Comparison of the Carcinogenic Effects of Five Nitrosamines in Guinea Pigs

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ABSTRACT

Five nitrosamines which are potent carcinogens in rats or hamsters were administered by gavage in olive oil solution twice a week to 20 male strain 2 guinea pigs. Nitroso-2,6-dimethylmorpholine given at 80 mg/kg/week for 12 weeks or at 32 mg/kg/week for 35 weeks gave rise to hemangioendothelial sarcomas of the liver in 6 and 19 animals, respectively. The same tumors were induced in 18 animals by dinitroso-2,6-dimethylpiperazine, together with hepatocellular carcinomas in six animals. Nitrosomethylododecylamine also induced mainly hemangioendothelial sarcomas of the liver (12 animals). A few bile duct carcinomas were also observed. Neither nitrosoheptamethyleneimine nor nitrosomethylidithyliurea seemed to induce tumors in guinea pigs under our conditions.

INTRODUCTION

Guinea pigs have been used sparingly in carcinogenesis studies, partly because it was believed for a time that they were resistant to the action of chemical carcinogens. The inability to produce tumors in guinea pigs with 2-acetylaminofluorene because of the absence of necessary enzymatic activation is a well-known phenomenon and was important in demonstrating the importance of interspecies metabolic variation and its effects on carcinogenesis.

Subsequent evaluation of older data and newer work has shown this species to be sensitive to the actions of some carcinogens (2, 3, 5, 16, 17). Nitrosamines are of special interest because of their carcinogenic potency in other rodent species and because of the likelihood of human exposure through in vivo formation of potentially hazardous nitrosated compounds.

In order to compare interspecies differences in carcinogenic effect by selected N-nitroso compounds, 5 compounds that have been tested in rats or hamsters were administered to guinea pigs. The doses were chosen to approximate those which gave rise to a significant incidence of tumors in the other species. Nitroso-2,6-dimethylmorpholine is a pancreatic carcinogen in Syrian hamsters (14) and an esophageal carcinogen in rats (8); dinitroso-2,6-dimethylpiperazine is an esophageal carcinogen in rats (7); nitrosomethylododecylamine induces transitional cell carcinomas of the urinary bladder in both rats (10) and Syrian hamsters (1); nitrosoheptamethyleneimine induces squamous lung tumors and esophageal tumors in rats (11, 15), lung tumors in European hamsters (13), and forestomach and esophageal tumors in Syrian hamsters (6); and nitrosomethylidithyliurea administered to rats in drinking water gives rise to tumors of the CNS (9).

MATERIALS AND METHODS

Each of the 5 nitrosamines was dissolved in olive oil at the concentrations indicated in Table 1, except for dimethylnitrosopiperazine, which was dissolved in ethyl acetate:olive oil (1:9). The animals were 8-week-old male strain 2 guinea pigs of the colony of the Frederick Cancer Research Center, that were maintained in a clean, conventional facility and fed Charles River guinea pig formula ad libitum. Each treatment group consisted of 20 animals, each of which was given the solution of the nitrosamine twice a week by gavage at a dose of 1.0 ml of solution per kg of body weight. The animals were weighed weekly, and the volume of solution given was adjusted once a month to compensate for increases in their body weights. The length of treatment (see Table 1) depended on monitoring the clinical progress of the animals and varied from one compound to another, since there were no preliminary studies of the toxicity of the compounds, in order to conserve animals and chemicals. Nitrosodimethylmorpholine proved to be toxic at the concentration used (40 mg/ml). Since some animals died at the 12th week of treatment, treatment was discontinued for the remaining animals, and the study was repeated at a lower concentration (16 mg/ml) with a new group of male guinea pigs. This treatment was stopped at 35 weeks. At the end of the treatment period, each group of animals was allowed to die spontaneously. Individuals were killed when moribund in order to minimize postmortem autolysis. Dead animals were subjected to complete gross necropsy with histopathological examination of selected organs and all grossly observed lesions.

RESULTS

The pattern of mortality of guinea pigs in all treatment groups and of controls given only olive oil is shown in Table 1. All of the animals in the groups treated with nitroso-2,6-dimethylmorpholine, dinitroso-2,6-dimethylpiperazine, and nitrosomethylododecylamine died with or from proliferative or neoplastic lesions which arose within the liver. No such liver lesions were encountered in animals treated with nitrosoheptamethyleneimine, nitrosomethylidithyliurea, or in olive oil controls.

Whereas the primary target organ for the 3 carcinogenic compounds was the liver, the patterns of lesions produced varied greatly from compound to compound and in one case was modified by dose-time relationships. Lesion incidences derived from all compounds are summarized in Table 2. The salient histopathological lesions are discussed below.

Histopathology. The most frequently encountered neoplasms were of vascular endothelial origin. Since the animals were allowed to die or become moribund, the tumors encountered were generally large and destructive. They were multicentric within the liver and metastasized often, mainly to the
Table 1
Mortality of guinea pigs treated with nitrosamines in olive oil

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total dose (mg/kg)</th>
<th>No. of survivors at week: 0 1 0 2 0 3 0 4 0 5 0 6 0 7 0 8 0 9 0 1 0 1 1 0 1 2 0</th>
<th>No. of animals with neoplasms/ no. of animals treated*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitroso-2,6-dimethylmorpholine, 16 mg/ml for 35 wk</td>
<td>1120 (8)</td>
<td>0 20 20 20 20 18 0</td>
<td>20/20</td>
</tr>
<tr>
<td>40 mg/ml for 12 wk</td>
<td>960 (7)</td>
<td>20 17 16 16 14 11 8 5 3 2 0</td>
<td>16/16</td>
</tr>
<tr>
<td>Dinitroso-2,6-dimethylpiperazine, 48 mg/ml for 50 wk</td>
<td>4900 (28)</td>
<td>20 20 20 20 20 20 13 13 13 2 0</td>
<td>20/20</td>
</tr>
<tr>
<td>Nitrosomethylidodecylamine, 100 mg/ml for 40 wk</td>
<td>8000 (35)</td>
<td>20 20 20 20 20 20 13 8 2 2 0</td>
<td>20/20</td>
</tr>
<tr>
<td>Nitrosopentamethyleneimine, 20 mg/ml for 90 wk</td>
<td>3600 (25)</td>
<td>20 20 20 20 36 19 15 6 4 2 2 0</td>
<td>0/20</td>
</tr>
<tr>
<td>Nitrosomethyldihexylurea, 20 mg/ml for 32 wk</td>
<td>1200 (8)</td>
<td>20 20 20 20 14 5 3 0</td>
<td>3/20</td>
</tr>
<tr>
<td>Olive oil control</td>
<td>20 20 20 20 20 20 20 18 11 8 6 6 6 6 0</td>
<td>0/20</td>
<td></td>
</tr>
</tbody>
</table>

* Includes animals with cystic biliary fibroadenosis.

<table>
<thead>
<tr>
<th>No. of animals with liver lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weekly treatment</td>
</tr>
<tr>
<td>Hepato-</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>carbohydrate</td>
</tr>
<tr>
<td>Nitroso-2,6-dimethylmorpholine, 32 mg/kg for 35 wk</td>
</tr>
<tr>
<td>80 mg/kg for 12 wk</td>
</tr>
<tr>
<td>Dinitroso-2,6-dimethylpiperazine, 96 mg/kg for 50 wk</td>
</tr>
<tr>
<td>Nitrosomethylidodecylamine, 200 mg/kg for 40 wk</td>
</tr>
<tr>
<td>Nitrosopentamethyleneimine, 40 mg/kg for 90 wk</td>
</tr>
<tr>
<td>Nitrosomethyldihexylurea, 40 mg/kg for 32 wk</td>
</tr>
<tr>
<td>Olive oil control 2 ml/kg for 90 wk</td>
</tr>
</tbody>
</table>

* One of these sarcomas was producing osteoid and was considered to be osteosarcoma.

R. H. Cardy and W. Lijinsky

Table 2
Neoplasms and neoplasm-like lesions in male guinea pigs treated with nitrosamines in olive oil

l Lung, but also to the spleen, pancreatic lymph node, kidney, and urinary bladder.

The tumors were morphologically variable, with differing patterns of growth seen from one tumor to another and within different areas of the same tumor. In some cases, the pattern was that of a lacy meshwork of anastomosing vascular clefts formed by pleomorphic, elongated, but reasonably well-differentiated neoplastic endothelial cells. Other tumors were decidedly more solid with large, plump anaplastic cells forming the bulk of the tumor and arranged in a pattern giving rise to less obvious but discernible blood-filled vascular spaces. Giant cells, many with bizarre nuclear configurations and abnormal mitotic figures, were common (Fig. 1). Connective tissue stroma was variable in amount and in some cases so abundant as to virtually obliterate the vascular pattern of the tumor.

In some instances, malignant endothelial cells could be seen to proliferate from the lining of sizeable blood vessels. In others, there was a diffuse pattern of proliferation of sinusoidal lining cells, which often surrounded and sequestered groups of hepatocytes which themselves exhibited marked degrees of nuclear and cytoplasmic atypia. Along with this, there was a proliferation of bile ducts and oval cells and, in some cases, adenomatoid hepatocellular hyperplasia (Fig. 2). These areas often blended imperceptibly with vascular elements, so that in any given microscopic field it could be exceedingly difficult to identify a specific cell type of origin (Fig. 3).

Many liver sections showed massive, diffuse, cystic, biliary proliferation and fibrosis which was termed "cystic biliary fibroadenosis." Grossly, when fixed, these livers looked like sponges and on cut surfaces resembled fixed lung tissue. Microscopically, there was a tremendous variation, both cytologically and architecturally, from liver to liver and within different areas of the same liver. There were often tumor-like masses arising from massive areas of cystic ductal proliferation. Some of these tumor-like lesions were well-differentiated papillary cystic structures containing inspissated bile and abundant fibrous stroma, while others were more highly cellular. Associated with these lesions were varying degrees of chronic inflammation, characterized in the most advanced lesions by copious amounts of fibrous-connective tissue (Fig. 4).
In some instances, there was the development of recognizable bile duct carcinomas. These tumors were composed of large, cuboidal to columnar cells arranged in clumps, cords, and papillae and were associated with abundant fibrous connective tissue. In some, the cells were pleomorphic with large pale nuclei, prominent nucleoli, and clumped chromatin. Mitotic figures were numerous, and invasion of surrounding parenchyma was present (Fig. 5).

In the case of animals exposed to dinitroso-2,6-dimethylpiperazine, these cholangiocarcinomas arose in livers that were devoid of cystic biliary fibroadenosis. All others, however, arose within livers severely affected by this massive, neoplastic-like process.

Although the line between hyperplasia and neoplasia is not clearly distinguishable by morphological criteria alone within the genesis of the biliary lesions as seen here, the lesions referred to as biliary fibroadenosis were not reversible but instead progressed following the removal of the test substance. They caused the destruction of large areas of hepatic parenchyma and resulted ultimately in the death of the animals. Histological evidence of cancer was observed in some instances. For these reasons, it is believed that these lesions are representative of the genesis of cholangiocarcinomas as they occur in the strain 2 guinea pig in response to 2 of the compounds tested. Further experimentation will be necessary to establish whether the biological behavior of this proliferative lesion justifies a broad use of the term cholangiocarcinoma.

Since all animals were allowed to die from their disease, animals in the early developmental stages of cystic biliary fibroadenosis were not available for examination; however, microscopic fields that were selected to show a spectrum of stages in the development of the lesions suggest that the early stages were associated with diffuse inflammatory triaditis. There was a brisk portal chronic inflammatory response associated with perportal piecemeal necrosis of hepatocytes and obliteration of the portal-limiting plate (Fig. 6). In the areas immediately adjacent to these portal areas was hepatocellular megalocytosis with nuclear hyperchromicity, atypia, and multinucleated forms.

Eight carcinomas were seen which were of hepatocellular origin. These carcinomas were generally solid, poorly differentiated tumors that were composed of large, anaplastic hepatocytes and showed numerous bizarre cytological and nuclear forms. They were associated with varying degrees of bile duct and Kupffer cell proliferation in adjoining parenchyma. In spite of their histologically malignant nature, none were observed to metastasize. One can speculate that had the animals lived longer some might have metastasized.

In several instances, the proliferative elements within a given lesion were intermixed intimately in such a patternless fashion that the term "complex" was used to describe the lesion.

Pulmonary metastases were usually vascular and anaplastic and ranged from microemboli to large destructive metastatic nodules associated with necrosis and hemorrhage.

There were a few scattered nonhepatic neoplasms in 4 of the treatment groups (Table 2). Two of these were probably related to compound exposure. There were 4 animals with primary lung tumors (2 adenomas and 2 carcinomas) of the 20 animals treated with dinitroso-2,6-dimethylpiperazine. There was a primary CNS tumor seen in one of the 20 animals treated with nitrosomethylidihydrazone.

In addition to neoplasia, the livers of all animals given these 3 compounds showed chronic toxic changes that were characterized by mild to severe alteration of hepatic architecture. There was often diffuse cytomegaly, with great variation in size and shape of hepatocytes, resulting in compression of sinusoids and in some cases the appearance of a pseudolobular pattern. Regenerative nodules were occasionally seen. Nuclear polymorphism was commonly seen with many large atypical and multinucleate forms. In the immediate proximity of tumors, these cells were often bizarre.

**DISCUSSION**

Nitrosamines are well known for their propensity to produce tumors that differ in type and site according to species, route of administration, and dose. Of particular interest in this study was the relationship of chemical structure to target site. It was reasoned that the administration of these 5 compounds might result in the induction of tumors at different sites, as was the case with other rodent species. With the possible exception of a few lung tumors and one CNS tumor, however, the primary target organ system was the liver for the 3 compounds where there was a clearly demonstrated carcinogenic effect.

Although the most common type of tumor encountered in this study as a whole was of endothelial origin, each of the 3 effective carcinogens produced a different spectrum of lesion types, and one of them produced different results with differing dose-time of administration patterns. Whereas the administration of 32 mg/kg/week of nitroso-2,6-dimethylmorpholine for 35 weeks resulted in a 100% incidence of tumors of vascular endothelial origin (including complex), dinitro-2,6-dimethylpiperazine also produced nearly a 100% incidence of vascular tumors and, in addition, produced a number of carcinomas of bile duct and hepatocellular origin. Nitrosomethylidodecylamine, on the other hand, while inducing a high incidence of vascular tumors, produced a 100% incidence of proliferative bile duct lesions (3 of 19 lesions were carcinomas) as its most characteristic effect.

The reasons for these differences are not clearly discernible from the study above. Similar or different metabolic pathways may account for certain similarities or differences in effect. These may be related to chemical structure. In the case of the aliphatic nitrosomethylidodecylamine, most of the liver lesions arose from bile ducts. The same chemical, on the other hand, induces transitional cell carcinomas of the bladder in both rats and Syrian hamsters (1, 10).

The structurally similar compounds nitrosodimethylmorpholine and dinitrosodimethylpiperazine probably have similar metabolic pathways. In rats, both compounds caused esophageal tumors and did so with similar carcinogenic potency. In this study, both dinitrosodimethylpiperazine and nitrosodimethylmorpholine (the latter given at 32 mg/kg/week for 35 weeks) produced tumors primarily of endothelial origin. Nitrosodimethylmorpholine, on the other hand, when given at a higher dose (80 mg/kg/week) for only 12 weeks produced a pattern of tumorigenesis similar to that of nitrosomethylidodecylamine. It would appear that a higher dose rate of that chemical was necessary to produce proliferation or transformation of bile duct epithelium than was necessary to transform endothelium. It seems also that longer continuous administration was necessary to produce angiosarcomas. Whether this is a reflection...
of the time required for malignant transformation of bile duct epithelium compared to hepatocytes or other constituents cannot be determined from this study. Precedent severe diffuse biliary hyperplasia and other toxic effects, however, probably contributed to death regardless of when transformation occurred.

The incidence of spontaneous lung tumors in guinea pigs is extremely low. We believe that in the case of dinitroso-2,6-dimethylpiperazine the incidence of pulmonary neoplasia was probably related to the treatment by the chemical. Induction of lung tumors similar to those seen in this study has been reported with the administration of other chemicals to guinea pigs (5). The relationship is not clear because limited work has been done to separate the effects of spontaneous pulmonary disease conditions of guinea pigs (many of which result in lesions morphologically resembling neoplasia) (4, 5, 12, 18) from the specific effects of administered chemicals. Within all groups of animals in this study, there was a high incidence of inflammatory pulmonary lesions, ranging from abscessation to chronic interstitial proliferative pneumonitis and adenomatoid proliferation of alveolar and bronchiolar epithelium. Squamous metaplasia of bronchiolar epithelium as described with diethylnitrosamine (3) was not seen.

The finding of one CNS tumor in the group of animals treated with nitrosoethylidihydroxyaceta is interesting. This compound is a potent carcinogen for the CNS in rats (9), and the tumor seen in this study, an astrocytoma, is a rare spontaneous finding in guinea pigs; therefore, its occurrence in this group of 20 animals is believed to be related to exposure to the chemical. Survival of this group was poor, mostly because of causes unrelated to neoplasia and contributed to by a general, compound-related lack of resistance to infection. Better survival would have resulted in a clearer indication of whether this compound is tumorigenic for the CNS in guinea pigs.

Nitrosodihydropylethyleneimine, on the other hand, a potent lung and esophageal carcinogen in rats (11) and carcinogenic in Syrian hamsters (6) and in European hamsters (13), induced no tumors in 20 guinea pigs after the administration of 3.6 g/kg (25 mmol/kg as a solution of 20 mg/ml in olive oil) of the chemical in 90 weeks.

Complex hepatic tumors and proliferative reactions have been described previously in guinea pigs given diethylnitrosamine (3). These findings, which were common in this study as well, seem to be a prominent feature of nitrosamine-induced hepatocarcinogenesis in the guinea pig. With few exceptions in this study, metastasizing tumor elements were of vascular origin. The high rate of metastasis of this class of tumors is probably related to the vascularity of the tumors themselves.

Among the 3 carcinogens, nitrosodimethylmorpholine appeared to be the most potent, giving rise to tumors after the administration of a total dose of approximately 7 mmol/kg body weight. Animals treated with the other 2 carcinogenic compounds (at higher dose rates for a longer period) survived longer, although most of them died with tumors; the total dose received by these animals was approximately 30 mmol/kg.

It is not possible to compare with precision the effectiveness as carcinogens of these 3 nitrosamines in the guinea pig, the rat, and the hamster because the route of administration and the doses were different. It appears, however, that the guinea pig is less susceptible to all 3 compounds than are the other 2 species, based on the doses received. It is remarkable that the most responsive organ in the guinea pig was the liver, whereas rat and hamster livers were unaffected by any of the compounds.

The reasons for these pronounced differences in the response of the guinea pig compared with those of the rat and hamster to these nitrosamines are quite unclear but might concern the different pathways of metabolism in the several species. At the doses administered in this study, many guinea pigs died early of liver toxicity and cancer. Were doses reduced or exposure patterns altered to levels less toxic and carcinogenic to the liver, one can speculate that tumors might have been encountered in other organ systems later during the course of the study.

Note Added in Proof


REFERENCES

Fig. 1. Angiosarcoma, anaplastic with many bizarre giant cells. H & E, × 140.

Fig. 2. Bile duct proliferation (arrow) seen adjacent to adenomatoid proliferation of hepatocytes. H & E, × 140.

Fig. 3. Margin of angiosarcoma (arrow) seen in intimate proximity to other abnormal proliferative elements, creating a histologically confusing picture. H & E, × 140.

Fig. 4. Massive bile duct proliferation with abundant connective tissue stroma. H & E, × 140.
Fig. 5. Poorly differentiated, invasive bile duct carcinoma. H & E, x 140.

Fig. 6. Portal area showing chronic inflammation and bile ductular proliferation with necrosis and loss of the portal limiting plate. Hepatic megalocytosis is seen adjacent. H & E, x 140.
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