SUMMARY

From the response of Sprague-Dawley rats to (a) aminopyrine, sodium nitrite, and CCl₄; (b) aminopyrine and sodium nitrite only; or (c) dimethylnitrosamine only, it was determined that the combination of aminopyrine, sodium nitrite, and CCl₄ is highly hepatotoxic and cirrhogenous and that this combination of chemicals induces hepatocellular tumors. Of 18 animals given these 3 compounds, 6 developed hepatocellular tumors, 9 developed hemangioendothelial sarcomas (Kupffer cell sarcomas) of the liver, and all developed severe postnecrotic cirrhosis. All animals that received aminopyrine and nitrite or dimethylnitrosamine without CCl₄ developed only the vascular tumors of the liver. The absence of hepatocellular tumors after administration of dimethylnitrosamine differed from the response usually ascribed to this chemical. Gross and microscopic characteristics of hepatocellular and hemangioendothelial tumors were examined and compared.

INTRODUCTION

The carcinogen produced from AP² and nitrite is DMN (7, 8), and studies at this laboratory have shown that the combination of AP and nitrite in drinking water produces a high incidence of hemangioendothelial sarcomas in the livers of rats (9). No other type of tumor was produced and no other primary tumor location was observed, although others using DMN have reported both hepatocellular and hemangioendothelial tumors in rat liver (2—5, 10, 11, 13). Since these tumors of endothelial origin occurred only in the liver, while endothelium is present throughout almost all tissues of the body, it seemed that the liver might play some role in the genesis of these sarcomas and that alteration of liver function as well as mitotic rate might influence the type of tumor produced by AP and nitrite.

Carbon tetrachloride (CCl₄) provides a means by which hepatocellular mitosis can be increased and liver function can be altered, and it has been used to enhance liver tumor formation and chromosomal aberrations by carcinogenic and mutagenic agents (1, 4, 11, 12). CCl₄, when given alone, has not been shown to produce hepatocellular tumors in rats (11). Our experiments used the concurrent administration of CCl₄ with AP and nitrite only. We also administered DMN directly to rats for comparison.

MATERIALS AND METHODS

Experimental animals were Sprague-Dawley female rats born in a closed colony at the Biology Division and maintained under specific-pathogen-free conditions. The animals were housed 3 to a cage and fed Purina laboratory chow ad libitum. A solution of AP and sodium nitrite, each at a concentration of 1000 mg/liter of water, was given in 20-ml aliquots per rat as drinking solution 5 days/week for 30 weeks to a group of 18 rats. DMN at a concentration of 40 mg/liter was given as drinking solution to a 2nd group of 18 rats for the same period. The group that received AP and nitrite in drinking water was also given CCl₄ i.g. as 0.2 ml of CCl₄:olive oil (1:1) once every 2 weeks for the 30-week period. A 3rd group of 12 animals received CCl₄ only, at the same dosage and schedule. Two animals from this last CCl₄ group were killed at 6, 12, 18, and 30 weeks of the experiment 72 hr after their last dose of CCl₄ and 4 animals were killed at 68 weeks.

Animals from an earlier experiment (9), which used only AP and nitrite at the same dosage and schedule as this experiment, served as controls. Treatment on all groups started when the animals were approximately 10 weeks old.

Tumors were discovered at the time of postmortem examination, which were performed on animals immediately after natural death or, in the group that received CCl₄ only, immediately after they were killed. Microscope slides were prepared from paraffin-embedded tissues, using hematoxylin and eosin stains.

RESULTS

The combination of AP, nitrite, and CCl₄ produced a severe degree of postnecrotic cirrhosis in the 18 animals tested, and all animals were dead after 35 weeks (Table 1). Five of the rats that died before 25 weeks had no tumors, and death was attributed to cirrhosis and liver failure. Nine animals in the group developed hemangioendothelial sarcomas (Kupffer cell sarcomas) of the liver and 6 animals developed hepatocellular tumors. This included 2 animals with both types of tumors. Six of the 9 animals with endothelial tumors of the liver had metastatic foci of these tumors in the lungs, but no

1 This research was jointly sponsored by the Chemical Carcinogenesis Program of the National Cancer Institute and by the U.S. Atomic Energy Commission under contract with the Union Carbide Corporation.

2 The abbreviations used are: AP, aminopyrine; DMN, dimethylnitrosamine; i.g., intragastric.

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hepatocellular metastases occurred. Hemangioendothelial sarcomas were similar to those produced in the previous experiment (9), in which metastasis to the lung occurred in 10 of 15 tumor-bearing animals. Table 1 shows survival patterns and numbers of tumors of each type.

All 18 animals that received DMN developed Kupffer cell sarcomas of the liver and 12 of these had metastatic tumor foci in the lungs. There were no hepatocellular tumors in this group and no liver cirrhosis was observed. Survival and tumor incidence is shown in Table 1.

Mitosis was increased in the livers of animals that received CCl₄ only and that were killed 72 hr after their last dose. Up to 20 mitotic figures per high-power field were seen, and this was sometimes accompanied by slight cirrhotic fatty change. No lesions were seen in the livers of animals that were killed at 68 weeks of the experiment.

At postmortem examination, the animals with cirrhosis were icteric and anemic. Ascites, hydrothorax, and s.c. edema were constant features. The livers were nodular and fibrotic and cut with increased resistance. Endothelial tumors were seen grossly as thin-walled, multilocular, blood-filled cysts. Some contained organized clots and thrombi, and others contained unclothed blood (Fig. 1). Small tumors appeared as dark red nodules with umbilicated surfaces. These growths ranged in size from 2 mm to 2 cm and projected from both diaphragmatic and visceral surfaces of the liver. They were commonly attached to the diaphragm, the greater and lesser omentum, and the mesentery of the duodenum and colon. Metastatic foci were seen only in the lung and appeared as 1- to 5-mm dark red nodules, randomly scattered throughout the parenchyma. Only rarely was a large part of a lobe affected (Fig. 2).

Microscopically, the vascular tumors first appeared as proliferative foci of endothelial cells in sinusoids (Fig. 3). As tumors progressed, sinusoids became dilated and filled with tumor cells, with loss of normal parenchyma and recognizable liver architecture. Connective tissue stroma became visible in the tumors, and clefts and cysts that resembled new blood vessels were formed. These were filled with blood and lined with anaplastic endothelium (Fig. 4). The lining cells of some clefts were large and polyhedral with basophilic cytoplasm and large vesicular nuclei that contained fine strands of chromatin and large, sometimes multiple, nucleoli. In other areas, the tumors assumed a more sarcomatous nature, infiltrating along sinusoids and isolating small groups of hepatocytes (Fig. 5). The association with blood-filled cysts was a constant feature throughout the tumors. Many such vascular spaces were lined by projecting fronds, which consisted of hepatocytic cords enveloped by endothelial cells (Fig. 6). The lumina of these cavities often contained laminated thrombi and islands of large anaplastic cells.

Metastatic foci in lungs were first seen as free tumor cells in the lumina of pulmonary arterioles. More advanced tumors surrounded blood vessels and established themselves in the lung parenchyma. These metastatic growths formed "pseudo-vessels" filled with blood and lined by the large anaplastic endothelial cells (Fig. 7). Occasionally, these tumors also assumed a sarcomatous growth pattern.

Both macroscopically and microscopically, hepatocellular tumors were difficult to distinguish from hyperplastic nodules in the cirrhotic livers, and in some areas this distinction was impossible. Hepatocellular tumors more closely resembled normal liver, except that they were lighter in color, fatty, and friable. They lacked the blood-filled character of endothelial tumors. Microscopically, these tumors consisted of recognizable hepatocytes that grew in an array of trabecular patterns. Normal architecture was lacking, and triads and central veins were absent. Malignant hepatocytes were usually laden with fat vacuoles, which gave them a lacy, foamy appearance (Fig. 8). No metastases of hepatocellular tumors were seen.

**DISCUSSION**

The combination of AP, nitrite, and CCl₄ induced hepatocellular tumors, which were not observed with DMN treatment alone. Because of the difficulty of accurately distinguishing hepatocellular tumors from nodular hyperplasia in the cirrhotic livers, it is probable that the incidence of these tumors was greater than indicated. Tumors counted were those that were most obvious; questionable areas were not included. The addition of CCl₄ also proved to be extremely hepatotoxic.

**Table 1**

*Survival patterns and tumor incidences in Sprague-Dawley female rats receiving AP, nitrite, carbon tetrachloride, and DMN in drinking water*

Animals were given AP and DMN in drinking water over a 30-week period, and carbon tetrachloride was administered by i.g. intubation at 2-week intervals for this period. Tumors were discovered at necropsy examination and were determined by gross and microscopic examination of tissues.

<table>
<thead>
<tr>
<th>Treatment (30 wk)</th>
<th>No. of animals on test</th>
<th>Survivors at below wk after initiation of treatment</th>
<th>No. of animals with tumors of</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Kupffer cells</td>
</tr>
<tr>
<td>AP, sodium nitrite, and carbon tetrachloride</td>
<td>18</td>
<td>18 16 15 13 5 2 0</td>
<td>6 9</td>
</tr>
<tr>
<td>AP and sodium nitrite</td>
<td>15</td>
<td>15 15 15 15 11 9 4 2 1 0</td>
<td>15</td>
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<td>DMN</td>
<td>18</td>
<td>18 18 18 18 18 6 2 0</td>
<td>18</td>
</tr>
</tbody>
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* a All animals were allowed to die naturally.
* b Two animals had tumors arising from both Kupffer cells and hepatocytes.
* c Data from a previously reported experiment (9).
and cirrhogenous. AP and nitrite in earlier studies (9) and DMN or CCl₄ alone in this study produced no cirrhosis.

The fact that DMN did not produce hepatocellular tumors is not consistent with commonly reported results. The difference in response could be due to a number of variables, such as strain of rat, influence of different diets, immediate and cumulative doses, and life-span after receiving DMN. This disparity may be due to differences in diagnostic interpretation of these tumors. Perhaps better documentation of tumors reported in future literature would be in order. Certainly, there should be some unanimity reached on classification of these tumors of liver origin, especially in view of the many metabolic studies involving DMN.

From our studies, it appears that the Kupffer cells are more responsive to the action of DMN than are liver cells themselves; however, hepatocytes did respond to the carcinogenic stimulus in the presence of CCl₄. The inducement of mitosis in liver cells by CCl₄ provides a situation not unlike that reported in many instances where carcinogenesis is greatly enhanced by mitotic activity of target cells (3, 14). Besides alteration of mitotic states, the effect of CCl₄ on liver cell enzymes and membranes that influence the metabolism of DMN is no doubt of great importance also in the initiation of these tumors.

The fact that Kupffer cells became malignant, while other endothelial and phagocytic cells elsewhere in the body did not, may be due to the exposure of the former cells to high concentrations of DMN in the portal circulation after intestinal absorption. DMN in circulating blood would be diluted after mixing with blood from the anterior and posterior vena cavae before reaching capillary beds in other major organs. The greater response of Kupffer cells could also be due to metabolism of DMN in nearby hepatocytes. Some unidentified immediate carcinogen may be produced, which the Kupffer cells could receive by pinocytosis from intact liver cells or by phagocytosis of debris from dead or dying liver cells. Unfortunately, little is known about the metabolism of DMN in Kupffer cells or about the metabolic interrelations between hepatocytes and Kupffer cells. Certainly, in view of our work, this is an area that needs further study.

REFERENCES

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Altered Tumor Response by CCl₄

4. Liver from rat that received DMN. Formation of “pseudovessel” lined by multilayered anaplastic endothelium. H & E, x 500.
5. Liver from rat that received DMN. Sarcomatous growth pattern of endothelial tumor. Vascular spaces are apparent at bottom. H & E, x 500.
6. Liver from rat that received DMN. Kupffer cell tumor in lung parenchyma that formed a vessel partially lined by anaplastic cells. Arrows outline 1 vessel. H & E, x 500.
7. Lung from rat that received DMN. Kupffer cell tumor in lung parenchyma that formed a vessel partially lined by anaplastic cells. Arrows outline 1 vessel. H & E, x 500.
8. Liver from rat that received AP, nitrite, and CCl₄. Hepatocellular tumor composed of fat-laden cells that grew in disorganized arrangement. H & E, x 500.
Altered Tumor Response by CCl₄
Alteration of Tumor Response in Rat Liver by Carbon Tetrachloride


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