Tumorigenicity of N-Nitrosohexamethyleneimine

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SUMMARY
A new carcinogen, N-nitrosohexamethyleneimine (N-nitrosoperhydroazepine), was synthesized and given to rats of both sexes. The incidence of mixed malignant liver cell carcinomas and endothelial sarcomas was virtually 100% when the drug was given at 200 mg/liter for 8 weeks, while benign and malignant epitheliomas of the tongue or esophagus occurred in about 30%. When the drug concentration was 50 mg/liter given continuously over the life-span of the animals, the incidence pattern of tumors in liver and upper alimentary tract was reversed. The possible relevance of the types and distribution of these cancers to interpretations of the mechanism of action is discussed.

INTRODUCTION
Much of the interest in the tumorigenic activity of the N-nitrosoamines centered on the aliphatic nitrosamines, especially dimethylnitrosamine and diethylnitrosamine. Druckrey (2) has suggested that a marked organotropic effect is evident in the many carcinogenic nitrosoamines that he has tested. One of the proposed mechanisms of carcinogenic action of the aliphatic nitrosamines involved conversion of the nitrosamine to an alkylating intermediate, which alkylates the guanylic acid moieties of DNA, this altered DNA then being the initiator of the neoplastic process. It might be surmised that the differences in tumorigenic effect of the various nitrosamines could be ascribed to differences in extent of metabolic conversion into an alkylating agent in different species and in different organs. Although some workers doubted that such a mechanism (involving production of an alkylating intermediate) could operate in the case of cyclic N-nitrosoamines, we have found that nitrosomorpholine, nitrosopyrrolidine, and nitrosopiperidine did give rise to an alkylated guanylic acid (5). As part of this study of the mechanism of action of cyclic N-nitrosoamines, several hitherto unexamined members of this group of compounds were tested in animals. Among these were nitrosazetidine (8) and the compound discussed here, nitrosohexamethyleneimine (NHM; N-nitrosoperhydroazepine).

MATERIALS AND METHODS
N-Nitrosohexamethyleneimine. Fifty grams (58 ml) of hexamethyleneimine (Eastman Organic Chemicals, Rochester, N.Y.) were dissolved in 120 ml of 25% sulfuric acid, cooled in ice. Approximately 100 grams of sodium nitrite were added in small portions and the solution allowed to stand overnight. The upper layer of light brown oil was removed and combined with an ether extract of the aqueous layer. The ether solution was washed twice with a little water and dried with anhydrous potassium carbonate. The ether was removed under reduced pressure at room temperature; the residue weighed 60 grams. This was distilled from an oil bath under reduced pressure, discarding the first 4-5 ml of distillate. The main bulk distilled as a pale yellow oil, b.p. 120-121°C at 15 mm. The yield was 49 grams (75% of the theoretical yield).

Animals. Male and female MRC rats, bred randomly in this laboratory, were used. The animals, 8-9 weeks old at the beginning of the experiment, were housed in plastic cages on sterilized litter in groups of five according to sex and fed Rock-
land food in pellets and water ad libitum. Durations stated are counted from the beginning of treatment.

Acute Toxicity. In a preliminary experiment, NHM dissolved in olive oil was given intraperitoneally. Most of the animals injected died shortly afterwards in convulsions, and the indicated LD$_{50}$ was near 300 mg/kg. A second group of 16 male rats was divided into four subgroups. The subgroups received, respectively, 100, 200, 400, and 800 mg/kg NHM in olive oil by gavage. Six of the animals in the two higher dose groups died with convulsions a few minutes after drug administration. Another two rats died 24 and 48 hours after administration of the drug.

Chronic Toxicity. NHM was dissolved in water at two concentrations, 50 mg/liter and 200 mg/liter. One hundred milliliters of each solution was given to each cage of five animals each night, and almost all of this volume was consumed by morning, after which the animals were given tap water in the daylight hours. The solution was contained in brown bottles and night feeding was selected because of the light sensitivity of the nitrosamine solution. Treatment groups were composed of 15 male and 15 female rats on each solution.

In Group I, which received the higher dose (200 mg/liter), two animals died after six weeks, and treatment was suspended for one month to allow the survivors to recover. After the month interval, treatment was resumed. Since the animals appeared ill and were losing weight, the treatment was suspended after a total of eight weeks, and the animals were then observed until death.

The animals receiving the lower dose (50 mg/liter, Group II) suffered no short-term ill effects and were treated continuously until death.

Complete autopsies were done on all animals, and tissues were fixed in Helly's fluid or formol-saline for histologic examination.

RESULTS

Acute Toxicity. The LD$_{50}$ was estimated according to the method of Weil (11) as 336 mg/kg.

In the animals that died shortly after treatment, the gross findings were acute stasis of the liver and peritoneal organs, meningeal congestion, and edema of the brain. Histologically, the only noteworthy findings were acute stasis in the liver with mild degeneration of the liver cells in the centrilobular areas, marked dilation of meningeal vessels, and edema of the brain. In the animals which died 24-48 hours after the administration of NHM, the gross findings were essentially similar, except for the liver which was soft and friable. Histologically, there was an extensive necrosis of the liver cells, mostly in the centrilobular areas, but often involving almost the entire lobule; congestion and edema of the brain were also seen.

Chronic Toxicity. Survival rates and tumor incidence in the animals of the two groups are summarized in Table 1.

Although the total number of tumor-bearing animals and total number of tumors were essentially comparable in the two groups, there was a notable difference in the type of tumors resulting from the two dose levels used. A high incidence of tumors of the liver and of the nasal cavity was registered in Group I where the higher dose of NHM (200 mg/liter) was given for only eight weeks, while Group II (50 mg/liter, continuously) showed a higher incidence of esophageal and tongue tumors.

In Group I two females died at the sixth week and one at the 13th week. Histologically, there were severe lesions of the liver; the normal architecture had completely disappeared and was replaced by nodular regeneration areas surrounded by hyperplastic connective tissue, where an abundant proliferation of bile duct cells and of elongated endothelial cells was quite prominent (Fig. 1). The endothelial cells were lining thin sinusoids or were facing each other in opposite rows, possibly lining obliterated sinuses (Fig. 2). The regeneration nodules were composed of parenchymal cells lying in distorted and abnormal trabeculae, mostly very thick with rare mitosis and large nucleoli.

The first liver tumor in Group I was observed in a female dead 16 weeks after the beginning of treatment. In the males the first liver tumor was observed in an animal dead at the 36th week.

In Group II the first liver tumor was observed in a female dead 41 weeks after the beginning of the experiment. The first liver tumor in the males was observed in an animal dead at the 48th week.

All other tumors in both groups were observed in animals dead or killed between the 48th and 68th week.

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats at start</th>
<th>Survivors (weeks)</th>
<th>Total No. of tumors</th>
<th>Number of rats with various tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10 20 30 40 50 60 TBA</td>
<td></td>
<td>Liver† Tongue Esophagus Nasal cavity Other sites</td>
</tr>
<tr>
<td>Group I</td>
<td>15 0</td>
<td>12 10 8 7 5 4 0</td>
<td>11 16 11 2 2 1</td>
<td></td>
</tr>
<tr>
<td>4 mg/day, 5 days a week for 8 weeks</td>
<td>15 0</td>
<td>15 15 15 13 8 5 0</td>
<td>15 23 15 1 4 2</td>
<td></td>
</tr>
<tr>
<td>Group II</td>
<td>1 mg/day, 5 days a week, life span</td>
<td>15 0</td>
<td>15 15 15 14 6 0</td>
<td>14 21 7 3 11</td>
</tr>
</tbody>
</table>

Survival rates and distribution pattern of tumors in rats treated with nitrosohexamethyleneimine. TBA, tumor-bearing animals.

† Liver cell carcinomas and endothelial sarcomas.

* One with carcinoma of the stomach.

‡ Two with squamous carcinoma of the stomach, one with carcinoma of the pancreas.
Because of the mixed nature of these tumors, the histologic identification of the tumors posed some difficulty. In almost every instance, liver cell tumors, either anaplastic or trabecular, were combined with proliferation of atypical endothelial cells which lined dilated sinusoids, often filling the widened sinusoids and occasionally forming solid masses (Fig. 3). While in some areas there appeared to be a close intermingling of neoplastic liver cells and atypical endothelial cells (Fig. 4), in other areas the atypical endothelial cells invaded the sinusoids leaving the liver cords, composed of normal liver cells, intact (Figs. 5, 6). The growth of endothelial cells in some areas was predominant, replacing large parenchymatous areas and showing sarcomatous patterns (Fig. 7). The term endothelial sarcomas of the liver is proposed for these tumors. Liver cell carcinomas and endothelial sarcomas of the liver are grouped under the common heading “Liver Tumor” in Table 1. The two types of tumor were always mixed, often with one or the other type predominating. Metastases were often seen in the lungs, while the tumors more rarely spread directly to the peritoneum and adjacent tissues.

The tumors of the nasal cavity were either highly keratinized squamous cell carcinomas, undifferentiated carcinomas with occasional tubular arrangements of the cells (Fig. 8), or neuroepithelial tumors. They apparently originated from the posterior and upper nasal cavity, were highly invasive, eroding the bone tissue, and progressing to the orbit, or, through the ethmoid, to the brain.

The tumors of the esophagus were in most instances multiple, often distributed along almost the entire length of the organ. In Group I three of the six animals bearing esophageal tumors had multiple squamous papillomas: one had one squamous papilloma, while the other two had multiple squamous papillomas plus multiple squamous cell carcinomas. In Group II, of the 24 rats bearing esophageal tumors, two had multiple squamous papillomas and the remaining 22 had multiple papillomas and a single or multiple squamous cell carcinoma. While the carcinomas of the esophagus were markedly invasive, metastases were only occasionally seen. There was one squamous papilloma of the tongue in a rat of Group II; all other tumors of the tongue were squamous cell carcinomas.

DISCUSSION

N-Nitrosodimethylamine is a new hepatotoxin and a powerful carcinogen in the rat for liver and other epithelial tissues, as well as inducer of malignant tumors of the sinusoidal endothelium in the liver. The type and distribution of the tumors induced may have some bearing on the problem of organotropism in the carcinogenic nitrosamines.

In the animals treated with the higher concentration of NHM for a short period, there was an almost 100% incidence of liver tumors, together with about one quarter of these tumors in the esophagus and nasal cavity, several animals bearing tumors at more than one site. By contrast, in the rats treated with the lower concentration of the drug for a longer period, the percentage incidence pattern between liver and esophagus was almost exactly reversed. In addition, these animals showed significantly higher incidence of carcinoma in the posterior third of the tongue, a tumor found only once in the first group.

The selective production of tumors in different tissues after treatment with various substances of different chemical classes has long been a difficult problem in experimental oncology, recently emphasized again by the striking organotropism of the carcinogenic nitrosamines [Druckrey et al. (2)] whose activity is presumably due to the nitroso group common to the series. The apparent specificity of the tumor distribution pattern for different chemicals has led to many hypotheses, some of them discussed here, usually based upon supposed differences in absorption, permeability, metabolism, and activation of the drugs administered. In the present studies of a new carcinogen, we have given the same drug by the same route but at different dose rates and for different periods of exposure, and the consequent differences in the patterns of induced tumors seem just as striking as those previously observed in comparisons of different carcinogenic substances.

Since the survival rates for animals of both groups were very similar, the differing pattern of tumor incidence cannot be ascribed simply to a differing life span or period of risk. As can be seen from Table 1, the cumulative dose in animals of Group I was 160 mg, while in Group II, which received the lower concentration, the cumulative dose varied from 150 to about 250 mg. It is difficult to believe the different tumor patterns could be due to such relatively minor differences in total dose.

The liver tumors were of multicentric origin, as were those of the upper alimentary tract, and they were of two histologically distinct types occurring simultaneously; namely, liver cell carcinomas and endothelial sarcomas of the liver. Without a detailed histogenetic study, it is not possible to trace with certainty the site of origin and the progression of the neoplastic process involving the liver as a whole. The occurrence of liver cell carcinomas is not unexpected in view of previous results from many laboratories with other nitrosamines, but the widespread involvement of the liver endothelium in the absence of endothelial tumors or other lesions of the endothelium elsewhere suggests several possible explanations for the mechanism of action of the carcinogen. Endothelial cells in general may be susceptible to the carcinogen, but tumors may appear only where the concentration of the carcinogen is sufficiently high, as in the liver. The proximate carcinogenic molecule may be formed metabolically in the parenchymal cells of the liver and have a brief half-life, thus being able to exert its action on neighboring cells, both parenchymal and endothelial, while being ineffective distantly. The possibility that endothelial cells of the liver might be more susceptible than endothelial cells of other organs seems very remote, but cannot be ruled out.

Experiments done with nitrosodiethylamine have shown that the route of administration does not alter the selection of particular organs for its carcinogenic action (1). It has been suggested (4) that this carcinogen enters the circulating blood and thereafter exerts its action on susceptible organs, either as such, or after being metabolized in loco. Our observations...
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of tumors in the tongue, esophagus, stomach, and nasal cavity would be in keeping with these suggestions of the importance of direct access of the drug (10) to a susceptible tissue, with conversion to the proximate agent by local metabolism. These are the tissues most directly exposed to the drug in the drinking solution, and the much higher incidence of such tumors resulting from prolonged continuous exposure in Group II makes this interpretation more probable. However, there is no direct evidence as yet of NHM having carcinogenic power by local application, and we have no evidence at present that these epithelial tissues are in fact capable of metabolizing NHM or other nitrosamines. Knowledge of the anatomical distribution of enzyme systems capable of converting nitrosamines into alkylating intermediates might contribute to an explanation of the organ pattern of the induced tumors, but it seems likely that other complex factors will also be involved. Although the conversion to an unidentified alkylating agent seems certainly to be a key metabolic event in the toxicity of nitrosamines (9) and while some correlation has been found between alkylation of nucleic acids and eventual tumor formation in susceptible organs in contrast to the absence of alkylation in resistant tissues (6), there is still no convincing evidence that alkylation is directly responsible for carcinogenesis (3, 7, 10). Obviously the site at which tumors are induced by a particular carcinogen is dependent upon multiple unknown factors, which must include the cumulative dose, the dose rate, and the time during which tissues are exposed, even when the drug is administered by a single route.

ACKNOWLEDGMENTS

We wish to thank Mr. A. Washington for the photographs and Mr. B. Paliulis and Miss P. Johns for technical assistance.

REFERENCES


Fig. 1. Liver of a rat dead six weeks after the beginning of treatment. Large regeneration nodules with complete disruption of the normal architecture. H & E, × 22.

Fig. 2. Liver of a rat dead 13 weeks after the beginning of the treatment. Large regeneration nodules, bile ducts, proliferation and hyperplasia of the endothelial cells. H & E, × 80.

Fig. 3. Atypical endothelial cells lining the liver cords along highly dilated sinusoids. In several areas the endothelial cells form clusters protruding into the sinusoids and blood lakes. H & E, × 150.

Fig. 4. Trabecular carcinoma with some pseudoglandular structure and proliferation of atypical endothelial cells which line the liver cords and spread through the dilated sinusoids. H & E, × 100.

Fig. 5. Endothelial sarcoma of the liver. H & E, × 80.

Fig. 6. Higher magnification of Fig. 5. Malignant endothelial cells are filling dilated sinusoids, lining thin liver cords composed of normal liver cells. H & E, × 450.

Fig. 7. Endothelial sarcoma of the liver. Frank sarcomatous appearance. H & E, × 150.

Fig. 8. Tumor of the nasal cavity in a rat dead 56 weeks after the beginning of the experiment. The cartilage divides an area where the tumor has the appearance of a squamous cell carcinoma highly keratinized, from another area where it has the appearance of an undifferentiated carcinoma with some glandular structure. H & E, × 31.

CANCER RESEARCH VOL. 28
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