell Biol. 29, 11 (1966).
2. Stich, H.F., San, R.H.C., and Kawazoe, Y. Nature 229, 416 (1971).
3. Stich, H.F., San, R.H.C., Miller, J.A., and Miller, E.C. Nature New Biology 238, 9 (1972).
4. Stich, H.F., and San, R.H.C. Proc. Soc. Exp. Biol. and Med. 142, 155 (1973).
5. Lieberman, M.W., Baney, R.N., Lee, R.E., Sell, S., and Farber, E. Cancer Res. 31, 1297 (1971).
6. Stich, H.F. In: Topics in Chemical Carcinogenesis, Takayama, S., Sugimura, T., and Odashima, S. (Editors). University Park Press, Baltimore, pp 17-28 (1972)
7. Garner, R.C., Miller, E.C., Miller, J.A., Garner, J.V., and Hanson, R.S. Biochem. Biophys. Res. Commun. 45, 774 (1971).
8. Malling, H.V. Mutation Res. 13, 425 (1971).
9. McGrath, R.A., and Williams, R.W. Nature 212, 534 (1966).
10. Lett, J.T., Caldwell, I., Dean, C.J., and Alexander, P. Nature 214, 790 (1967).
11. Laishes, B.A., and Stich, H.F. Can. J. Biochem. (1973, in press).
12. Stich, H.F., and San, R.H.C. Mutation Res. 10, 389 (1970).