The Development of Carcinoma in Liver of Rats Treated with m-Toluylene diamine and the Synergistic and Antagonistic Effects with Other Chemicals

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SUMMARY

Carcinoma of the liver with invasion and metastases has been observed in rats fed diets containing 0.1 or 0.06 percent m-toluylene diamine. Histologically all the tumors were hepato-cellular carcinomas closely resembling those following the administration of other hepatic chemical carcinogens. The simultaneous administration of DL-ethionine had a synergistic effect with m-toluylene diamine on liver tumor formation. In contrast, cancer induction was completely prevented by the concomitant feeding of 3-methylcholanthrene, alpha-naphthyl isothiocyanate, or p-hydroxypropiophenone and m-toluylene diamine.

INTRODUCTION

In 1937, Nagata (22, 23) reported that rats fed a diet containing m-toluylene diamine for about 170 days developed no hepatomas. Recently, subcutaneous injections of mTD into rats were observed by Umeda (35) to lead to one hundred percent development of sarcomata in surviving rats. However, no notable changes in internal organs and, especially, no liver cirrhosis and no hepatoma were found in these rats.

In the present study, long-term observations on rats fed with mTD revealed the induction of hepatic carcinomas. The effects of DL-ethionine, 3-MC, ANI, and PHP on that of mTD were also examined. The results of these studies are reported here.

MATERIALS AND METHODS

Wistar strain male rats (Fuji Animal Farm), weighing from 150 to 180 gm, were used.

First Experimental Series

Thirty rats were divided into three groups of 12, 12, and 6 rats respectively. Group 1 was fed the semisynthetic basal diet described previously (12) and composed of polished rice powder, 75 percent; casein, 10 percent; salt mixture #2 (Nutritional Biochem. Co.), 4 percent; corn oil, 10 percent; vitamin mixture, 1 percent; supplemented with 0.1 percent mTD. Group 2 was fed the same basal diet supplemented with 0.06 percent mTD, and Group 3 was kept as control on the basal diet. The mTD was purchased from The Matheson Coleman & Bell Co. Inc.

Second Experimental Series

Eighty-seven rats were divided into 8 groups. These groups of rats were fed various combinations of mTD, ethionine, basal, or stock diets as outlined in Table 3. The stock diet was from Oriental Food Co., and ethionine was obtained from Calbiochem.

Third Experimental Series

Eighty-one rats were divided into 8 groups. These rats were fed various combinations of mTD, 3-MC, ANI, or PHP as outlined in Table 5. The 3-MC was purchased from Wako Pure Chem. Ltd., the AN! from Tokyo Kasei Chem. Co., and the PHP from Eastman Organic Chem.

Animals were housed three to a cage in an air-conditioned room kept at 24°C and were weighed once a week. The animals of the first and third experimental series were fed the experimental diets for about 35 weeks. Those of the second experimental series were fed the experimental diets for 20 weeks followed by about 4 weeks of stock diet. The animals were observed until death or they became moribund, at which time they were killed by ether. All experimental animals were necropsied and examined for tumors and metastases. The liver of each animal was weighed, and pieces taken for histologic study. The tissues were fixed in Stieve's solution and in 10 percent formaldehyde solution. All tissues were stained with hematoxylin and eosin, and selected tissues were stained with Mallory's, van Gieson's, and periodic acid-Schiff stains.

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1 The following abbreviations are used: mTD, m-toluylene diamine, 2,4-toluenediamine; DL-ethionine, ethionine; 3-MC, 3-methylcholanthrene; ANI, alpha-naphtyl isothiocyanate; PHP, p-hydroxypropiophenone; 3'-Me-DAB, 3'-methyl-4-dimethylaminoazobenzene; 2-AAF, 2-acetylaminofluorene; DAB, p-dimethylaminoazobenzene.
RESULTS

First Experimental Series

Growth Curves. Animals on diets containing mTD showed much less weight gain than control animals (Chart 1). They ceased to gain weight between the 4th to 8th week, and then their weight gradually decreased until they died or were killed.

Gross Findings. A summary of the mean survival periods and changes in body weight of animals in the three groups in the first series are shown in Table 1. In the first group, all 9 rats maintained on the 0.1 percent mTD diet had multiple neoplasms in the liver measuring from 1 to 4 cm in diameter. The tumors were yellowish and contained many areas of hemorrhage. Six of these 9 rats had multiple metastatic tumors (Fig. 1) in the lymph nodes, omentum, lungs, and epididymis. They also had multiple areas of nodules in the liver. In the second group, 7 of the 11 rats had grossly visible multiple liver neoplasms measuring from 0.5 to 1.0 cm in diameter. The tumors were yellowish and showed areas of metastases in the lymph nodes and omentum. The livers of the control rats on the basal diet without mTD were essentially normal.

Microscopic Findings. A summary of microscopic findings in this series is shown in Table 2. In rats on diet supplemented with mTD, oval cell infiltration in the periportal areas, fatty changes of the liver parenchymal cells, bile duct proliferation, cirrhosis, nodular hyperplasia, cholangiofibrosis, and hepatic carcinoma were found. Areas of hepatic carcinoma presented the appearance of hepatocellular carcinoma (Fig. 2). Most of the areas of cancer were discrete, and not encapsulated, and many showed invasion of the surrounding liver tissue. Vascular invasion was frequently seen. Most of the cords in the hepatocellular carcinoma were two or more cells thick (Fig. 3). The nuclei of tumor cells were prominent and were often multiple. Most of the tumor cells showed greater cytoplasmic basophilia than non-neoplastic liver cells. Mitotic figures were frequent in all tumor areas. In the non-neoplastic portion of the liver, there was extensive proliferation of oval cells with differentiation to bile duct epithelium. Numerous areas of nodular hyperplasia were observed (Fig. 4) in the liver. Areas of cholangio-
fibrosis were also present in the liver (Fig. 5). Occasionally, foci of extramedullary hematopoiesis were seen in the sinusoids of the liver. Metastatic areas in lymph nodes, omentum, epididymis (Fig. 6), and lung (Figs. 7, 8) showed the same histologic pattern of hepatocellular carcinoma with large irregular nuclei and many mitotic figures. Animals treated with mTD had no primary neoplasms in organs other than the liver.

Second Experimental Series

Body Weight Changes. The mean survival in each experimental group and the average body and liver weights of the eight groups are summarized in Table 3. The livers of the animals fed on diets containing mTD or ethionine showed more weight gain than those of groups fed mTD alone.

Liver Changes. In Group 1, no hepatoma was found. The livers of the animals in this group showed some oval cell infiltration and 3 of the organs had nodular hyperplasia. In Group 2, a few animals had oval cell infiltration in the periportal areas. One animal had nodular hyperplasia, but no hepatoma was seen. In Group 3, 5 of the 15 animals had hepatocellular carcinoma, but no animal in this group had any metastasis. The livers of some animals in this group showed moderate to marked degree of oval cell infiltration. In one rat there was a small area of atypical cells wholly within the confines of a large nodular hyperplasia, but no atypical areas were found outside the nodule of this liver. Four animals had areas of cholangiofibrosis in the liver. In Group 4, 4 of the 11 rats had hepatocellular carcinoma. Almost all the rats in this group had oval cell infiltration in the liver. In Group 5, no hepatoma or cirrhosis was seen. The livers of all rats had oval cell infiltration and one rat had nodular hyperplasia. In Group 6, no hepatoma or cirrhosis was seen. All rats had oval cell infiltration and two rats had nodular hyperplasia. In Group 7, no hepatoma was seen. All animals in this group had many nodular hyperplasia and cirrhosis. In Group 8, 6 of the 12 rats had hepatocellular carcinoma. Numerous areas of nodular hyperplasia were found in the livers of all rats in this group, and 2 of the 6 animals had extensive intraperitoneal seeding of metastatic carcinoma. The liver of all rats on diets containing mTD showed fatty changes in liver parenchymal cells. The histologic findings in livers in the second experimental series are summarized in Table 4.

Third Experimental Series

Body Weight Changes. The mean survival in this series and the average changes in body and liver weights of rats are re-
recorded in Table 5. The body weight of rats on diets supplemented with PHP alone increased markedly. All rats receiving mTD lost weight. However, the increase in liver weight in the group on diet supplemented with mTD only is noteworthy; an even greater increase in liver weight was noted in animals receiving mTD plus 3-MC, ANI, or PHP.

**Liver Changes.** The histologic changes and incidence of cancer in the livers of animals on the various diets are recorded in Table 6. 3-MC, ANI, or PHP completely prevented induction of cancer by mTD under the conditions of the present study. Histologically, 3-MC, ANI, and PHP slightly reduced oval cell infiltration. However, these compounds did not prevent the fatty changes of the liver parenchymal cells induced by mTD (Figs. 9–12). Numerous areas of nodular hyperplasia were seen in the group given mTD. A few areas of nodular hyperplasia were seen in the group on 3-MC or ANI and mTD.

**DISCUSSION**

This study confirms that experimental liver cancer can be induced in rats by mTD. Histologically, all the liver tumors seen after mTD administration were hepatocellular carcinomas. The histologic characteristics of the hepatic cell carcinomas were similar to those seen with ethionine (4–7), DAB (13, 16, 34), 3′-Me-DAB (8, 18, 34), or 2-AAF (33, 36). In the present investigation, the incidence of cancer in rat liver was

### Table 4

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet schedule (weeks)</th>
<th>Histopathologic findings of liver</th>
<th>Cancer (at 25 weeks)</th>
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<tr>
<td></td>
<td>4</td>
<td>8</td>
<td>12</td>
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<tr>
<td></td>
<td>Oval cells</td>
<td>Cirrhosis</td>
<td>Cholangiofibrosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
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</tr>
<tr>
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<td>+</td>
<td>−</td>
</tr>
<tr>
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<tr>
<td>8</td>
<td>ST Eth Eth</td>
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<td>+</td>
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</table>

Histopathologic findings in rats treated with ethionine and mTD. mTD, basal diet with 0.1% m-toluylenediamine; BD, basal diet; St, stock diet; Eth, basal diet with 0.25% ethionine.

### Table 5

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats</th>
<th>Av. duration of expt. (weeks)</th>
<th>Body weight (gm)</th>
<th>Liver</th>
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<td></td>
<td>Initial</td>
<td>Final</td>
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<td>32.2 ± 4.3</td>
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<td>206.3</td>
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<tr>
<td>3-MC</td>
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<td>36.0 ± 0.0</td>
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<tr>
<td>ANI</td>
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<td>36.0 ± 5.0</td>
<td>160.0</td>
<td>388.3</td>
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<tr>
<td>PHP</td>
<td>11</td>
<td>36.2 ± 0.8</td>
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<td>543.5</td>
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<tr>
<td>mTD + 3-MC</td>
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<td>33.7 ± 7.3</td>
<td>152.4</td>
<td>203.6</td>
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<tr>
<td>mTD + ANI</td>
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<td>217.2</td>
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<tr>
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<td>35.0 ± 3.0</td>
<td>165.4</td>
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<tr>
<td>Control</td>
<td>6</td>
<td>36.0 ± 0.0</td>
<td>157.5</td>
<td>403.8</td>
</tr>
</tbody>
</table>

Changes in body and liver weights in rats treated with mTD, 3-MC, ANI, and PHP. mTD, basal diet with 0.1% m-toluylenediamine; 3-MC, basal diet with 0.0067% 3-methylcholanthrene; ANI, basal diet with 0.1% alpha-naphthal isothiocyanate; PHP, basal diet with 1.0% p-hydroxypropiophenone.

### Table 6

<table>
<thead>
<tr>
<th>Group</th>
<th>Histopathologic findings in liver</th>
<th>Cancer (at 36 weeks)</th>
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</thead>
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<td>Fatty changes</td>
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</tr>
<tr>
<td>3-MC</td>
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<td>−</td>
</tr>
<tr>
<td>ANI</td>
<td>+++</td>
<td>−</td>
</tr>
<tr>
<td>PHP</td>
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<td>mTD + ANI</td>
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<tr>
<td>mTD + PHP</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Control</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

Histopathologic findings in rats treated with mTD, 3-MC, ANI, and PHP. mTD, basal diet with 0.1% m-toluylenediamine; 3-MC, basal diet with 0.0067% 3-methylcholanthrene; ANI, basal diet with 0.1% alpha-naphthal isothiocyanate; PHP, basal diet with 1.0% p-hydroxypropiophenone.
higher on treatment with 0.1 percent mTD than on treatment with 0.06 percent mTD. The number of areas on nodular hyperplasia were also more with 0.1 percent than 0.06 percent mTD.

Nagata (22, 23) has reported that no hepatoma or nodular hyperplasia was observed in rat liver after treatment with mTD, but, at the same time, diffuse biliary cirrhosis of the liver was seen. Recently, Umela (35) has reported that rhabdomyosarcoma was produced by repeated subcutaneous injection of a 0.4 percent solution of mTD in propylene glycol into normal rats, but liver cirrhosis was found in these rats.

There is some evidence that one site of origin of liver carcinoma is a nodular hyperplasia (2, 6–9, 11, 24, 25, 28, 34). Biochemical and histochemical observations of nodular hyperplasia in the hepatocarcinogenesis in rats have been reported (3, 9). In the present work, nodular hyperplasia was already present in rats which were killed at 20 weeks. Nodular hyperplasia may be necessary before the neoplastic changes of mTD carcinogenesis occur in the rat liver.

It is now well established that various chemical carcinogens act synergistically. For example, there is a synergism between 2-AAF and 3'-Me-DAB (14), between 2-AAF and tannic acid (21), and between DAB and ethionine (19). The results of the second experimental series have also shown that the effects of ethionine and mTD are synergistic. So the development of nodular hyperplasia induced by mTD may be continued by ethionine. Moreover, ethionine-induced nodular hyperplasia may be continuously developed by mTD. Similar findings have been observed in the case of ethionine and DAB or 2-AAF.

It is evident from the third experimental series that the administration of ANI, PHP, or 3-MC has a significant inhibitory effect on the induction of hepatic cancer by mTD. Antagonistic actions have been reported between azo dyes and ANI (29, 30) or PHP (1, 27), ethionine and PHP or ANI (31, 32), 2-AAF and 3-MC (15, 17, 20), and 3'-Me-DAB and 3-MC (15, 26) (Table 7).

Some chemicals which inhibit liver carcinogenesis, such as 3-MC, appear to accelerate the destruction of the carcinogen by induction of liver enzymes (17), and ANI or PHP may have similar actions. However, 3-MC had no evident influence upon the development of preneoplastic or neoplastic changes in the liver induced by ethionine (15). These findings are consistent with the conclusion that the metabolism of ethionine by microsomal enzymes is not essential for liver carcinogenesis with ethionine.

From the present study, it would seem, therefore, that 3'-Me-DAB, 2-AAF, and mTD have rather similar actions and may be grouped together, whereas the action of ethionine is different.

REFERENCES

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Figs. 1—20. mTD, m-toluylenediamine; 3-MC, 3-methylcholanthrene; ANI, alpha-naphthyl isothiocyanate; PHP, p-hydroxypropiophenone. Fig. 1. Metastases of the lung and mediastinal lymph nodes in a rat fed on basal diet supplemented with 0.1 percent mTD for 36 weeks.

Fig. 2. An area of hepatocellular carcinoma in the liver of a rat treated with 0.1 percent mTD diet for 36 weeks. The cells are arranged in columns two or more cells thick, separated by canals lined with epithelium. H & E, × 100.

Fig. 3. High-power magnification of Fig. 2 showing many large irregular cells. H & E, × 400.

Fig. 4. Nodular hyperplasia in the liver of a rat treated with 0.1 percent mTD diet for 31 weeks. There is some compression of the surrounding liver by the nodule. H & E, × 100.

Fig. 5. An area of cholangiofibrosis in the liver of a rat treated with 0.1 percent mTD diet for 32 weeks. H & E, × 100.

Fig. 6. Metastatic cancer cells in epithidiosis of a rat treated with 0.1 percent mTD diet for 36 weeks. H & E, × 100.

Fig. 7. Metastatic areas in the lung of a rat treated with 0.1 percent mTD diet for 36 weeks showing small foci in the alveoli. H & E, × 100.

Fig. 8. High-power magnification of Fig. 7 showing irregular cancer cells in alveolar space. H & E, × 400.

Fig. 9. An area of fatty changes of liver parenchymal cells in a rat treated with 0.1 percent mTD diet for 32 weeks. H & E, × 100.

Fig. 10. An area of fatty changes in liver parenchymal cells in a rat treated with 0.1 percent mTD plus 0.0067 percent 3-MC diet for 34 weeks. H & E × 100.

Fig. 11. An area of fatty changes of liver parenchymal cells and bile duct proliferation in a rat treated with 0.1 percent mTD plus 0.1 percent ANI diet for 35 weeks. H & E, × 100.

Fig. 12. Diffuse fatty change of liver parenchymal cells in a rat treated with 0.1 percent mTD plus 1.0 percent PHP for 35 weeks. H & E, × 100.
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