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DNA REPAIR AND SENSITIVITY OF MOUSE EMBRYO FIBROBLASTS TO METHYL METHANESULPHONATE AND N-METHYL-Ν'-NITRO-N-NITROSOGUANIDINE *

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DNA repair synthesis and cytotoxicity were evaluated in early passage mouse embryo fibroblasts from five inbred strains (B10, CBA, C3H/A, DBA/2, BALB/c) and in BALB/3T3 IL-2 cells after the cultures had been treated for 3 h with methyl methanesulphonate (MMS) or N-methyl-Ν'-nitro-N-nitrosoguanidine (MNNG). In the presence of hydroxyurea, the incorporation of tritiated thymidine into the MMS- or MNNG-treated cells derived from B10, CBA, C3H/A or DBA/2 mice, was, at the concentrations used, significantly higher than into controls untreated with the mutagens. Under analogous experimental conditions there was no detectable DNA repair synthesis in two kinds of cells derived from BALB/c mice. MNNG was more cytotoxic to the cells derived from BALB/c mice than to those of the four remaining strains. The sensitivity of all kinds of early passage mouse fibroblasts to MMS was similar at each MMS concentration tested. Cloning efficiency of BALB/3T3 IL-2 cells exposed to MMS at the concentration of 10^{-3} or 10^{-4} M did not differ from that of untreated controls. The latter cells treated with MNNG at the concentration of 10^{-4} or 2 \times 10^{-4} M did not develop colonies.

In microbial systems cell survival after exposure to DNA damaging mutagens may depend on their competence of excision repair (Bridges, 1976; Green & Muriel, 1977; Witkin, 1976). In xeroderma pigmentosum cells impairment of DNA repair is correlated with their hypersensitivity to UV irradiation and to the action of several DNA damaging chemicals (Macher et al., 1975, 1977). The magnitude of DNA repair in mammalian cells varies not only among different species (Hart & Setlow, 1974; Koval et al., 1978; 1978; Yagi, 1982) but also among the cells or tissues of the same species (Stich & Kieser, 1974; Meek

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et al., 1980; Elliott & Johnson, 1985). There is evidence that chick embryo cells of retino-glial origin are deficient in repair competence (Karan et al., 1977). According to the findings of Peleg et al. (1976), early passage cells cultured from 13- to 15-day embryos are repair proficient, whereas similar passage cells from 17- to 19-day embryos are not.

The aim of this study was an evaluation of the interdependence between the competence of DNA repair synthesis and the sensitivity of mouse embryonic cells to DNA damaging agents. The experiments were performed with cells cultured from five different strains of mice and cells of a clone obtained in this laboratory from BALB/3T3 cell line (Chłopkiewicz & Koziorowska, 1983). As DNA damaging agents were chosen MNNG and MMS. They are ultimate mutagens and were shown to induce DNA repair in several systems (Michael & Williams, 1974; San & Stich, 1975).

MATERIALS AND METHODS

Chemicals. MNNG from Fluka (Switzerland) was dissolved in dimethylsulphoxide (Sigma Chem. Co., St. Louis, MO, U.S.A.); MMS from Merck-Schuchhardt (München, F.R.G.) was dissolved in water. Hydroxyurea was purchased from Sigma, [3H]dThd (2.0 Ci/mmol) from the Radiochemical Centre (Amersham, England), trichloroacetic acid from Reanal (Budapest, Hungary), culture media from the Serum and Vaccines Factory (Lublin, Poland). PPO and POPOP were from Koch-Light Labs. Ltd (Colnbrook, Bucks., England).

Primary cultures and successive transfers of mouse embryonic cells. Mouse embryos from five inbred strains (B10, CBA, DBA/2, C3H/A and BALB/C) were used. Aseptically collected embryos (on day 19 of gestation) were decapitated and eviscerated, cut into fragments and disaggregated with a magnetic stirrer in a 0.25% trypsin solution. The cell suspension was transferred to a tube containing a few drops of calf serum and centrifuged at 1000 rpm for 10 min. The pellet was resuspended in EMEM supplemented with 10% calf serum and antibiotics (streptomycin 0.1 mg/ml, penicillin 120 U/ml). Cell cultures were initiated by seeding 3.5 × 10^6 cells in 80 ml medium per Roux bottle. Cultures were transferred every 5 - 6 days. Fibroblasts at early passages (2nd to 4th) were used in all experiments.

BALB/3T3 IL-2 cells. These cells were described previously in detail (Chłopkiewicz & Koziorowska, 1983). They were cultured in Falcon flasks in EMEM containing 10% fetal calf serum. In these experiments cells between the 4th and 8th passage were used. At this time period they displayed

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1 Abbreviations used: N,N-methyl-N′-nitro-N-nitrosoguanidine; MMS, methyl methanesulphonate; [3H]dThd, [3H]thymidine; EMEM, Eagle’s minimal essential medium with Earle’s salts.
density-dependent inhibition of growth and did not give rise to multilayered foci.

*Measurement of repair synthesis.* A modification of the method described by Trosko & Yager (1974) was used. Cells were seeded at a density of $1.5 \times 10^5$ cells/ml in Leighton flasks and were grown to confluence by incubation for 48 h in EMEM + 10% calf serum + antibiotics. After a further 1 h incubation with 10 mM-hydroxyurea, MMS ($10^{-4}$, $10^{-3}$ M or MNNG ($10^{-4}$, $2 \times 10^{-5}$ M) and $[^3H]dTdh$ (1 µCi/ml) were added and incubated for 3 h. Incorporation of $[^3H]dTdh$ was stopped by addition of cold thymidine at the concentration of 50 µg/ml. After incubation with MMS or MNNG and $[^3H]dTdh$ the cells were washed several times with cold phosphate buffered saline and dried in air. To determine the incorporation of $[^3H]dTdh$ into DNA, the cells were treated with 1 M-NaOH for 1 h at 37°C to digest RNA. After incubation the suspension was cooled and 10% of trichloroacetic acid was added. The cell fractions insoluble in trichloroacetic acid were collected onto millipore filters, dried and placed in vials with 8.0 ml of a mixture of toluene with POPOP and PPO for subsequent liquid scintillation counting.

Controls were run in the presence of hydroxyurea, without treatment with MMS or MNNG. DNA repair synthesis was expressed as percentage increase in $[^3H]dTdh$ incorporation as compared with controls.

*Cytotoxicity studies.* For estimating the ability of mouse embryonic cells to grow or survive after treatment with MNNG or MMS, growth curves or survival curves were made. About $1.5 \times 10^5$ cells/ml were seeded on Leighton flasks containing 2 ml of EMEM supplemented with 10% calf serum. After 24 h cell cultures were treated with MMS at concentrations of $10^{-3}$, $10^{-4}$ or $10^{-5}$ M, or with MNNG at concentrations of $2 \times 10^{-4}$, $10^{-4}$ or $10^{-5}$ M for 3 h, washed twice and kept in EMEM for 72 h. During this period viable cell number was determined at time intervals indicated in the text. Viability of the cells was determined by staining with 0.4% trypan blue. Cell counts of 5 samples, differing by not more than $\pm 10\%$, were averaged to plot the pattern of cell growth or cell survival. The data presented are means from experiments repeated 5-6 times each. When possible, after 72 h the viable cells were transferred at the same initial cell density ($1.5 \times 10^5$ cells/ml) and the growth rate was estimated according to the procedure described above. Cytotoxicity of the two chemical agents to BALB/3T3 IL-2 cells was measured by plating an appropriate number of cells per dish ($5 \times 10^4$), allowing 24 h for cell attachment and introducing MNNG or MMS at the specified concentration for 3 h. After this time the mutagen-containing medium was replaced by fresh culture medium. The cells were refed every two days until colonies developed to macroscopic size. After 10 days they were stained with crystal violet and counted. The average number of colonies
per dish (data from 8 dishes) divided by the number of cells inoculated gave the cloning efficiency for a particular mutagen concentration.

At the highest concentration used (1.0%) dimethylsulphoxide was not cytotoxic.

RESULTS

Prior to the main experiments a short-term toxicity test was performed by the method described by San & Stich (1975). The cultures were exposed for 3 h to various concentrations of MNNG or MMS and immediately afterwards they were examined for detachment and loss of cells or morphological cell alteration. The concentrations of MMS and MNNG chosen on this basis were in the concentration range used by San & Stich (1975).

All experiments concerning repair synthesis were carried out in the presence of hydroxyurea, following protocols of other workers (Lieberman et al., 1971; Ocken & Allen, 1975) and samples containing hydroxyurea in the absence of mutagens were used as controls. As shown in Table 1, the residual semiconservative DNA synthesis after treatment with hydroxyurea was for every kind of cells less than 5% of normal value. In the same Table are summarized the results on the response of all kinds of mouse

Table 1

Effect of MMS or MNNG treatment on tritiated thymidine incorporation

<table>
<thead>
<tr>
<th>Mice</th>
<th>Inhibition of semi-conservative synthesis (%)</th>
<th>Repair incorporation after treatment with MMS (percent of control ± SD)</th>
<th>Repair incorporation after treatment with MNNG (percent of control ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10⁻³ M</td>
<td>10⁻⁴ M</td>
</tr>
<tr>
<td>B10</td>
<td>95.2</td>
<td>293.6 ± 28.8</td>
<td>126.0 ± 9.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n = 9</td>
<td>n = 8</td>
</tr>
<tr>
<td>CBA</td>
<td>97.3</td>
<td>233.9 ± 23.3</td>
<td>147.6 ± 14.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n = 17</td>
<td>n = 10</td>
</tr>
<tr>
<td>DBA/2</td>
<td>96.8</td>
<td>270.5 ± 22.1</td>
<td>143.8 ± 13.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n = 7</td>
<td>n = 7</td>
</tr>
<tr>
<td>C3H/A</td>
<td>97.0</td>
<td>240.3 ± 33.8</td>
<td>114.0 ± 9.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n = 10</td>
<td>n = 10</td>
</tr>
<tr>
<td>BALB/c</td>
<td>95.4</td>
<td>100.0 ± 8.2</td>
<td>98.4 ± 13.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n = 10</td>
<td>n = 10</td>
</tr>
</tbody>
</table>
embryonic cells studied to treatment with MMS or MNNG. As revealed by statistical analysis, the incorporation of $[^3]$HdT into the cells derived from B10, CBA, C3H/A or DBA/2 mice, treated first with hydroxyurea and then with MMS or MNNG was, at the mutagen concentrations used, significantly greater than into control cells. In parallel experiments

![Graphs](image)

**Fig. 1.** a. Growth curves of untreated and MMS-treated cells from DBA/2 mice. Inoculum was about $1.5 \times 10^5$ cells/ml. Zero time refers to 24 h cultures. b. Growth curves of untreated and MMS-treated cells from DBA/2 mice transferred after 72 h at the same initial cell density. For details see Materials and Methods. Mutagen concentration: none (control), (-----); $10^{-5}$ M (x-x-x), $10^{-4}$ M (-----); $10^{-3}$ M (-----)

**Fig. 2.** a. Growth curves of untreated and MMS-treated cells from BALB/c mice. Inoculum was about $1.5 \times 10^5$ cells/ml. Zero time refers to 24 h cultures. b. Growth curves of untreated and MMS-treated cells from BALB/c mice transferred after 72 h at the same initial cell density. Mutagen concentration: none, control (-----); $10^{-5}$ M (x-x-x); $10^{-4}$ M (-----); $10^{-3}$ M (-----)
with cells from BALB/c mice no unscheduled DNA repair synthesis was shown to occur indicating that these cells are deficient in detectable DNA repair not only in the case of UV light induced damage to DNA (Peleg et al., 1976) but also of the damage induced by the two mutagens investigated in this study.

A lower level of \(^{3}\text{H}\)dThd incorporation into cells from BALB/c mice after treatment with MNNG at the concentration of \(2 \times 10^{-4}\) M may reflect inhibition of residual DNA synthesis by MNNG itself. A similar suggestion was presented by Karran et al. (1977) in studies pertaining to repair-deficient cells of retinoid origin.

Figures 1 and 2 show growth curves of the mutagen-untreated and MMS treated cells from DBA/2 and BALB/c mice. There was no significant difference between the shape of the curves for both kinds of cells before and after passaging. Under analogous experimental conditions growth curves of cultures from B10, C3H/A and CBA mice were similar to those from DBA/2 and BALB/c mice. The effects of MNNG at the concentration of \(10^{-5}\), \(10^{-4}\) and \(2 \times 10^{-4}\) M on cell growth and survival are presented in Figs. 3 and 4. The survival curves of these cells reflect that what could be predicted from the DNA repair capacities of each. The cells from BALB/c mice exposed to MNNG at the concentration of \(10^{-4}\) of \(2 \times 10^{-4}\) M died 24 h earlier that the cells from DBA/2 mice, showing a well pronounced increase in the sensitivity to the killing action of this chemical agent. The survival of cells cultured from embryos of three other strains of mice (C3H/A, CBA and B10) was similar to that of cells from DBA/2 mice.

Measurement of DNA repair induced by those concentrations of MMS and MNNG which elicited repair in cells from all kinds of mice with the exception of cells from BALB/c mice, were carried out on cultures of BALB/3T3 IL-2 cells in parallel with cytotoxicity studies.

As shown in Table 2, the cells of this clone were repair-deficient like the early passage cells from the same strain of mice. After 10 days the number of colonies in dishes treated with MMS at the concentrations of \(10^{-3}\) or \(10^{-4}\) M did not differ from that in untreated cultures. The cells treated with MNNG at the concentrations of \(10^{-4}\) or \(2 \times 10^{-4}\) M did not develop colonies.

**DISCUSSION**

The results presented in this study are consistent with the findings of others (Peleg et al., 1976) that cells cultured from BALB/c mice embryos at the late stage of development are repair-deficient. On the other hand,
these results indicate that this feature distinguishes the BALB/c cells from those derived from the other mouse strains studied now.

There is evidence that in some systems a correlation exists between the impaired ability of cells to replicate DNA and their inability to carry out DNA repair (Peleg et al., 1976; Karran et al., 1977). Since in our study the rate of growth of cells cultured from BALB/c mice was similar to the growth rate of other early passage mouse embryo cells it might be assumed that the observed repair-deficiency was a feature of BALB/c
mice. This assumption finds support in the results obtained with the BALB/3T3 IL-2 cell line since, like the early passage cells, they were repair-deficient.

It is of interest that the repair-deficient early passage cells from BALB/c mice showed an increased sensitivity to the killing by MNNG; on the other hand they possessed capacities to cope with MMS induced DNA damage similar to those of repair proficient cells. They differed therefore

**Table 2**

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration (M)</th>
<th>$[^3]H\text{dThd incorporation}$ (% of control ± SD)</th>
<th>No. of colonies/dish</th>
<th>Cloning efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMS</td>
<td>$10^{-4}$</td>
<td>$100.0$</td>
<td>216</td>
<td>43.2</td>
</tr>
<tr>
<td></td>
<td>$10^{-3}$</td>
<td>$116.8 ± 28.2$</td>
<td>198</td>
<td>39.0</td>
</tr>
<tr>
<td></td>
<td>$10^{-3}$</td>
<td>$95.6 ± 17.9$</td>
<td>202</td>
<td>40.0</td>
</tr>
<tr>
<td>MNNG</td>
<td>$10^{-4}$</td>
<td>$100.0$</td>
<td>190</td>
<td>38.1</td>
</tr>
<tr>
<td></td>
<td>$2 × 10^{-4}$</td>
<td>$118.3 ± 11.8$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$10^{-3}$</td>
<td>$74.7 ± 13.1$</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

in the latter feature from repair-deficient bacterial cells (Green & Muriel, 1977) and xeroderma pigmentosum cells (Maher et al., 1977). MNNG may produce up to fifteen different reaction products with DNA while MMS produces only single major adducts in DNA (Singer, 1976; Schendel & Michaeli, 1984). It is therefore possible that mouse embryo cells exposed to MMS can accumulate a certain amount of damage without that damage being expressed as a lethal event. A similar suggestion has been presented to explain survival of cells exposed to low doses of X-rays (Terzaghi & Little, 1976). This explanation is in accordance with the observation that cloning efficiency of BALB/3T3 IL-2 cells exposed to MMS was similar to that of untreated cells in spite of the fact that MMS at the concentrations used did induce repair synthesis in cells of other strains of mice indicating the induction of a damage to DNA.

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REFERENCES


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