Effect of Nitrofurans Antagonistic to 3'-Methyl-4-dimethylaminoazobenzene in Hepatocarcinogenesis and RNA Polymerase Activity of Liver Cell Nuclei in Rats

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SUMMARY

The purpose of this study was to determine (a) whether the hepatocarcinogenesis in rats fed 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB) could be inhibited by the simultaneous and followed feeding of two nitrofurans; (b) whether 3'-Me-DAB feeding inhibited DNA-dependent RNA polymerase activity of liver nuclei; and (c) whether the nitrofurans ameliorated 3'-Me-DAB-induced changes in RNA polymerase activity. The nitrofurans were 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide and 2-amino-5-[2-(5-nitro-2-furyl)-1-(2-furyl)vinyl]-1-1, 3, 4-oxadiazole. Male Donryu rats were fed 1 or 0.5 g of 3'-Me-DAB by being maintained on a diet containing 0.06% 3'-Me-DAB or 3'-Me-DAB plus 0.2% of a nitrofuran, and then they were maintained on basal diet for 60 or 300 days, respectively. The incidence of hepatomas induced by 3'-Me-DAB was reduced to about one-fifth by the simultaneous feeding of a nitrofuran. Feeding of 3'-Me-DAB brought gradual reductions of RNA polymerase activities of liver nuclei; 65 to 80% reduction of Mn⁺⁺-(NH₄)₂SO₄-activated RNA polymerase activity and 50% reduction of the Mg⁺⁺-activated one were observed in the rats fed 3'-Me-DAB diet for more than 27 days. The reduction of RNA polymerase activity was delayed or prevented by the simultaneous feeding of a nitrofuran. The induction of hepatomas in rats fed 0.5 g 3'-Me-DAB was also retarded by the followed feeding of a nitrofuran. The followed feeding of a nitrofuran promoted the recovery of 3'-Me-DAB-induced deleterious changes in RNA polymerase activity and liver nucleic acid contents. These and associated findings indicated that the nitrofurans retarded 3'-Me-DAB carcinogenesis by exhibiting their own effect, antagonistic to 3'-Me-DAB, on rat liver.

INTRODUCTION

In the previous study (2), we found that DAB, fed to the rats for 10 days at a level of 0.06% in the diet, caused inhibition of DNA-dependent RNA polymerase (EC 2.7.7.6) activity of liver nuclei. In order to provide further evidence for the assumption that carcinogenic azo dyes cause inhibition of RNA polymerase activity of liver nuclei, the present study was made on 3'-Me-DAB, because 3'-Me-DAB is more potent in the carcinogenic activity than DAB and causes nucleolar segregation of liver nuclei (12, 18, 19), which is well associated with inhibition of RNA polymerase activity of liver nuclei specifically with regard to aflatoxin B₁, one of the most potent hepatocarcinogens (13, 19, 20, 23, 24).

Another purpose of this study is to provide some information that might be useful for assessing whether inhibition of RNA polymerase activity is an important event in azo dye hepatocarcinogenesis. In this respect, it is worthwhile to follow RNA polymerase activity throughout the carcinogenic process and also to examine whether RNA polymerase activity would be influenced by the substances that inhibit azo dye carcinogenesis. As such inhibitors, we chose N₁ and N₂ for this study, because either nitrofuran exhibits a strong inhibitory effect on DAB carcinogenesis when it was fed to rats simultaneously with DAB (1, 10, 11). The 3rd purpose is to demonstrate the possibility that these nitrofurans would inhibit azo dye carcinogenesis by exhibiting their own effect on rat liver, because the nitrofurans, in contrast to DAB, stimulate RNA polymerase activity of liver nuclei. As an approach to demonstrate this possibility, studies were also made to determine whether the hepatocarcinogenesis induced by 3'-Me-DAB could be inhibited not only by the simultaneous feeding of the nitrofurans but also by the followed feeding of them and then to inquire whether such aspects in the inhibition of hepatocarcinogenesis would be reflected on RNA polymerase activity of liver nuclei.

MATERIALS AND METHODS

Animals used were male and female Donryu rats, obtained from Nippon Rat Co., Saitama, Japan. They were maintained on a semisynthetic diet, CE-2 (Ref. 11; consisting of protein, 24%; fat, 3.5%; carbohydrate, 56%; minerals, 6%), obtained from CLEA Japan Inc., Tokyo, Japan. At 8 to 9 weeks of age, they were divided into groups and fed one of the experimental diets, namely 3'-Me-DAB...
diet (CE-2 diet containing 0.06% 3'-Me-DAB), 3'-Me-DAB plus N1 diet (3'-Me-DAB diet containing 0.2% N1), 3'-Me-DAB plus N2 diet (3'-Me-DAB diet containing 0.2% N2).

Autopsies were performed on both the animals dead in the course of feeding experiments and those sacrificed at the end of the feeding schedules. Tissue samples for microscopy were fixed in 10% formaldehyde solution, and paraffin sections were stained with hematoxylin and eosin. For electron microscopy, tissue samples were minced into pieces of about 1 cm³, fixed in 2% glutaraldehyde for 2 hr, and postfixed with 1% OsO₄ for 3 hr. They were then dehydrated in a graded series of alcohol and propylene oxide and embedded in Epon 812. Sections were double stained with uranyl acetate and lead citrate and examined with a Hitachi HU-11DS electron microscope.

Isolation of liver nuclei was made by a modification of the method of Dingman and Sporn (3); details of the procedure are described in the previous report (2). The mean RNA/DNA ratio of the isolated liver nuclei from the animals fed basal diet was 0.17, and the recovery of the nuclei in the nuclear fraction from the liver homogenate was more than 50%, based on DNA analysis. RNA polymerase activity of the isolated liver nuclei was assayed in the systems underactivation by Mg⁺⁺ at low ionic strength and by Mn⁺⁺ at high ionic strength with ammonium sulfate, according to the procedure of Widnell and Tata (21, 22). Radioactivity was measured in Beckman LS-150 liquid scintillation system and the extent of quenching was estimated by the internal standardization technique using a toluene-¹⁴C standard. The mean efficiency of counting was 52%.

The quantity of protein bound dye was determined according to the method of Miller and Miller (8). The quantitative analyses of RNA and DNA were made by the methods of Fleck and Begg (4) and Giles and Myers (5), respectively. Protein was analyzed according to the method of Lowry et al. (7), with crystalline bovine albumin as standard. UTP-¹⁴C was obtained from the Radiochemical Centre, Amersham, England; and GTP, CTP, ATP, and UTP were obtained from Böehringer und Söhne GmbH, Mannheim, Germany. Nitrofurans were donations from the Ueno Pharmaceutical Co., Ltd., Osaka, Japan.

**RESULTS**

The effect of the simultaneous and followed feeding on 3'-Me-DAB hepatocarcinogenesis was examined in rats fed 1 or 0.5 g of 3'-Me-DAB and is summarized in Table 1. Rats of Groups 1A to 1C' were fed an experimental diet, described in "Materials and Methods," until they consumed 1 g of 3'-Me-DAB or 1 g of 3'-Me-DAB and 3.34 g of a nitrofuran. This required about 100 days; thereafter, each group was fed basal diet for 60 days (male) or 90 days (female). Ten of 11 male rats fed 3'-Me-DAB alone (Group 1A) developed hepatomas. The simultaneous feeding of a nitrofuran with 3'-Me-DAB reduced the incidence of hepatomas to 2 among 11 animals (Groups IB and 1C). Female rats were somewhat resistant to the carcinogenic activity of 3'-Me-DAB. Hepatomas were found in 5 of 12 female animals fed 3'-Me-DAB alone (Group 1A') and none among 10 animals fed 3'-Me-DAB plus N2 diet (Group 1C'). Rats of Groups 2A, 2B, and 2C were fed 0.5 g 3'-Me-DAB or 0.5 g 3'-Me-DAB and 1.67 g of a nitrofuran by being maintained on an experimental diet. The rats of Groups 2D and 2E were first fed 0.5 g of 3'-Me-DAB, and then they were fed 1.67 g of a nitrofuran by being maintained on diet containing 0.2% of a nitrofuran. Thereafter, each of Groups 2A to 2E was maintained on basal diet and sacrificed at 1 year after the start of feeding with experimental diets.

<table>
<thead>
<tr>
<th>Intake (g/rat) of</th>
<th>Associated liver lesions</th>
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<tbody>
<tr>
<td></td>
<td>Adenoma and adenomatous growth</td>
</tr>
<tr>
<td><strong>Group</strong></td>
<td><strong>No. and sex of rats</strong></td>
</tr>
<tr>
<td>1A</td>
<td>11 M</td>
</tr>
<tr>
<td>1B</td>
<td>11 M</td>
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<tr>
<td>1C</td>
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<tr>
<td>1A'</td>
<td>12 F</td>
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<td>1C'</td>
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<td>2A</td>
<td>11 M</td>
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<td>2B</td>
<td>4 M</td>
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<td>2C</td>
<td>8 M</td>
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<tr>
<td>2D</td>
<td>5 M</td>
</tr>
<tr>
<td>2E</td>
<td>11 M</td>
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* Followed feeding of a nitrofuran.
Groups 2D and 2E were first fed 0.5 g 3'-Me-DAB, and then they were fed 1.67 g of a nitrofuran by being maintained on basal diet containing 0.2% nitrofuran. Thereafter, all groups were maintained on basal diet and sacrificed 1 year after the start of feeding with experimental diets. Feeding of 0.5 g 3'-Me-DAB was enough for the induction of hepatomas in male rats, and 10 of 11 animals fed 3'-Me-DAB alone (Group 2A) developed hepatomas. The incidence of hepatomas was reduced not only by the simultaneous feeding of a nitrofuran (Groups 2B and 2C) but also by the followed feeding of a nitrofuran (Groups 2D and 2E).

Feeding 3'-Me-DAB to male rats brought about reduction of 2 kinds of activities of DNA-dependent RNA polymerase of liver nuclei (Chart 1). Mg**+-activated RNA polymerase activity was inferred to be associated with the synthesis of rRNA, and the Mn**+(NH₄)₂SO₄-activated one was inferred to be associated with the synthesis of DNA-like RNA (22). The reduction of Mn**+(NH₄)₂SO₄-activated RNA polymerase activity was more remarkable: 70 to 80% reduction was seen in the rats fed 3'-Me-DAB diet for 27 and 50 days, while the reduction of the Mg**+-

Activated RNA polymerase activity was about 50%. The simultaneous feeding of a nitrofuran brought an elevation of Mg**+-activated RNA polymerase activity, which was seen at 7 and 15 days in the animals fed 3'-Me-DAB plus N1 (or N2) diet, and prevented this RNA polymerase activity from being reduced by 3'-Me-DAB at 27 and 50 days. On the other hand, the effect of 3'-Me-DAB on Mn**+(NH₄)₂SO₄-activated RNA polymerase activity was so strong that the simultaneous feeding of a nitrofuran prevented the activity reduction only during the early days (7 and 15 days) of feeding. In Table 2 are summarized the repeated experiments on RNA polymerase activities and liver nucleic acid contents with 10 animals fed one of the experimental diets for 50 days. In agreement with the findings in RNA polymerase activities, 3'-Me-DAB feeding reduced RNA/DNA ratios of liver and liver nuclei. The simultaneous

### Table 2

<table>
<thead>
<tr>
<th>Diet</th>
<th>RNA polymerase activity (pmoles UTP/mg DNA)</th>
<th>RNA/DNA ratio</th>
<th>Liver DNA (mg/g liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mg**+- activated</td>
<td>Mn**+(NH₄)₂SO₄-activated</td>
<td>Liver nuclei</td>
</tr>
<tr>
<td>Basal</td>
<td>666 ± 102e</td>
<td>1760 ± 376e</td>
<td>0.171 ± 0.009f</td>
</tr>
<tr>
<td>3'-Me-DAB</td>
<td>337 ± 77</td>
<td>637 ± 301</td>
<td>0.154 ± 0.021</td>
</tr>
<tr>
<td>3'-Me-DAB + N1</td>
<td>596 ± 181f</td>
<td>603 ± 378</td>
<td>0.204 ± 0.0225</td>
</tr>
<tr>
<td>3'-Me-DAB + N2</td>
<td>722 ± 205f</td>
<td>850 ± 568</td>
<td>0.204 ± 0.021</td>
</tr>
</tbody>
</table>

* 3'-Me-DAB diet, diet containing 0.06% 3'-Me-DAB; 3'-Me-DAB + N1 diet, 3'-Me-DAB diet containing 0.2% N1; 3'-Me-DAB + N2 diet, 3'-Me-DAB diet containing N2.

* Mean of the values for 10 animals ± S.D.

†p < 0.01, relative to the rats fed 3'-Me-DAB diet.

‡p < 0.05, relative to the rats fed 3'-Me-DAB diet.
feeding of a nitrofuran prevented the reduction of liver RNA/DNA ratio and elevated the RNA/DNA ratio of liver nuclei. The reductions of RNA polymerase activities and liver RNA/DNA ratio associated with the ingestion of 3'-Me-DAB were recovered by the followed maintenance of the rats on basal diet (Chart 2). The followed feeding of a nitrofuran promoted the recovery of RNA polymerase activities (Chart 2) and the levels of liver nucleic acid contents (Table 3). The effect of nitrofurans against 3'-Me-DAB was also noted in the histological examinations of liver. The marked proliferation of ductal cells caused by 3'-Me-DAB was effectively reduced by the simultaneous feeding of a nitrofuran (Figs. 1 to 3). The condensation and local segregation or light capping of the nucleolus were often observed in the liver cells of the rats fed 3'-Me-DAB diet (Fig. 4). Either nitrofuran caused a hypertrophy of the nucleolus (Fig. 6) and preserved the loose structure of the nucleolus against the attack of 3'-Me-DAB (Fig. 5). It is conceivable that nitrofurans would exhibit influences on the metabolic fate of 3'-Me-DAB and prevent the carcinogen from interacting with the constituents of liver. Therefore, the level of the protein-bound dye in the liver was estimated as a measure of the interaction of 3'-Me-DAB with liver constituents (Chart 3). However, the simultaneous feeding of a nitrofuran with 3'-Me-DAB brought no significant effect on the level of the protein-bound dye.

DISCUSSION

Feeding 3'-Me-DAB to rats brought reductions of RNA polymerase activities of liver nuclei, and the reduction of Mn++-(NH₄)₂SO₄-activated one was more remarkable than that of Mg++-activated one. Such selectivity in the reduction of RNA polymerase activity was more clearly indicated in our previous observation on DAB (2). Kizer and Clouse (6) presented a similar finding that the rats fed 3'-Me-DAB showed decreased incorporation of orotic acid-6-¹⁴C into nonnucleolar nRNA, but incorporation into nucleolar RNA was decreased to a lesser extent. The reduction of RNA polymerase activities was accompanied by the reduction of RNA/DNA ratios of liver and liver nuclei (17) and also with nucleolar segregation of liver cell nuclei (12, 18, 19). These effects of 3'-Me-DAB were stronger than those of DAB (2) in agreement with the fact that 3'-Me-DAB is stronger in carcinogenic activity than is DAB (9).

The simultaneous feeding of the present nitrofurans delayed or retarded the induction of hepatomas in rats fed

![Chart 3. Effect of the simultaneous feeding of a nitrofuran on the protein-bound dye level in the liver of male rats fed 3'-Me-DAB. Each point represents the mean of the protein-bound dye levels of 4 rats, estimated by the absorbance at 520 nm of the acid solution of polar dye from 50 mg liver protein. The mean values of the control rats fed 3'-Me-DAB diet for 7, 15, 27, and 50 days were 0.254, 0.261, 0.197, and 0.184, respectively. The values of the rats fed 3'-Me-DAB plus a nitrofuran diet are expressed in fractions of the control values ±S.D. A, fed 3'-Me-DAB plus N1 diet; Δ, fed 3'-Me-DAB plus N2 diet.](chart3.png)
3'-Me-DAB. These nitrofurans, in contrast to the azo dye carcinogens, can enlarge rat liver and stimulate RNA polymerase activities of liver nuclei. These effects and activities of the nitrofurans were also noted in the rats fed 3'-Me-DAB and a nitrofuran simultaneously, and they reflected on RNA polymerase activities, fine structures of liver nuclei, and the contents of liver nucleic acids. On the other hand, the level of the protein-bound dye, which was estimated as a measure of the reaction of 3'-Me-DAB with liver constituents, was not significantly influenced by the simultaneous feeding of a nitrofuran. Therefore, it is assumed that the nitrofurans retarded the induction of hepatomas by exhibiting their own effects, antagonistic to 3'-Me-DAB, on rat liver. This assumption was further confirmed by the finding that the followed feeding of a nitrofuran could also retard the induction of hepatomas in the rats fed 3'-Me-DAB, promoting the recovery of RNA polymerase activities and the levels of liver nucleic acids.

The inhibition of RNA polymerase activity of liver nuclei has been well associated with nucleolar segregation in some hepatotoxins such as aflatoxin B, and actinomycin D (13, 15, 23). Therefore, the reduction of RNA polymerase activities associated with the ingestion of 3'-Me-DAB can be attributed to the toxic effect of 3'-Me-DAB on liver cells. However, there remains the possibility that the reduction of RNA polymerase activities was also due to the changes in liver cell population, especially in the late stage of azo dye feeding (14, 16). Detailed studies are necessary to follow the fate of the cells that were attacked by 3'-Me-DAB and to determine the meanings of the effect of 3'-Me-DAB and the present nitrofurans on RNA polymerase activities of liver nuclei in the processes of hepatocarcinogenesis.

REFERENCES


*The histological examination of rat livers indicated that adenoma or adenomatous growth of hepatocytes was frequently found in the livers of the rats fed both 3'-Me-DAB and a nitrofuran (Table 1). Thus, the protection of a nitrofuran from 3'-Me-DAB-induced neoplastic transformation was consistently exerted only against hepatoma induction.
Figs. 1 to 3. Micrographs of livers of male rats fed an experimental diet for 24 days. P, peripheral parts; C, central veins. H & E, × 80.

Fig. 1. Liver of a rat fed 3'-Me-DAB diet. The extensive proliferation of ductal cells is seen in the peripheral parts of liver lobule. The hepatocytes around the central veins are hypertrophic and degenerative.

Fig. 2. Liver of a rat fed 3'-Me-DAB plus N1 diet. The anisocytosis and atrophy of hepatocytes are seen. However, the proliferation of ductal cells is not evident.

Fig. 3. Liver of a rat fed 3'-Me-DAB plus N2 diet. A finding similar to that of Fig. 2.

Figs. 4 to 6. Electron micrographs of liver cell nuclei of male rats fed an experimental diet for 24 days. Bar, 1 μm. Uranyl acetate and lead citrate, × 13,000.

Fig. 4. Liver nucleus of a rat fed 3'-Me-DAB diet. The nucleoli are small. An assembly of granular components (G) is seen at the center of the nucleolus at the right side. The nucleolus at the left side exhibits a light nucleolar cap (LC). The chromatin around the nucleolus is diffuse and thin.

Fig. 5. Liver nucleus of a rat fed 3'-Me-DAB plus N2 diet. The nucleolus is comparatively large and round. However, a light nucleolar cap (LC) is seen at the right corner of the nucleolus.

Fig. 6. Liver nucleus of a rat fed N2 diet. The nucleolus is hypertrophic and the structure of nucleolonema is loose. The areas of pars amorpha (PA) are well extended, and the nucleolus-associated chromatin (NAC) is thin.
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