Carcinogenic Effects of Sequential Administration of Two Nitrosamines in Fischer 344 Rats

C. J. Michejda, M. B. Kroeger-Koepeke, and R. M. Kovatch

ABSTRACT

The carcinogenic effects of sequential treatment of female F344 rats with two nitrosamines were studied. The animals received either methylnitrosourea (NMEA), a strong liver carcinogen, N-nitrosomethylamline (NMA), a moderately strong esophageal carcinogen, or N-nitrosopyrrolidine, (NPyr), a weaker liver carcinogen. The sequentially treated groups were given NMEA followed by NMA and vice versa, NPYr followed by NMEA and vice versa. The dose and duration for each chemical in the sequentially treated groups were identical for the individual treatments. The animals were allowed to die or were killed when moribund. The animals surviving longer than 110 weeks were sacrificed.

The NMEA-NPyr and NPYr-NMEA groups had a tumor spectrum characteristic for NMEA alone (a mixture of hepatic carcinomas and sarcomas with extensive metastases to the lungs). The survival was reduced in the NMEA-NPyr group compared to the NMEA alone group. The time to death of the NMA-NMGA group was not affected by the NMGA treatment, but many of the animals had esophageal neoplasms. The NMEA-NMGA group survival was reduced when compared to the NMEA alone group but the tumor spectrum was dominated by NMEA.

The data indicate that when the target organ is the same, the effect of two nitrosamines is additive with the stronger carcinogen dominating the tumor spectrum. When the target organs are different, the initial exposure influences the tumor spectrum, although the treatment with the second nitrosamine enhances the tumorigenicity of the initial nitrosamine.

INTRODUCTION

Most carcinogen testing has been carried out by treating animals with a single carcinogen, or, in the case of initiation-promotion studies, by a carcinogen followed by a promoting agent. Humans, on the other hand, are probably exposed to an assortment of carcinogens and it is reasonable to assume that the final outcome of human chemical carcinogenesis is some poorly defined function of the sum of carcinogenic insults.

Several groups have recognized a need to assess the effects of exposure to multiple carcinogens, either administered together or sequentially. Thus, in 1959, Odashima (1) observed that exposure to multiple carcinogens, either administered to an assay against of altered cells. Recently, Williams and Furuya (8) showed that DEN and 2-AAF, irrespective of the order of application, enhanced liver carcinogenesis, while phenobarbital given before 2-AAF failed to produce enhancement.

Another aspect of sequential administration of carcinogens was investigated by Habs et al. (9), who treated rats with three different doses of the chemotherapeutic cyclophosphamide-methotrexate-5-fluorouracil regimen. It was shown that the cyclophosphamide-methotrexate-5-fluorouracil regimen produced a carcinogenic response in several organs of the rat and therefore represents a significant carcinogenic risk to the treated patients.

The goals of the experiments described in this paper are: (a) to determine whether the carcinogenic effect of the sequential treatment by two nitrosamines, whose target organ is the liver, was additive, as had been observed with other carcinogens; and (b) to determine whether the additivity principle held if the two nitrosamines had different target organs. Accordingly, we describe the results obtained from sequential administration of NMEA, primarily a liver carcinogen, either before or after the administration of NMA, an esophageal carcinogen, and sequential administration of NMEA either before, or after another liver carcinogen, NPYr.

MATERIALS AND METHODS

All three nitrosamines (NMEA, NMA, and NPYr) were purchased from Sigma Chemical Co. (St. Louis, MO). NMEA and NPYr were found to be >99% pure. NMA was purified by column chromatography on Florisil to remove traces of disoloration.

Female 8-week-old Fischer 344 rats were obtained from the colony of the Frederick Cancer Research Facility. They were bred and housed...
within a barrier. The animals were housed, four to a cage, in plastic cages with wire bottoms. They were fed Rockland rat diets in pellets ad libitum. The 160 rats were divided into groups of 20 animals, seven groups received treatment while one group was an untreated control. The chemicals were administered to the rats in drinking water. Each cage was provided with 80 ml of solution of a nitrosamine (20 ml/rat/day) on each of 5 days of the week. On the other 2 days tap water was given ad libitum to compensate for any water deficit. The treatment protocol for the seven treatment groups is summarized in Fig. 1. At the end of the treatment periods the animals received water and chow ad libitum, as did the control rats. The animals were allowed to die naturally or were killed when found to be moribund. All of the surviving animals were killed at week 110. At death, all of the animals were necropsied. All lesions and major organs and tissues were fixed in formalin, then sectioned and stained with hematoxylin and eosin for histological examination.

Statistical analyses of the data included calculation of survival curves by the Kaplan-Meier method, adjusted for censored observations. The survival curves were compared using Cox's test (10). The probability values quoted in the figure legends are derived from Cox's test. The tumor incidence in the NMA-NMEA and NMEA-NMA groups as a function of age were analyzed by calculating the cumulative age-specific prevalence rates (11).

RESULTS

Three groups of 20 animals each were treated with each of the test chemicals alone. The animals treated with NMA survived about as long as the untreated controls, while the NPy treated animals had slightly reduced survival when compared to controls. The NMA treated animals received a total dose of 100 mg/animal and the NPy treated animals received 270 mg/animal. In contrast, the animals treated with NMEA (total dose of 120 mg/animal) had a significantly reduced survival time when compared to the untreated controls (mean survival time was about 45 weeks). Histopathological examination showed that these animals died with neoplasms similar to those described by Lijinsky and Reuber (12) (Table 1). The dose chosen for NMEA in the present study was somewhat lower than in the previous work (12), and the duration of treatment was 15 instead of 30 weeks. The intent was to achieve a dose which would not produce malignant tumors within 100 weeks. However, the chosen dose gave rise to a high incidence of tumors. The doses chosen for NMA and NPy were significantly less effective in inducing cancers within the first 100 weeks of treatment. The NPy dose was about the same as that reported by Lijinsky and Reuber (13), but it was given for 30 instead of 50 weeks. The dose of NMA was equivalent to the dose used in our earlier carcinogenesis studies (14), but the animals were treated for 20 instead of 50 weeks. All of the NMA and NPy treated animals were examined histopathologically. The NMA treated animals had tumors typical for an aging F344 rat (15) (Table 1), while the NPy treated animals had those tumors as well as hepatocellular carcinomas (Table 1). These animals survived almost as long as did the untreated controls. The sequential administration involved 4 groups of 20 rats each, as denoted in Fig. 1. The animals in the NMEA-NMA group all died with malignant neoplasms. The survival curves of this group, for the NMEA alone group, and the untreated controls are shown in Fig. 2. The sequentially treated animals survived for a shorter time than those treated with NMEA alone. The tumor incidence of this group and the reverse treatment group (NMA-NMEA) is shown in Table 1. It can be seen that the tumor incidence of the NMEA-NMA group is similar to that of the NMEA alone group, except that the combined group produced a few esophageal carcinomas which were absent in the NMEA alone group. The tumors induced by NMEA, at the dose used, were mainly hepatocellular carcinomas and hemangiosarcomas of the liver. These tumors were usually aggressive and frequently metastasized into other organs, particularly the lung (13). NMEA at higher doses also induces some esophageal tumors, but in the present study they were usually limited to relatively small squamous cell papillomas. NMA, on the other hand, exclusively induces tumors of the upper gastrointestinal tract, particularly the esophagus (14). It is interesting to note that 3 animals in the NMEA-NMA group had squamous cell carcinomas of the esophagus, along with hepatic tumors; no esophageal carcinomas were found in the NMEA alone group.

Fig. 2 also shows the survival curve of the group treated first with NMA followed by NMEA. These animals had a shorter survival time than those treated with NMA alone, but this time was considerably longer than that of the NMEA-NMA group. All of the animals died with malignant tumors, the incidence of which is shown in Table 1. It is readily seen that these animals had more malignant esophageal tumors than did the NMEA-NMA group, and somewhat fewer liver tumors. The major differences between these two groups are best seen by comparing the tumor incidence of the individual animals as a function of time to death. The salient features, shown in Table 2, are that all of the animals in the NMEA-NMA group had hepatic tumors, with malignant tumors of the esophagus appearing later. The NMA-NMEA animals, on the other hand, died primarily from esophageal tumors in roughly the first one-half of the animals that died, while the hepatic tumors became a more important cause of death in the other half. It should be emphasized that the first named tumor in Table 2 for each animal was discerned to be the principal lesion leading to death.

The third and fourth groups of sequentially treated rats received NMEA, at the same dose as in the other groups, and NPy was given at the same dose and for the same duration as in the group treated with NPy alone. The animals which were first treated with NMEA followed by NPy had a significantly shorter survival time than those treated with NMEA alone (Fig. 3). The tumor distribution in that group, shown in Table 1, is similar to that for NMEA alone. The reverse treatment, NPy followed by NMEA, also resulted in all of the animals dying with tumors, but the mean time to death was longer than in the NMEA-NPy group (60 weeks versus 37 weeks). The animals, however, had significantly reduced survival as compared to those treated with NPy alone (Fig. 3). The tumor spectrum of the NPy-NMEA group was virtually identical to that in the NMEA-NPy group (Table 1).
Table 1 Tumor incidence in sequentially treated groups (20 female F344 rats per group)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total no. of tumor bearing animals</th>
<th>Liver</th>
<th>Esophagus</th>
<th>Metastases of liver tumors to lung</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HC*</td>
<td>HS</td>
<td>NN</td>
<td>CC</td>
</tr>
<tr>
<td>NMEA-NMA</td>
<td>20</td>
<td>1 14</td>
<td>14 13</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>NMA-NMA</td>
<td>20</td>
<td>10 6</td>
<td>10 9</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>NPyr-NMA</td>
<td>20</td>
<td>19 14</td>
<td>5 1</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>NMEA-NPyr</td>
<td>20</td>
<td>20 15</td>
<td>9</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>NMEA</td>
<td>20</td>
<td>12 10</td>
<td>8 5</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>NMA</td>
<td>18</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>NPyr</td>
<td>20</td>
<td>20 6</td>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Control</td>
<td>19</td>
<td>1</td>
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</tr>
</tbody>
</table>

*HC, hepatocellular carcinoma; HS, hemangiosarcoma; NN, neoplastic nodule; CC, hepatocellular-cholangiocellular carcinoma; SCC, squamous cell carcinoma; SCP, squamous cell papilloma; TCC, tubular cell carcinoma; TCA, tubular cell adenoma.

**Metastatic sarcomas in the mesentery (1); spleen (1) and esophagus (1); kidney sarcoma (1); adrenal sarcoma (1); and mononuclear cell leukemia (1).**

***Forrestomach SCP (1); trachea SCP (1); tongue SCP (2) and SCC (1); hard palate SCC (1); oral pharynx SCC (1); adrenal pheochromocytoma (1); and mononuclear cell leukemia (4).***

****Adenocarcinoma of the ileum and metastatic HC in the pancreas.****

*****Metastatic HS of the kidney (1); adrenal pheochromocytoma (1); HS of pancreas (1); Forrestomach SCP (1); and metastatic ovarian carcinoma (1).*****

******Mononuclear cell leukemia (2); SCP Forrestomach (1); metastatic HS spleen (1); and metastatic HS mesentery (1).******

*******Tongue SCC (1); anterior pituitary adenoma (7); brain astrocytoma (2); cerebellar meninges fibrosarcoma (1); adrenal medulla pheochromocytoma (3); mammary fibroadenoma (8); leukemia (1); thyroid adenoma (1); skin fibroma (1); uterus hematoma (2); adrenal cortex carcinoma (1); adrenal cortex adenoma (1); thymus SC (1); mandibular lymph node C (1); and Zymbal's gland carcinoma (1).*******

********Adrenal medulla pheochromocytoma (8); leukemia (9); brain carcinoma (1); anterior pituitary carcinoma (1); anterior pituitary adenoma (2); adrenal cortex adenoma (1); oral mucosa soft palate SCC (1); tongue SCP (1); lung mesothelioma (1); peritoneal mesothelioma (1); thyroid follicular cell adenoma (1); uterine adenoma (1); pancreas HS (1); and mesentery HS (1).********

**********Pituitary adenoma (13); pituitary carcinoma (1); leukemia (4); adrenal medulla pheochromocytoma (1); mammary fibroadenoma (7); adenocarcinoma (1); skin fibroma (1); skin hemangiosarcoma (1); subcutis fibroadenoma (1); subcutis hemangioma (1); spleen sarcoma (1); Forrestomach squamous cell papilloma (1); thyroid follicular cell carcinoma (1); thyroid C-cell adenoma (3); and lung follicular cell carcinoma (1).**********

— absence of tumors.

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Fig. 2. Kaplan-Meier survival curves for NMEA alone, for NMEA followed by NMA, for NMA alone, for NMA followed by NMEA, and for untreated controls. The difference between the NMEA followed by NMA group and the NMEA group was significant (P < 0.03). The difference between the NMA alone group and the NMA followed by NMEA group was significant (P < 0.001). The difference between the untreated control group and the NMA alone group was not significant (P > 0.15). The surviving control animals were sacrificed at week 110.

**DISCUSSION**

Over the last 30 years, the carcinogenicity of many nitrosamines has been examined. However, no experiments involving sequential administration of more than one nitrosamine have been carried out in order to assess the possibility of either additivity or synergism of the two compounds. To this end, we examined the effect first of two liver carcinogens and then of a liver and an esophageal carcinogen.

Sequential treatment of the rats with the two liver carcinogens, NMEA and NPyr, produced tumors which were characteristic of NMEA, irrespective of whether NPyr treatment preceded or followed the NMEA treatment. These were mainly highly aggressive epithelial and endothelial tumors of the liver, which frequently metastasized to the lungs and other organs. The animals died, often from massive bleeding caused by the rupture of the blood vessels in the endothelial tumors, or of the metastatic lesions in the lung. NPyr, on the other hand, produces predominantly hepatocellular carcinomas and a few cholangiocarcinomas in the Fischer 344 rats (13) (Table 1).

According to a classification system of Habs and Schmähl (16), the NPyr-NMEA treatment can be termed an independent additive risk, in agreement with earlier results obtained from sequential treatment of F344 rats with 2-AAF and DEN (6, 8). The mechanism of this additive effect remains unclear; however, it is clear that the more powerful carcinogen, NMEA, had a dominant effect on the tumor spectrum, even when the first carcinogen to which the animals were exposed was NPyr. The combination of the two chemicals elicited a greater carcinogenic response than did either of the two chemicals alone. This is readily seen in the survival curves of the NMEA-NPyr group (Fig. 3), but is less readily apparent from the survival curve of the NPyr-NMEA group (Fig. 3). In order to make a comparison between the two groups more readily apparent, the NPyr-NMEA survival curve was shifted down the abscissa by 31 weeks. This period corresponded to the duration of NPyr treatment plus 1 week. This curve is shown in Fig. 4, together with the survival curve of the animals treated with NMEA alone. These curves suggest that the NPyr-NMEA treatment was significantly more effective than was the NMEA treatment alone.

The survival curve for the group treated with NMEA first, followed by NMA (Fig. 2), indicates that the combined treatment reduced survival as compared to the NMEA alone treatment. The tumors produced by this combined treatment were similar to those produced by NMEA alone (Table 1), except that three animals developed squamous cell carcinomas of the esophagus, which were absent in the group treated only with NMEA. NMA is readily metabolized by rat liver enzymes (17, 18); however, it is possible that NMEA pretreatment could result in a decrease in the metabolism of NMA in the liver so...
that more of this carcinogen reaches the esophagus, in a manner
similar to the effect of ethanol on the metabolism and toxicity
of N-nitrosodimethylamine (19). No liver tumors have ever
been observed in rats treated with NMA (14, 20, 21), but it is
clear from these data that NMA is able to enhance the carcin-
genicity of NMEA in that organ, possibly by synergistically
enhancing the growth of the NMEA altered liver cells.

The rats treated with NMA first, followed by NMEA (Fig.
2), did not survive as long as the rats treated with NMA alone.
The latter, in fact, lived almost as long as the untreated controls,
and the differences between the NMA treated group and the
controls were not statistically significant. The NMA-NMEA
group had a mean survival time of 70 weeks, considerably longer
than the 33-week mean survival time following the reverse
treatment (Fig. 2). When the survival curve for the NMA-
NMEA group was adjusted by shifting it down the abscissa by
21 weeks (duration of the NMA treatment plus 1 week) in a
manner similar to that shown in the NPyr-NMEA curve, and
the result was compared with the survival curve of NMEA alone
(Fig. 4), it was clear that the NMA treatment did not diminish
the survival of the rats more than the effect produced by NMEA
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ACKNOWLEDGMENTS

We are especially grateful to Dr. Shu-Mei Guo for the statistical analysis of the data, and to Barbara Thomas for her expert assistance with the animal experiments.

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