Toxicity, Tissue Changes, and Tumor Induction in Inbred Swiss Mice by MethylNitrosamine and -amide Compounds

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SUMMARY

The toxic and carcinogenic actions of four methyl-nitrosamine or -amide compounds, given as a single intraperitoneal injection to newborn and adult inbred Swiss mice, were examined.

Toxicity was approximately directly proportional to the stability of the three compounds capable of methylating macromolecules in vitro.

Methylnitrosourea induced a high incidence of thymic lymphomas in adults and newborns of either sex. Dimethyl-nitrosamine induced a high incidence of hepatomas in newborns only. These two drugs, as well as methylnitrosourethan and methylnitroso-p-tolylsulfonamide, induced a high incidence of pulmonary adenomas.

Mice given a single sublethal intraperitoneal injection of methylnitrosourea underwent cell depletion of the thymolymphoid and myeloid systems, followed by recovery. These events occurred more rapidly in the myeloid systems than in the thymolymphoid system. These events may represent steps in the genesis of lymphomas induced by methylnitrosourea.

INTRODUCTION

Alkylnitrosamine compounds in general are good or even powerful carcinogens causing tumors in many species and in many organs (3, 8). Their additional potential advantages for the design of critical experiments are that they alkylate cell components, thus providing a marker of their action, and that they are unstable or rapidly metabolized, providing close to instantaneous effects (3, 7—9) in comparison with other potent carcinogens such as the polycyclic hydrocarbons.

In order to begin to exploit these potential advantages, 4 of these drugs, administered as a single intraperitoneal dose, were tested for their toxicity in an inbred strain of Swiss mice and for their carcinogenicity in adult and newborn mice of the same strain.

Swiss mice are known to be susceptible to the development of thymic lymphomas following the administration of chemical carcinogens such as 7,12-dimethylbenz(a)-anthracene (1) or methylnitrosourea (6, 15). In the former instance, bone marrow from treated mice is capable of enhancing the yield of thymic lymphomas when injected into mice given a low dose of the carcinogen, which of itself induces a low yield of such tumors (1). As bone marrow has been shown to repopulate a radiation-injured thymus (4, 5, 10, 16, 17), bone marrow and thymus changes in methylnitrosourea-treated inbred Swiss mice may be of interest.

Changes induced in lymphoid and myeloid tissues by a single near LD_{50} dose of methylnitrosourea were therefore studied in adult mice of our inbred Swiss strain.

MATERIALS AND METHODS

Inbred CFW/D mice, derived from Swiss mice obtained from Carworth Farms, New City, N. J., and bred for more than 45 generations by strict brother X sister matings (2), were used for these experiments. When adult mice were used, they were 6 to 8 weeks old at the beginning of the experiments, whereas newborn mice were used within 24 hr of delivery. The mice were kept in air-conditioned quarters with an artificial light cycle and fed Purina fox chow (TheRalston-Purina Company, St. Louis, Mo.) and tap water ad libitum. They were kept a maximum of 6 animals to a cage.

Four methylnitrosos compounds were tested. Dimethyl-nitrosamine and methylnitroso-p-tolylsulfonamide were obtained from Eastman Organic Chemicals, Rochester, N. Y., and methylnitrosourea and methylnitrosourethan were obtained from K & K Laboratories, Plainview, N. Y. The drugs were stored at 5°C and were used without further purification.

The drugs were administered to adult or to newborn mice by a single intraperitoneal injection. The solutions for injection were prepared immediately before use and injected within 1 hr after dissolving of the drug was begun. The solutions were prepared and injected in only enough light to make the maneuvers feasible. DMN\(^2\) and MNUA were dissolved in 0.15 M sodium chloride-0.015 M sodium citrate; MNUN was dissolved in 0.5% ethanol; MNTS was dissolved in 80% ethanol.

The most unstable of these drugs, MNUA (Table 1) was tested for stability in SSC by observing the decay of the

\(^{1}\)Supported by grants of the National Cancer Institute of Canada. Received January 30, 1969; accepted April 28, 1969.

\(^{2}\)The abbreviations used are: DMN, dimethylnitrosamine; MNUA, methylnitrosourea; MNUN, methylnitrosourethan; MNTS, methylnitroso-p-tolylsulfonamide; SSC, 0.15 M sodium chloride-0.015 M sodium citrate.
and at 1- to 5-min intervals for 50 min. The operations were done in subdued light and the spectral decay observed at 37° and at 20°. The final pH of the blank and test solutions was determined at the end of the runs. The half-life of the nitroso group was calculated from the data.

Toxicity of all 4 drugs was tested in adult mice of either sex over a wide range of dosages. Groups of at least 6 mice were used for each dose, and the LD₅₀ dose was determined from the acute lethal effects observed. Acute lethal effects were defined as those causing death of an animal within 3 weeks of injection. An insignificant number of deaths occurred in all groups between 3 weeks and 2 months following injection. After 2 months some mice began to die of tumors.

Tumor induction by all 4 drugs was tested in adult mice of either sex and in mixed sex litters of newborn mice. In all but 1 control group, 30 or more mice were given injections at the beginning of the experiments. The control groups were untreated and 1 contained only 23 mice. The mice were kept for 1 year following injection. Those mice that died later than 3 weeks after injection, or were sacrificed at the end of the experiment, were carefully autopsied and all tumor-like lesions were examined histologically. Only those lesions shown to be tumors microscopically were used in the final accounting. In 1 control group and 1 experimental group the mice were observed for 250 days only.

Thymolymphoid and myeloid tissue changes were studied following the injection of MNUA at 0.25 mmole/kg, 0.75 mmole/kg, or 1.00 mmole/kg, fresh body weight, to adult males and at 0.75 mmole/kg, fresh body weight, to adult females. The following parameters were determined: the number of cells present in the entire shaft and lower epiphysis of 1 femur counted in a hemocytometer; the microhematocrit, the white cell count, and the differential count on blood obtained from the retroorbital sinus; the fresh weights of the spleen and of the thymus dissected as free of fat as possible in a standardized manner, the histological examination of these 2 organs; the body weight of the mouse. These examinations were performed at various intervals for up to 50 days. Groups of 10 mice were examined at each interval. Means and standard deviations were calculated, and appropriate groups were analyzed for significant differences at the 1% level with the Student's t test.

RESULTS

Stability of Methylnitrosourea in Solution. The decrease of the absorbance of MNUA in SSC at 3900 Â in a recording spectrophotometer. A 68 mM solution of MNUA in dimethylformamide was prepared and diluted 10 times in a cuvet with SSC, and the spectra were recorded immediately and at 1- to 5-min intervals for 50 min. The operations were done in subdued light and the spectral decay observed at 37° and at 20°. The final pH of the blank and test solutions was determined at the end of the runs. The half-life of the nitroso group was calculated from the data.

Toxicity of Four Methylnitroso Compounds Given to Adult Mice. The results of the toxicity experiments are summarized in Table 1, in which the data on the stability of the 4 compounds available in the literature are also listed. The known order of increasing stability is MNUA, MNUN, MNTS, DMN. The order of increasing toxicity as determined in these experiments is MNUA, MNUN, DMN, MNTS.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Solvent</th>
<th>Approximate LD₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMN</td>
<td>SSC</td>
<td>0.26</td>
</tr>
<tr>
<td>MNUA</td>
<td>SSC</td>
<td>1.4</td>
</tr>
<tr>
<td>MNUN</td>
<td>0.5% ethanol</td>
<td>0.28</td>
</tr>
<tr>
<td>MNTS</td>
<td>80% ethanol</td>
<td>0.09</td>
</tr>
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Table 1

Nitroso compound stability and toxicity

<table>
<thead>
<tr>
<th>Drug</th>
<th>Half-life</th>
<th>Approximate LD₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>in buffer (pH 7)(hr)</td>
<td>Solvent</td>
</tr>
<tr>
<td>M.W.</td>
<td>Solvent</td>
<td>Approximate LD₅₀</td>
</tr>
<tr>
<td></td>
<td>20°</td>
<td>37°</td>
</tr>
<tr>
<td>DMN</td>
<td>c</td>
<td>74.1</td>
</tr>
<tr>
<td>MNUA</td>
<td>1.2</td>
<td>103.1</td>
</tr>
<tr>
<td>MNUN</td>
<td>80</td>
<td>24.2</td>
</tr>
<tr>
<td>MNTS</td>
<td>94</td>
<td>214.2</td>
</tr>
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</table>

aDruckrey et al. (3).
bMcCalla et al. (9).
cListed by Druckrey et al. as "stable" (3).

Tumor Induction by Four Methylnitroso Compounds in Adult and Newborn Mice. The results of these experiments are listed in Tables 2 and 3. All 4 drugs caused an increased incidence of pulmonary adenomas in adult and in newborn mice.

MNUA in addition caused a 63 to 72% incidence of lymphomas both in adults and newborns of either sex. In the adult mice a small but insignificant difference was observed between the responses of males and females. In the females the tumors appeared somewhat earlier and at a somewhat greater incidence. Both male and female newborns developed lymphomas, but because of small numbers any difference between the sexes was insignificant and they are listed together. Of a total of 60 lymphomas induced, 8 (13%) involved the spleen or the spleen and lymph nodes or liver, but not the thymus. The remainder involved the thymus alone, or together with the spleen or lymph nodes. Histologically, these tumors were predominantly of the undifferentiated cell type, except for those tumors that caused late deaths or those that were discovered 1 year after injection, some of which were of the lymphocytic type.

Fourteen DMN-treated newborns developed 26 hepatomas. These hepatomas were all of the hepatocellular type, although of varying degrees of loss of differentiation, and all were locally invasive. DMN-treated adults developed no hepatomas.

Other malignant tumors occasionally were seen following the injection of any of the 4 drugs. Most of these (9 tumors) in 36 mice were seen following the injection of MNUA into adult female mice observed for 1 year. Such tumors were not observed in MNUA-treated males which were observed for 250 days or in newborns.

These mice are highly susceptible to the induction of squamous cell papillomas (2), and a few such benign tumors were observed in control and experimental mice.
Nitroso Compound Carcinogenesis

Table 2
Nitroso compound carcinogenesis after a single near LD$_{50}$ dose i.p. to adult mice

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mmoles/kg)</th>
<th>Early deaths (%)</th>
<th>Effective No. of mice$^a$</th>
<th>No. of First tumor (days)</th>
<th>Average survival (days)$^b$</th>
<th>Lung adenomas No. of other tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lung adenomas</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Per mouse Benign Malignant</td>
</tr>
<tr>
<td>MNUA</td>
<td>0.19</td>
<td>0</td>
<td>23$^a$</td>
<td>0</td>
<td>0</td>
<td>3$^c$ 0</td>
</tr>
<tr>
<td>MNUA</td>
<td>1.20</td>
<td>14</td>
<td>42$^a$</td>
<td>2</td>
<td>365</td>
<td>365+$^b$</td>
</tr>
<tr>
<td>MNUN</td>
<td>0.19</td>
<td>14</td>
<td>36$^a$</td>
<td>2</td>
<td>202</td>
<td>219+</td>
</tr>
<tr>
<td>MNTS</td>
<td>0.07</td>
<td>38</td>
<td>26$^a$</td>
<td>1</td>
<td>199</td>
<td>199+</td>
</tr>
<tr>
<td>MNUA</td>
<td>0.75</td>
<td>0</td>
<td>30$^a$</td>
<td></td>
<td></td>
<td>58.7</td>
</tr>
<tr>
<td>MNUN</td>
<td>0.19</td>
<td>14</td>
<td>36$^a$</td>
<td>2</td>
<td>202</td>
<td>219+</td>
</tr>
<tr>
<td>MNTS</td>
<td>0.07</td>
<td>38</td>
<td>26$^a$</td>
<td>1</td>
<td>199</td>
<td>199+</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td>3.50</td>
</tr>
</tbody>
</table>

$^a$Mice living longer than 3 weeks after injection.
$^b$Survival of mice with lymphomas only.
$^c$Squamous cell papillomas of skin.
$^d$Experiment terminated 250 days after injection.
$^e$Two breast carcinomas, 1 carcinoma of lung.
$^f$Four fibrosarcomas, 2 squamous cell carcinomas of skin, 2 angiosarcomas of spleen, 1 squamous cell carcinoma of stomach.
$^g$Carcinoma of lung.
$^h$Carcinomas of breast.

Table 3
Nitroso compound carcinogenesis after a single near LD$_{50}$ dose i.p. to newborn mice

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mmole/kg)</th>
<th>Early deaths (%)</th>
<th>Effective No. of mice$^a$</th>
<th>No. of First tumor (days)</th>
<th>Average survival (days)$^b$</th>
<th>Lung adenomas No. of other tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Lung adenomas</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Per mouse Benign Malignant</td>
</tr>
<tr>
<td>DMN</td>
<td>0.11</td>
<td>0</td>
<td>50</td>
<td>3</td>
<td>358</td>
<td>365+$^b$</td>
</tr>
<tr>
<td>MNUN</td>
<td>1.00</td>
<td>34</td>
<td>23</td>
<td>1</td>
<td>365</td>
<td>365+$^b$</td>
</tr>
<tr>
<td>MNUN</td>
<td>0.08</td>
<td>0</td>
<td>36</td>
<td>1</td>
<td>365</td>
<td>365+$^b$</td>
</tr>
<tr>
<td>MNTS</td>
<td>0.04</td>
<td>45</td>
<td>24</td>
<td>2</td>
<td>243</td>
<td>305</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.50</td>
</tr>
</tbody>
</table>

$^a$Mice living longer than 3 weeks after injection.
$^b$Survival of mice with lymphomas only.
$^c$Squamous cell papillomas of skin.
$^d$Cavernous hemangiomas.
$^e$26 hepatomas.
$^f$Not examined.
$^g$One adenocarcinoma of stomach, 1 fibrosarcoma.
$^h$Adenocarcinoma of lung.
$^i$Myelogenous leukemia.

Thymolymphoid and Myeloid Tissue Changes following Methylnitrosourea Injection. As an indication of the widespread effects of MNUA, it was found that all the parameters examined were affected by the treatment. These effects were temporary in all instances, with a return to control values at some point during the 50-day observation period of the experiments (Charts 1 to 7). The least-affected parameter was the body weight of the animals, where a significant difference was obtained only in males treated with 1.0 mmole MNUA/kg 7 days after the injection of the drug. There was a return to control levels by about Day 20. Similarly, a significant depression of the hematocrit was observed in the same mice with a low point at Day 14 (81% of control value) with a return to control values again by Day 20.

The next affected were the bone marrow cell counts and the weights of the spleens (Charts 1, 2, 6, 7). In these 2 instances there was an early marked decrease in these 2 parameters with minimal values achieved on Days 1 to 3. These minimal values were significantly different from control values at all 3 dosage levels and the degree and duration of this effect was in proportion to the dose in males. These 2 parameters returned back to control levels quickly, the weights of the spleens by Day 10 and the bone marrow cell counts by Day 14.
Chart 1. Counts of bone marrow cells from single femurs of adult male mice given a single i.p. injection of 0.25 mmole, 0.75 mmole, or 1.0 mmole methylnitrosourea/kg body weight.

Chart 2. Weights of spleens of adult male mice given a single i.p. injection of 0.25 mmole, 0.75 mmole, or 1.0 mmole methylnitrosourea/kg body weight.

Chart 3. Peripheral white blood counts of adult male mice given a single i.p. injection of 0.25 mmole, 0.75 mmole, or 1.0 mmole methylnitrosourea/kg body weight.

Chart 4. Peripheral differential white blood cell counts of adult male mice given a single i.p. injection of 0.75 mmole methylnitrosourea/kg body weight. Open symbols, controls; solid symbols, experimental mice. •, lymphocytes; ▲, △, neutrophils; □, □, other cells.

Within 14 days after MNUA, a decrease in spleen weight was observed, and this decrease was more pronounced at higher doses. By Day 30, spleen weight had returned to control levels, except for the 0.75 mmole MNUA/kg group, which remained significantly lower than controls. By Day 50, spleen weight in all groups was comparable to controls.

With the lower doses, the return to normal was somewhat more rapid. With 0.75 mmole MNUA/kg, spleen weights were above control levels on Day 15, but this was not significantly different from controls.
Chart 5. Thymus weights of adult male mice given a single i.p.
 injection of 0.25 mmole, 0.75 mmole, or 1.0 mmole methyl
 nitrosourea/kg body weight.

Histological examination of the spleens of males given 0.75
 mmole MNUA/kg indicated that the normal cell population of
 the white pulp (lymphoid tissue) and of the red pulp
 (myeloid tissue) was markedly depleted by Day 3, but the
 stromal elements were not involved in the process. By Day 6,
 earliest regenerative changes in the red pulp were noted.
 At Days 10 and 15 there was massive immature myeloid
 proliferation in the red pulp and the original foci of
 proliferation became confluent. Increasing maturation of this
 myelopoietic tissue was observed by Day 20, and by Day 30
 the spleens were indistinguishable from control spleens. The
 first recovery of the white pulp, observed as repopulation of
 the germinal centers by a small number of large cells
 resembling reticulum cells, was seen at Day 6, with marked
 enlargement of these foci by Day 10 and a complete
 conversion of these foci to lymphocyte-like cells by Day 15.
 Normal germinal centers reappeared in the center of these
 lymphoid collections by Day 20, and recovery to normal
 appearance was present by Day 30. Analogous results were
 seen in spleens of the other groups.

The peripheral neutrophil counts of males given 0.75 mmole
 MNUA/kg (Chart 4) fell below control values on Day 3, but
 this decrease was not statistically significant. There was a
 return to control levels by Day 10.

With respect to the myeloid system of the mouse, there
 fore, as reflected in the hematocrit, the bone marrow cell
 counts, the differential peripheral white counts, and the
 weights and histological appearance of the spleens, a marked
 depression was observed about 3 days after the admin-
 istration of the drug, and complete recovery, largely by
 undifferentiated cells, was achieved by Day 20, with full
 differentiation, at least in the spleens, by Day 30.

The most affected were the parameters reflecting the
 thymolymphoid system: the thymus weights, peripheral
 white blood counts, and peripheral lymphocyte counts
 (Charts 3 to 7). The lowest values observed in these 3
 parameters were also reached rapidly, by Days 3 to 5 in
 most instances. This was 1 or 2 days later than the minimal
 value points in the primarily myeloid parameters. All the
 minimal values observed in the thymolymphoid parameters
 were significantly different from control values. The effects
 were again in proportion to the dose, as was the recovery.
 The recovery occurred later than that observed for the
 myeloid system. Thymus weights returned to normal early
 (by Day 10) only following the lowest dose, but took
 between 20 and 30 days to recover with the 2 high doses.
 With all 3 doses there was a temporary supernormal average
thymus weight in males, but only with 0.75 mmole MNUA/kg was this weight significantly different from controls. This was not observed in females.

Histological examination of thymuses of males given 0.75 mmole MNUA/kg dose showed the most marked generalized cell depletion of the cortical and of the medullary zones by Day 3. By Day 6 small thymocytes began reappearing in the cortical zones in a diffuse, rather than focal, distribution. This diffuse repopulation by thymocytes continued until a normal size of the cortex was achieved by about Day 20. At no time was a zone of immature cells seen in the regenerating cortex, such as was observed following the injection of DMBA into newborn mice (11). The first evidence of recovery in the medullary regions was seen by Day 20. This was occurring in a focal manner, and consisted of collections of cells resembling reticulum cells with some large epithelial cells present. These foci were larger by Day 30, and mitoses were observed in them, especially in proximity to the large epithelial cells, which also contained mitotic figures. By Day 50 there was a beginning of confluence of these medullary foci and a decrease in mitotic activity in them. Analogous changes were seen in thymuses from animals of the other groups. These observations were similar to the pattern of recovery seen in the white pulp of the spleen.

DISCUSSION

The reviews of the induction of tumors by nitroso compounds by Magee and Barnes (8) and by Druckrey (3) indicate that little work has been done with single doses of these compounds in mice. Terracini and Stramignoni (15) and Kelly et al. (6) have demonstrated the induction of thymic lymphomas in newborn and adult mice following a single injection of methylnitrosourea. Kelly et al. (6) also observed a high incidence of pulmonary adenomas in the same mice. Terracini et al. (14) have demonstrated the induction of hepatomas and a high incidence of pulmonary adenomas in newborn mice given a single injection of dimethylnitrosamine. In addition to confirming these observations, we noted a high incidence of pulmonary adenomas following single injections to adult and newborn mice of methylnitrosourethan and methylnitroso-p-tolylsulfonamide.

The use of 4 different methylnitroso compounds made it possible to compare the known properties of these compounds with their toxicity and their capacity to induce tumors. Three of the 4 compounds tested, MNUA, MNUN, and MNTS, are capable of alkylating biological materials in vitro. The toxicity of these 3 compounds is directly proportional to their stability (Table 1). Only the least toxic and least stable MNUA induced a significant incidence of thymic lymphomas. No significant sex difference in the incidence of thymic lymphomas was seen.

Dimethylnitrosamine is incapable of alkylating biological compounds in vitro, and requires an enzyme system resident in the livers and lungs of mice and rats to do so (8). This may be why, in spite of its in vitro stability, it thus proved to be relatively toxic to mice and to induce pulmonary adenomas and hepatomas in the newborn mice.

A single injection of methylnitrosourea into adult mice in a range of dosages below the LD50 level produced widespread effects in the thymolymphoid and myeloid systems of the mice. Cell depletion followed by repopulation was observed. In general the myeloid system was affected somewhat more rapidly and recovered sooner than the thymolymphoid system.

In view of the fact that methylnitrosourea induces in these mice lymphomas originating predominantly in the thymus (6, 15), these observations of early changes in the thymolymphoid system following the injection of the drugs are of interest. The situation could be analogous to that demonstrated for dimethylbenzanthracene-induced (1) thymic lymphomas in mice. In this instance the source of the ultimate lymphoma seems to migrate from the bone marrow to the thymus, much as bone marrow repopulates the thymus of irradiated mice (4, 5, 10, 16, 17). The observation that following treatment with methylnitrosourea the recovery from injury of the bone marrow precedes the recovery of the thymolymphoid system is compatible with that possibility. Further studies will be aimed at elucidating the role of the thymolymphoid and myeloid systems in the genesis of the methylnitrosourea-induced lymphomas.

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