[CANCER RESEARCH 43, 2942–2946, June 1983]
0008-5472/83/0043-0000$02.00

Susceptibility of Germ-free Rats to the Hepatotoxic Effects of Dimethylnitrosamine or Dimethylamine plus Sodium Nitrite Administered Orally[1]

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ABSTRACT

The influence of intestinal microflora on the hepatotoxic effects of dimethylnitrosamine (DMN) or dimethylamine (DMA) plus $NaNO_2$ was studied by comparing the degree of liver necrosis and the levels of serum alanine aminotransferase (GPT) and aspartate aminotransferase (GOT) in germ-free and conventional male Wistar rats (320 to 340 g). In one experiment, both germ-free and conventional rats were intubated with DMN in respective doses of 8, 9, and 10 mg/kg of body weight, while in another experiment, both groups were intubated with DMA (1500 mg/kg) plus $NaNO_2$ (100 mg/kg). In both experiments, 48 hr after intubation, there was a marked difference in the degree of liver necrosis and the levels of serum GPT and GOT between the groups. In particular, a dose of 8 mg of DMN or 1500 mg of DMA plus 100 mg of $NaNO_2$ produced severe liver necrosis in the majority of germ-free rats, while the same dose did not produce any detectable liver necrosis in the majority of conventional rats. At a dose of 8 mg, serum GPT and GOT levels were raised to 22 and 15 times normal values, respectively, in germ-free rats, but only to about twice the normal values for both levels in conventional rats. At the combination dose of DMA plus $NaNO_2$, the levels of serum GPT and GOT were raised to 40 and 30 times normal values, respectively, in germ-free rats, while both levels remained almost normal in conventional rats. Thus, the results indicated that the liver of the germ-free state was far more susceptible to the acute toxic effects of DMN as well as DMA plus $NaNO_2$ administration at a certain dose range than was the liver of the conventional state, suggesting the influence of the absence of microflora.

INTRODUCTION

The discovery by Barnes and Magee (3) in 1954 that DMN[3] is acutely hepatotoxic to some laboratory animal species has been followed by a considerable amount of experimental work on DMN (4, 15–18, 26). It has become apparent that nitrite can interact with secondary amine in the rodent stomach at defined pH to form nitrosamine (2, 8, 25). Furthermore, it has been found that some tertiary amines react with nitrite in certain conditions, yielding DMN (11, 12, 14). Since DMA is a natural constituent of fish and nitrate or nitrite is plentifully available in vegetables, the hepatotoxic activity of DMN in connection with tumorigenesis has been of increasing importance (6, 13, 24). We were greatly interested in whether or not intestinal microflora has any influence on the hepatotoxic effect of DMN or DMA plus $NaNO_2$, since it is quite likely that DMN is yielded in the gastrointestinal tract among a large amount of microorganisms. For the purpose of finding any clue to this problem, we used germ-free rats. As far as we are aware, a comparative study using germ-free rats treated with DMN alone has not been done previously. However, regarding studies with DMA plus $NaNO_2$, Pollard *et al.* (20) reported that germ-free CFW mice were more sensitive to a toxic effect of $NaNO_2$ prior to the synthesis of nitrosamine than were conventional CFW mice. But they did not say much about the sensitivity of the liver to a toxic effect of DMN alone.

MATERIALS AND METHODS

Germ-free and conventional Wistar male rats weighing 320 to 340 g were used as experimental and control rats. Germ-free rats were those which had been produced by our own technique (19) through more than 30 generations in our laboratory. The germ-free rats were maintained in stainless steel isolators. Their germ-free status was checked as described by Wagner (29). The conventional rats were those which were obtained by a conventionalization method exposing the germ-free rats into a temperature- and humidity-controlled clean room; they had been produced through about 10 generations in the same laboratory. The composition of pellet diet used was reported previously (19). The pellet diet was steam sterilized at 121° and given to both germ-free and conventional rats. No necrotic lesions have ever been spontaneously observed before in the parenchymatous organs of both germ-free and conventional rats on our sterilized pellet diet.

DMN and $NaNO_2$ were products of Wako Pure Chemical Industries Ltd., Osaka, Japan. DMA, as a hydrochloride, was obtained from Tokyo Kasei Manufacturing Co., Tokyo, Japan. Each of the compounds was dissolved in distilled water and passed through a Millipore filter into sterile containers. The containers were introduced into an autoclave attached to the germ-free isolator, and their surfaces were sterilized under peracetic acid spray. Then, one container was transferred into the isolator for germ-free rats, and the other was taken out of the autoclave for conventional rats.

In the first experiment, 38 germ-free and 60 conventional experimental male rats were divided into 3 groups, respectively. After 6-hr fasting, they were given DMN by stomach tube in respective doses of 8, 9, and 10 mg/kg of body weight, each dissolved in 1 ml of distilled water. Forty-eight hr after the intubation, blood was collected from the abdominal aorta under ether anesthesia, allowed to clot, and analyzed for serum GPT and GOT by the method of Reitman-Franklin. For controls, 14 germ-free and 25 conventional male rats intubated with distilled water instead of DMN were used.

In the second experiment, 17 germ-free and 20 conventional experimental male rats were given DMA (1500 mg/kg body weight) in 1 ml distilled water together with $NaNO_2$ (100 mg/kg) in 0.5 ml distilled water by stomach intubation after 6-hr fasting. This combination was determined after preliminary tests of various combinations of doses. Forty-eight hr after the intubation, blood was similarly collected and analyzed

[1] This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science, and Culture of Japan.

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[3] The abbreviations used are: DMN, dimethylnitrosamine; DMA, dimethylamine; GPT, alanine aminotransferase; GOT, aspartate aminotransferase.

Received May 3, 1982; accepted March 8, 1983.

for serum GPT and GOT. For controls, 5 germ-free and 6 conventional male rats were given 1500 mg of DMA per kg of body wieght alone, and 5 germ-free and 6 conventional male rats were given 100 mg of $NaNO_2$ per kg of body weight alone.

In both experiments, all animals were autopsied immediately after blood collection, and their parenchymatous organs, such as the liver, kidney, heart, brain, and adrenals, were removed for pathohistological examination. Parenthetically, in the preliminary experiment, hepatic lesions were meausred biochemically and examined histologically at 3 time points (24, 48, and 72 hr) after DMN or DMA plus $NaNO_2$ treatment. However, liver necroses were too minute to be graded at 24 hr after the treatment, and the liver necroses at 72 hr after the treatment were almost similar in grade to those at 48 hr in both germ-free and conventional rats. In view of these results, we chose 48 hr as the time point. For light microscopy, these tissues were fixed in 10% formalin and embedded in paraffin. Sections were examined by the hematoxylin-eosin staining method. The liver was graded for necrosis according to the following criteria: absent; slightly centrilobular; moderately centrilobular; and zonal or more extensive.

RESULTS

In the first experiment, all of the experimental rats tolerated DMN up to 10 mg and none died within 48 hr. The necrotic lesions were observed only in the liver. The grade of hepatic necrosis and the levels of serum GPT and GOT at 48 hr after DMN intubation are shown in Tables 1 and 2. When 8 mg of DMN per kg of body weight were given, most of the treated germ-free rats showed a slightly or moderately centrilobular necrosis in the liver, and 3 cases even showed zonal necrosis (Fig. 1), there being no cases without liver necrosis, while 75% of conventional rats were free of detectable liver necrosis (Fig. 2), and 25% of them showed a slightly centrilobular necrosis. At a dose of 8 mg, serum GPT and GOT levels were raised to 22 and 15 times normal value, respectively, in germ-free rats, while both levels were only about twice that of normal values in conventional rats.

When 9 mg of DMN per kg of body weight were given, treated germ-free rats showed either moderately centrilobular or zonal necrosis in the liver, while 30% of the treated conventional rats showed no detectable liver necrosis, and 60% showed slightly centrilobular necrosis, there being only 2 cases with moderately centrilobular necrosis. At a dose of 9 mg, serum GPT and GOT levels showed 34 and 27 times the normal value in germ-free rats, respectively, while both levels showed only 4 times the normal values in conventional rats.

When 10 mg of DMN per kg of body weight were given, 67% of germ-free treated rats showed a severe degree of necrosis, *i.e.*, zonal or more extensive necrosis, while 50% of conventional treated rats showed slightly and 35% showed moderately centrilobular necrosis, there even being 3 cases without necrosis. At a dose of 10 mg, serum GPT and GOT levels were raised to 42 and 40 times normal values in germ-free rats, respectively, while in conventional rats, the former was raised 13 times and the latter about 11 times that of normal values.

In the second experiment, 14 of 17 germ-free rats and all conventional rats tolerated 1500 mg of DMA plus 100 mg of $NaNO_2$ at 48 hr after the intubation. As in the first experiment, the necrotic lesions were observed only in the liver. The grade of liver necrosis and the levels of serum GPT and GOT at 48 hr after the intubation are shown in Tables 3 and 4. Zonal or more extensive necrosis in the liver was very common in treated germ-free rats (Fig. 3), while such a severe necrosis was never observed in treated conventional rats, there being only 3 cases with slightly centrilobular necrosis (Fig. 4). The levels of serum GPT and GOT were raised to 40 and 30 times the normal value, respectively, in treated germ-free rats, while both levels remained almost normal in treated conventional rats. No hepatic necrosis was ever observed in control rats treated with DMA alone or $NaNO_2$ alone.

Table 1

Grade of hepatic necrosis in germ-free and conventional rats 48 hr after the intubation of DMN at the doses of 8, 9, and 10 mg/kg of body weight, respectively

Dose (mg)	Status	No. of rats examined	Incidence of graded necrosis			
			NON[a]	SC	MC	Z
8	GF	14	0	5 (36)[b]	6 (43)	3 (21)
	CV	20	15 (75)	5 (25)	0	0
9	GF	12	0	0	6 (50)	6 (50)
	CV	20	6 (30)	12 (60)	2 (10)	0
10	GF	12	0	0	4 (33)	8 (67)
	CV	20	3 (15)	10 (50)	7 (35)	0

[a] NON, absent; SC, slightly centrilobular; MC, moderately centrilobular; Z, zonal or more extensive; GF, germ-free; CV, conventional.
[b] Numbers in parentheses, percentage.

DISCUSSION

In this paper, we have clearly indicated that the liver of the germ-free rats used was far more susceptible to the acute toxic effect of DMN as well as DMA plus $NaNO_2$ administration at a certain dose range than was the liver of the conventional rats used. The results produced by 8 mg of DMN per kg of body weight alone corresponded with those produced by 1500 mg of DMA plus 100 mg of $NaNO_2$. That is, an 8-mg dose of DMN alone or DMA plus $NaNO_2$ produced a severe liver lesion in the majority of germ-free rats at 48 hr after the intubation, while the

Table 2

Serum GPT and GOT activities in germ-free and conventional rats 48 hr after the intubation of DMN

p values, calculated from the statistic *t* for serum GPT and GOT values between germ-free and conventional rats at the doses of 8, 9, and 10 mg of DMN, are <0.001.

Dose (mg/kg of body wt)	Serum GPT (Karmen units)		Serum GOT (Karmen units)	
	Germ-free	Conventional	Germ-free	Conventional
0	30 ± 4[a] (14)[b]	29 ± 7 (25)	75 ± 7 (14)	62 ± 9 (25)
8	660 ± 96 (14)	50 ± 15 (20)	1156 ± 98 (14)	120 ± 22 (20)
9	1023 ± 96 (12)	119 ± 44 (20)	2023 ± 178 (12)	247 ± 78 (20)
10	1254 ± 195 (12)	378 ± 180 (20)	2981 ± 353 (12)	709 ± 237 (20)

[a] Mean ± S.D.
[b] Numbers in parentheses, number of rats examined.

Table 3

Grade of hepatic necrosis in germ-free and conventional rats 48 hr after the intubation of $NaNO_2$ (100 mg/kg) plus DMA (1500 mg/kg)

Status	No. of rats examined	Incidence of graded necrosis			
		NON[a]	SC	MC	Z
Germ-free	14	0	0	3 (21)[b]	11 (79)
Conventional	20	17 (85)	3 (15)	0	0

[a] NON, absent; SC, slightly centrilobular; MC, moderately centrilobular; Z, zonal or more extensive.
[b] Numbers in parentheses, percentage.

Table 4

Serum GPT and GOT levels in germ-free and conventional rats 48 hr after the intubation of $NaNO_2$ (100 mg/kg) plus DMA (1500 mg/kg)

p values, calculated from the statistic *t* for serum GPT and GOT values between germ-free and conventional rats intubated with $NaNO_2$ (100 mg/kg) plus DMA (1500 mg/kg), are <0.001.

Status	No. of rats examined	Serum GPT (Karmen units)	Serum GOT (Karmen units)
Germ-free	14	1186 ± 296[a]	2253 ± 452
Conventional	20	34 ± 18	76 ± 30

[a] Mean ± S.D.

same dose did not produce any detectable liver necrosis in the majority of conventional rats, thus revealing a distinct difference between germ-free and conventional rats.

Points to consider regarding the difference in response to DMN or DMA plus $NaNO_2$ between germ-free and conventional rats are as follows. (*a*) The question as to whether the microbial degradation of the chemical carcinogen given p.o. is possible in conventional rats can be raised first. The subject of the microbial degradation seemed likely to have become a minor problem in light of the experimental results that DMN in certain doses produces liver necrosis in rats whether given by p.o., i.v., i.p., or s.c. injection in conventional rats (16). However, Rowland and Grasso (23) reported that a major proportion of bacterial types, common in the intestinal tract of many animals and in humans, was active in degrading diphenylnitrosamine and DMN. However, we cannot say much about this subject, because we have no evidence supporting such microbial degradation of DMN presently. (*b*) Other differences between germ-free and conventional rats as regards DMN, such as absorption, hepatic uptake, microsomal activation, metabolite conjugation, and susceptibility of hepatocytes to necrosis, are all equally valid considerations. Utili *et al.* (28) described that bacterial endotoxins, lipopolysaccharides, exert a wide variety of biological effects on the liver, such as changes in bile secretion, hepatic blood flow, energy production, and carbohydrate metabolism. If such changes of biological activities in conventional animals incessantly exposed to bacterial endotoxins are related to the enhancement of hepatic resistance against toxic substances, this phenomenon might help to explain the difference in hepatic injury by DMN between germ-free and conventional rats. (*c*) Relevant to the items described above, the differences in the structure and function between both groups, such as the mucosal structure (7), renewal of intestinal epithelium (1), and activities of intestinal (5, 22) and hepatic enzymes (10), also would be noteworthy as a fundamental problem. (*d*) The relationship between acute liver injury and carcinogenesis has been discussed by several investigators (14, 16, 17, 27). Ying *et al.* (30) reported upon an essential role of liver cell necrosis in the development of early putative preneoplastic liver lesions induced by a necrogenic dose of diethylnitrosamine. In recent years, a hyperplastic nodule in the liver has become the object of attention as a precancerous change (9, 21). However, hepatotoxic necrosis is not necessarily related to carcinogenicity. Therefore, further comparative investigation would be needed on the formation of such hepatocyte foci resistant to necrosis due to DMN or DMA plus $NaNO_2$ administration in germ-free and conventional rats.

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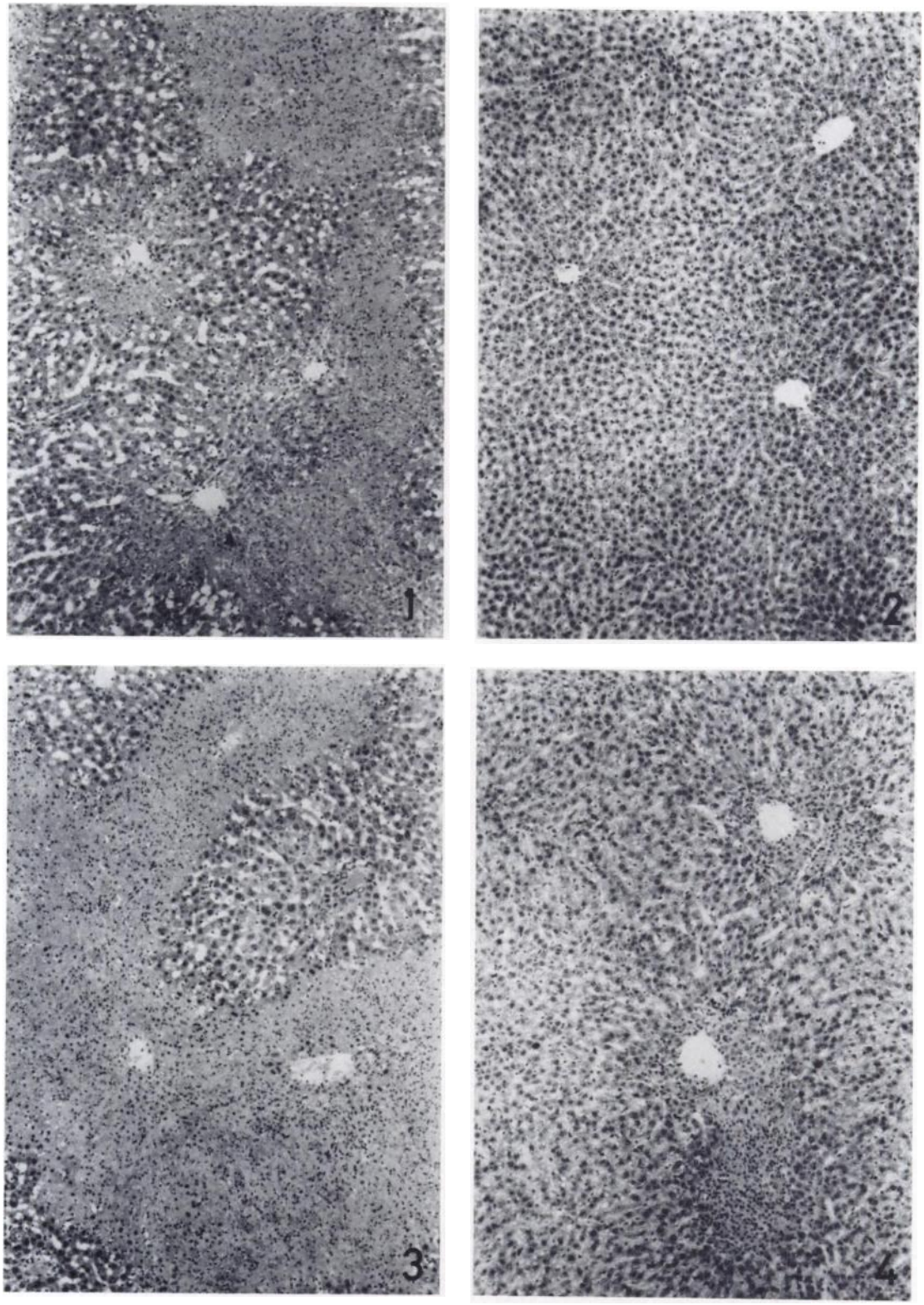

Fig. 1. Photomicrograph of zonal liver necrosis observed in a germ-free rat given 8 mg of DMN. H & E, × 80.
Fig. 2. Photomicrograph of absence of detectable liver necrosis observed in a conventional rat given 8 mg of DMN. H & E, × 80.
Fig. 3. Photomicrograph of a severe degree of liver necrosis observed in a germ-free rat given 1500 mg of DMA plus 100 mg of $NaNO_2$. H & E, × 80.
Fig. 4. Photomicrograph of slightly centrilobular liver necrosis observed in a conventional rat given 1500 mg of DMA plus 100 mg of $NaNO_2$. H & E, × 80.