Nitrogen Mustard Inhibition of Azo Dye Carcinogenesis

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Cellular growth and development may be altered appreciably by administration of the nitrogen mustards. The chemotherapeutic value of these compounds in the treatment of leukemia and other neoplasms has been well established (5). In addition, the studies reported by Auerbach, Tatum, Karnofsky, and others have indicated that these agents are mutagenic for Drosophila (1, 2), Neurospora (21), bacteria (20), and chick embryonic tissue (13). Recent reports from this and other laboratories provide conclusive proof that the radiomimetic nitrogen mustards are active carcinogens (8, 10, 12).

Berenblum (4) demonstrated that the addition of mustard gas in a 1 per cent concentration inhibited the production of warts by tar. Further studies established that the inhibition was a direct effect of the chemical on the animal. The carcinogen, 1,2,5,6-dibenzanthracene, gave a similar effect. Recently, Riegel and associates (19) have shown that administration of 1,2,5,6-dibenzofluorene reduced the tumor incidence resulting from methylcholanthrene. In a somewhat comparable study, Richardson and Cunningham (18) found that the application of methylcholanthrene resulted in a lowering of the tumor incidence in rats fed diets containing 8'-methyl-4-dimethylaminoazobenzene (3'MeDAB). There have been several instances, however, when the administration of one carcinogenic agent did not alter the action of a second carcinogenic agent (8). The incidence of ear tumors in Swiss mice exposed to ultraviolet light was not altered by weekly injections of nitrogen mustard.¹

Since the nitrogen mustards are capable of inhibiting cellular development and also of inducing somatic mutations and tumors, it appeared worthwhile to study the effect of these agents on the process of cancer induction by other compounds. An alteration of this process by the nitrogen mustards may provide information bearing on the mechanism of carcinogenesis. In the present study, methylbis(β-chloroethyl)amine (HN₂) was injected subcutaneously into albino rats fed diets containing 3'MeDAB. The effect of this treatment on the resulting incidence of liver tumors is reported.

METHODS

Male albino rats of the Holtzman Sprague-Dawley strain were maintained on a purified diet (9) containing 0.06 per cent 3'MeDAB until tumors were evident by palpation or by laparotomy of control animals. At this time, the rats were placed on a purified basal diet containing no dye for an additional 4–5 weeks to allow the tumors to grow to recognizable size (15). The animals treated with nitrogen mustard received subcutaneous injections once weekly of 0.5 mg HN₂/kg of body weight; the drug was administered as a 0.01 per cent solution of the hydrochloride salt in physiological saline. All animals were weighed and food intakes for the various groups were determined throughout the study. The food intake was lower with the 3'MeDAB diet than with the basal diet alone. Animals fed the dye-containing diet, however, maintained or gained weight slowly. Administration of HN₂ to dye-fed rats did not appreciably change the food intake or the maintenance of body weight.

Initially, two series of experiments were carried out. The first series, conducted over a 10-week period, comprised three groups: 3'MeDAB controls fed the dye as described above; animals fed the dye and treated weekly with HN₂; and HN₂ controls fed basal diet and similarly treated. The second series was a repetition of the first and also included groups to show the effect of varying the injection period of HN₂ during the feeding of the carcinogen. This series included: 3'MeDAB controls fed the dye for 12 weeks, animals treated with HN₂ the first 2 weeks of a 10-week dye-feeding period, rats treated with HN₂ the last 4 weeks of the feeding period (sixth to tenth week on experiment), animals treated weekly during a dye-feeding period of 10 weeks, animals treated throughout a 12-week feeding period, and control animals...
maintained on the dye-free diet and treated with HN2 for 10 weeks. A third confirmatory series included: rats maintained on this diet and treated with HN2 the first and second week, and animals fed the dye and treated weekly during the 12-week period.

At the end of 15–16 weeks, when control animals fed the diets containing S'MeDAB showed a high percentage of palpable tumors, the rats were sacrificed by decapitation and exsanguination. At this time, gross observations of the condition of livers were made, and each liver was designated as being tumorous, probably tumorous (containing areas resembling tumor tissue and severely cirrhotic), cirrhotic, or normal. While the distinction between these groups was slight, all were judged valid. Where chemical determinations of nucleic acids were to be made, the livers were perfused in situ with cold saline, removed, and stored at 0°C for several hours. The liver tissues were homogenized; phosphorus, nitrogen, desoxyribonucleic acid and ribonucleic acid were determined as described in a previous publication (11).

RESULTS

The tumor incidence in animals fed the carcinogenic azo dye S'MeDAB and treated with nitrogen mustard, as compared to control dye-fed animals, is summarized in Table 1. In determining the percentage of tumor formation, the “probable” tumors were counted as tumors. Cirrhotic livers were considered nontumorous unless definite tumors lesions were evident.

Control animals fed the diet containing S'MeDAB for 10 or 12 weeks showed a 90–100 per cent incidence of liver tumors (Table 1). Both untreated and nitrogen mustard-treated control animals maintained on the basal diet alone had normal livers. The greatest inhibition of tumor development was found in animals fed the dye for 10 weeks and treated once weekly with HN2 throughout the feeding period. In this group, less than one-third of the animals exhibited tumors, and most of the remaining rats had livers of normal appearance.

An initial observation was made on one rat in the first series of experiments which, because of in-

### Table 1

<table>
<thead>
<tr>
<th>Diet</th>
<th>No. weeks</th>
<th>Treatment</th>
<th>Description of liver at termination of experiment</th>
<th>Survival at end of dye-feeding period</th>
<th>Survivors without tumors</th>
<th>Tumor incidence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>12</td>
<td>HN2, 10 wks.</td>
<td>All livers normal</td>
<td>9/9</td>
<td>9</td>
<td>0/9</td>
</tr>
<tr>
<td>S'MeDAB†</td>
<td>10</td>
<td>None</td>
<td>Tumors present in all livers. Most of surrounding tissue cirrhotic.</td>
<td>7/8</td>
<td>4</td>
<td>3/7</td>
</tr>
<tr>
<td>Basal</td>
<td>6</td>
<td>HN2, first 2 weeks</td>
<td>One with definite tumor, two probable. Four livers were nearly normal.</td>
<td>7/9</td>
<td>5</td>
<td>2/7</td>
</tr>
<tr>
<td>S'MeDAB</td>
<td>6</td>
<td>HN2, last 4 weeks</td>
<td>Tumors present in two livers. Others normal or nearly normal.</td>
<td>7/8</td>
<td>0</td>
<td>7/7</td>
</tr>
<tr>
<td>Basal</td>
<td>12</td>
<td>None</td>
<td>Tumors in all livers. Livers enlarged and severely cirrhotic.</td>
<td>12/14</td>
<td>1</td>
<td>11/12</td>
</tr>
<tr>
<td>S'MeDAB</td>
<td>12</td>
<td>HN2, first 2 weeks</td>
<td>One normal, tumors present in all others. Livers enlarged, cirrhotic.</td>
<td>8/12</td>
<td>0</td>
<td>5/8</td>
</tr>
<tr>
<td>Basal</td>
<td>12</td>
<td>HN2, 12 wks.</td>
<td>Tumors in eight livers. Most livers were somewhat enlarged and cirrhotic.</td>
<td>14/15</td>
<td>0</td>
<td>8/14</td>
</tr>
</tbody>
</table>

* Tumor incidence: number with tumors over number surviving at end of dye-feeding period.
† HN2: methylbis(chloroethyl)amine hydrochloride. Administered subcutaneously, 0.5 mg/kg body weight.
‡ S'MeDAB: S’methyl-4-dimethylaminoazobenzene. Incorporated in basal diet at a level of 0.06 per cent.
incidence obtained when \( \text{HN}_2 \) was injected the last 4 weeks prior to the basal diet period. In addition, treatment with \( \text{HN}_2 \) of several rats bearing small liver tumors did not appreciably modify the growth or development of the tumors.

From the data presented in Table 1, it is evident that administration of \( \text{HN}_2 \) does not completely inhibit liver tumor formation. Also, it may be noted that a higher incidence of tumors was obtained when the azo dye and \( \text{HN}_2 \) were administered for 12 weeks instead of 10 weeks. Thus, prolonged feeding of the dye might partially overcome the inhibiting effect of \( \text{HN}_2 \). Several animals from a group fed the diet containing \( 3^\prime\text{MeDAB} \) and also a group fed this same regimen but treated with \( \text{HN}_2 \) were sacrificed at 7 weeks so that the condi-

| Table 2 |

<table>
<thead>
<tr>
<th>Diet</th>
<th>No. weeks</th>
<th>Treatment</th>
<th>Description of liver</th>
<th>Desoxynucleic acid (mg. per cent)</th>
<th>Ribonucleic acid (as ribose) (mg. per cent)</th>
<th>Nitrogen (mg. per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>7</td>
<td>None</td>
<td>Normal</td>
<td>666</td>
<td>184</td>
<td>2,845</td>
</tr>
<tr>
<td>( 3^\prime\text{MeDAB} )</td>
<td>7</td>
<td>None</td>
<td>Cirrhotic</td>
<td>428</td>
<td>146</td>
<td>2,077</td>
</tr>
<tr>
<td>( 3^\prime\text{MeDAB} )</td>
<td>7</td>
<td>( \text{HN}_2 ), 7 wks.</td>
<td>Normal</td>
<td>379</td>
<td>144</td>
<td>1,845</td>
</tr>
<tr>
<td>Basal</td>
<td>10</td>
<td>( \text{HN}_2 ), 10 wks.</td>
<td>Normal</td>
<td>308</td>
<td>143</td>
<td>2,620</td>
</tr>
<tr>
<td>Basal</td>
<td>5</td>
<td>None</td>
<td>Tumorous</td>
<td>600</td>
<td>188</td>
<td>2,490</td>
</tr>
<tr>
<td>( 3^\prime\text{MeDAB} )</td>
<td>12</td>
<td>None</td>
<td>Cirrhotic</td>
<td>383</td>
<td>155</td>
<td>2,285</td>
</tr>
<tr>
<td>Basal</td>
<td>4</td>
<td>( \text{HN}_2 ), 12 wks.</td>
<td>Normal</td>
<td>396</td>
<td>152</td>
<td>2,415</td>
</tr>
<tr>
<td>( 3^\prime\text{MeDAB} )</td>
<td>10</td>
<td>( \text{HN}_2 ), first</td>
<td>Normal</td>
<td>358</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>6</td>
<td>( \text{HN}_2 ), 10 wks.</td>
<td>Normal</td>
<td>358</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

\* \( 3^\prime\text{MeDAB} \): \( 3^\prime\text{methyl-4-dimethylaminoazobenzene. Incorporated in basal diet at a level of 0.06 per cent.} \)

\( \dagger \text{HN}_2 \): methylibis(\( \beta \)-chlo襁ethyl)amine hydrochloride. Administered subcutaneously, 0.5 mg/kg body wt.

Each group was composed of tissue from three to seven animals.

DISCUSSION

The induction of liver tumors by the carcinogenic azo dye, \( 3^\prime\text{MeDAB} \), was inhibited by the simultaneous administration of the nitrogen mustard, \( \text{HN}_2 \). This was predicted from the nucleotoxic properties of the mustard. The mechanism of hepatoctoma formation may possibly be attributed to mitotic stimulation in the liver tissues in response to the azo dye. An increase in mitotic activity is evident from the histological and chemical studies of Price and associates (16, 17) and of Cunningham et al. (6, 7). An increase in the numbers of nuclei and a corresponding increase in DNA per gram of liver tissue was noted as the carcinogenic azo dyes were administered. The increase in DNA in the liver tumors produced is further evidence for this proposed mechanism of tumor formation, i.e., mitosis accompanied by or following upon increased synthesis of this nucleic acid. Conversely, studies with nitrogen mustard have shown
that this chemical causes a marked depression of DNA synthesis and an inhibition of mitosis. Results of the experiments in the present study, with regard to both tumor incidence and DNA concentrations, are evidence for the antagonistic effect of HN2 on the azo dye, and can be interpreted to further support the proposed mechanisms of action of these two compounds.

Since the azo dyes stimulate mitosis in liver tissues and also bring about an increase in the concentration of liver DNA, it might be postulated that nuclei isolated from the livers of animals injected with P22 would contain relatively large quantities of the isotope. Studies completed in this laboratory (11) have shown that, following P22 administration, the specific activity of phosphorus was from 5 to 10 times greater in the liver DNA of animals fed diets containing 3'MeDAB than in normal control tissues. Recently, we have demonstrated that HN2 administered subcutaneously to animals fed the azo dye greatly reduced this incorporation of P22 into the nuclear DNA. These observations are in agreement with the present findings and provide further evidence of the suppression of mitotic activity by the nitrogen mustard.

Two relationships between the dye-feeding period and nitrogen mustard injection were established. First, the nitrogen mustard apparently inhibits tumor formation only in the initial stage of azo dye administration; therefore, its preventive effect is exerted only in the beginning proliferative stage of tumor development. The results are in accord with experiments by Marshak on the effect of mustard gas (HS) on mitosis and P22 uptake in regenerating rat liver (14). He found mitosis to be greatly depressed when administration of HS immediately followed partial hepatectomy; inhibition of a lesser degree occurred if the treatment was initiated several hours after regeneration had begun. This suggested that if the mustard reaches the cells before mitosis begins, it prevents mitosis. Second, Marshak observed a quantitative relation between inhibition and the amount of mustard gas used, the inhibition decreasing with decreasing amounts of mustard gas. Studies reported here show a similar relationship between the amount of dye fed and the amount of nitrogen mustard injected, as was indicated by the higher tumor incidence in the 12-week feeding period.

This study provides further information concerning the mechanism of action of the nitrogen mustards. Thus, HN2 has been found to exert an inhibiting effect on the induction of tumors by a carcinogenic azo compound. The proposed mechanism involves an inhibition of mitosis caused in turn by a decreased synthesis of desoxyribonucleic acid in the individual cell.

SUMMARY

1. Albino rats were fed diets containing the carcinogenic azo dye, 3'-methyl-4-dimethylaminoazobenzene, and also given subcutaneous injections of methylbis(β-chloroethyl)amine (HN2) at weekly intervals.

2. Animals fed the diets containing the dye exhibited a 90–100 per cent incidence of liver tumors after 10–12 weeks. Animals fed the same diet but treated with HN2 showed a tumor incidence of 30–60 per cent.

3. This inhibition of azo dye carcinogenesis by HN2 was most effective if the mustard was administered in the initial stages of the dye-feeding period. Injection of HN2 during the last 4 weeks did not affect the tumor incidence.

4. From chemical studies of the liver tissue, evidence was obtained indicating that HN2 inhibits the formation of liver tumors by azo dyes by blocking mitosis and the synthesis of desoxyribonucleic acid.

REFERENCES


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* D. N. Ward, E. L. Brandt, and A. C. Griffin. The Effects of Nitrogen Mustard and a Pyridinium Compound on the Phosphorus Turnover in Rat Liver during Azo Dye Cancer Induction (manuscript submitted for publication).


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