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Mutagenicity of N-Nitrosodimethylamine in *Salmonella* / Microsome Assay and the Effect of Vitamin C on the Formation of N-Nitrosodimethylamine

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Abstract

The effective method to detect the mutagenicity of N-nitrosodimethylamine (NDMA) by using *Salmonella*/microsome assay was studied. The effect of vitamin C on the mutagenicity of the formed NDMA and during the formation of NDMA from nitrite and secondary amine was also investigated. Aroclor 1254-induced hamster S9 mix was more effective in activation NDMA than rat S9 mix induced by Aroclor 1254 or phenobarbital. Dimethyl sulfoxide and ethanol suppressed the mutagenic effect of NDMA, however, phosphate buffer (pH 7.4), distilled water, 95% methanol and Tween 80 + water (1 : 4) were the appropriate dissolving system in the mutagenicity test of NDMA. Vitamin C did not show any inhibitory effect on the mutagenicity of the formed NDMA. However, the revertants of *Salmonella typhimurium* TA100 were significantly reduced ($p< 0.05$) when vitamin C was added to the reaction mixture of nitrite and dimethylamine during the formation of NDMA. The amount of the formed NDMA was analyzed using HPLC and the level was decreased by about 95%. Thus it was concluded that vitamin C inhibited greatly the formation of NDMA from nitrite and dimethylamine.

Key words : N-nitrosodimethylamine, *Salmonella*/microsome assay, vitamin C

INTRODUCTION

Nitrosodimethylamine (NDMA) was one of the environmental carcinogen formed by the reaction of nitrite with secondary amine of dimethylamine under an endogenous acidic condition such as stomach.[1,2] It is also produced during processing of foods[3].

NDMA is a indirect mutagen which requires liver microsomal enzymes to activate to an ultimate mutagen. Yahagi et al[5]. reported that NDMA was mutagenic on *Salmonella typhimusium* TA100 rather than TA98 and dimethyl sulfoxide (DMSO), which is the most widely used solvent in *Salmonella* mutagenicity test, inhibited the mutagenic effect of NDMA. Thus, it required finding alternative dissolving solvents for the mutagenicity test of NDMA.

Vitamin C is believed to suppress the formation of carcinogenic N-nitroso compounds from nitrite and amines *in vivo* and *in vitro*[6-8]. Guttenplan[6] reported that vitamin C, which is the common form at physiological pH exhibited substantial nucleophilic character and vitamin C, might protect aganist electrophilic attack on cellular DNA by interception of alkylation agents.

In this study, we developed an appropriate S9 mix and NDMA dissolving solvent for the mutagenicity test of NDMA, which is difficult to activate in normal rat liver microsomal enzyme system to activate it and dissolving solvent of DMSO. The effect of vitamin C on the mutagenicity of the

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formed NDMA and during the formation of NDMA from nitrite and dimethylamine was also evaluated.

MATERIALS AND METHODS

Bacterial strain

Salmonella typhimurium TA100 strain, a histidine-requiring strain, was kindly supplied by Dr. B. N. Ames, University of California, Berkery, CA, USA. The genotypes of the tester strain were confirmed routinely for their histidine requirements, deep rough (*rfa*) mutation, *uvr*B mutation, and for the presence of the R factor.

Chemicals

N-nitrosodimethylamine (NDMA) was purchased from Sigma Chemical Co.(USA) and dissolved in distilled water. L-ascorbic acid was obtained from Hoffmann-La Roche, Nutley, NJ, USA. $NaNo_2$ (Junsei Chemical Co., Japan) and dimethylamine HCl (Sigma Chemical Co. USA) were used for the formation of NDMA in the model system.

Preparation of S9 fractions and S9 mixes

Sprague-Dawley male rats and Syrian golden hamsters weighing appoximately 200g were used as the source of livers to prepare S9 fractions. To induce the rat or hamster liver enzymes, Aroclor 1254 dissolved in corn oil (500mg/kg of body wt.) was injected intraperitioneally to each rat 5 days before sacrifice, and phenobarbital was added to the drinking water of the rats at the level of 1g / liter for 7days and then the rats and hamsters were sacrificed. Removed livers of the rats or hamsters were minced in 0.05M KCl, homogenized and centrifuged at $9000 \times g$ and the supernatant (S9 fraction) was saved and distributed in 2ml portions in plastic Nunc tubes, frozen quickly and stored immediately at -80℃ until used. The S9 mix was prepared by using the S9 fraction discribed by Maron and Ames[9].

Mutagenicity test

Preincubation test described by Matsushima et al.[10] in which 0.5ml of S9 mix, 0.1ml of a test strain from an overnight culture ($1 \sim 2 \times 10^9$ cells / ml) and 0.1ml of test compound preincubated at 37℃ for 30min were employed. 2ml of the top agar kept at 45℃ were added and vortexed for 3 seconds. The resulting entire mixture was poured on the minimal agar plate. The plates were incubated for 48 hrs at 37℃ and then revertant bacterial colonies on each plate were counted.

Model system for the formation of NDMA from nitrite and dimethylamine

For the formation of NDMA, the mixtures containing 5ml $NaNO_2$ (1M, 2M, 8M and 16M), 2.5ml dimethylamine hydrochloride (0.5M, 1M, 4M and 8M) and 25ml Sorensen's citrate I buffer were adjusted to pH 3 and then made up to 50ml with distilled water. The effect of vitamin C on the N-nitrosamine production was tested in mixtures containing 5ml of 4M $NaNO_2$, 2.5ml of 2M dimethylamine hydrochloride, 25ml of Sorensen's citrate I buffer, 5ml of 16M vitamin C and made up to 50ml with distilled water. These reaction mixtures were incubated in stoppered flasks at 37℃ for 3hrs.

NDMA determination

NDMA formed after the reaction of nitrite with dimethylamine was analyzed on HPLC (Waters, USA) using μ Bondapak™ C_{18} column with mobile phase of distilled water and CH_3CN (95 : 5 containing 0.2% K_2HPO_4) at a flow rate of 1.5ml per min. The effluents were monitored at 254nm.

Statistical analysis

Statistical analysis was performed by analysis of variance. Significant differences between treatment means were determined by using Duncan's multiple range test or Student's t test[11].

RESULTS AND DISCUSSION

Since rat S9 mix, which was the most widely used in Ames assay system, was ineffective to activate NDMA to an ultimate mutagen in a preparatory experiment, we performed experiments to detect the effective liver S9 fraction to activate the NDMA.

Aroclor 1254-induced, phenobarbital-induced rat liver S9 fractions and Aroclor 1254-induced hamster liver S9 fraction were prepared. As shown in

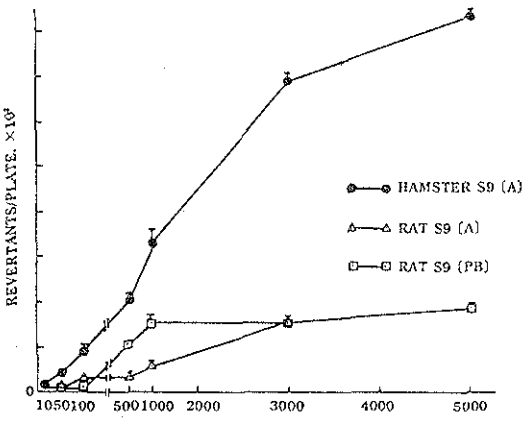

Fig. 1. Effect of different S9 mixtures on N-nitrosodimethylamine (NDMA) mutagenicity in *Salmonella typhimurium* TA100. (A) represents Aroclor 1254 induced, and(PB) means phenobarbital induced.

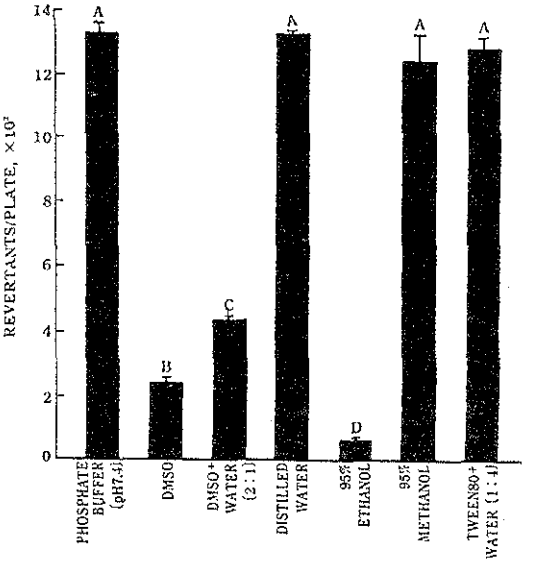

Fig. 2. Effect of different mutagen dissolving system on the hamster S9 mix induced N-nitrosodimethylamine (3000μ g/plate) mutagenicity in *Salmonella typhimurium* TA100. The different letters surmounted on the bars are significantly different at the 0.05 level of significance as determined by Duncan's multiple range test.

Fig. 1, phenobarbital-induced rat S9 mix was more effective to detect the mutagenicity of NDMA than Aroclor 1254-induced rat S9 mix. The revertant numbers of the TA100 were higher by almost 3 times in the presence of rat S9 mix induced by phenobarbital than those induced by Aroclor 1254 when 1000μ g of NDMA was added in the test system. However, the most effective S9 to activate NDMA was the hamster S9 mix. Revert-ant numbers were the highest when the hamster S9 mix was added to the NDMA mutagenicity system. The revertants increased proportionally with increase of NDMA concentrations per plate.

These results were in agreement with the report[12] which indicated that activation of NDMA required the presence of both microsomal and cytosolic fraction of liver and an inhibitor of NDMA activation was present in rat and mouse microsomes. Yoo et al[13]. reported the metabolic activation of NDMA in microsomal activation system of hamster and that rat liver was related to NDMA demethylase. These studies supported that hamster S9 mix was more effective than rat S9 mix for the mutagenicity test of NDMA.

DMSO, which is used in common as a dissolving solvent of mutagens in the test, inhibited the mutagenicity of NDMA. To find the appropriate solvents for NDMA and other chemicals used with in the Ames test, various dissolving solvents for the mutagenicity of NDMA were evaluated. Fig. 2 shows that phosphate buffer (pH 7.4), distilled water, 95% methanol and tween 80 +water (1: 4) were appropriate to dissolve NDMA; however, DMSO, DMSO + water (2 : 1) and 95% ethanol suppressed the mutagenicity of NDMA. These results are supported by the report[14] that the mutagenic activities of N-nitrosodialkylamine were decreased by the addition of dimethyl sulfoxide, dimethyl formamide, 95% ethanol or acetonitrile. These organic solvents did not appear to exert their influence by desmutagenic and antimutagenic actions, and the inhibitory effect is a result of interference with the process of metabolic activation by liver S9.

The effect of vitamin C on the mutagenicity of NDMA was studied by using the system developed as above. As shown in Fig. 3, vitamin C did not inhibit the mutagenicity of NDMA although the concentration of vitamin C increased up to 500μ g per plate in the system.

Table 1 shows that the amount of NDMA formed after 3hrs in the model reaction system contained various concentrations of nitrite and dimethylamine at 37℃ and pH 3. Increased concentrations of nitrite and dimethylamine in the reaction mixture formed higher levels of NDMA. When 1.6M of

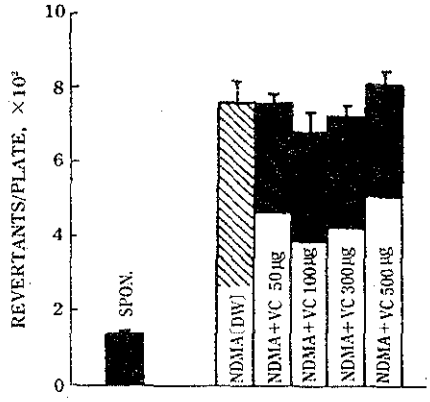

Fig. 3. Effect of vitamin C on the mutagenicity of N-nitrosodimethylamine (NDMA, 2000μg/plate) in the presence of hamster S9 in *Salmonella typhimurium* TA100. Vitamin C was dissolved in distilled water (DW).

Table 1. The amount of N-nitrosodimethylamine formed after reaction of nitrite with dimethylamine at pH 37℃ and 37 for 3hr

Reaction concentrations	N-nitrosodimethylamine(ppm)
0.1M-$NaNO_2$ + 0.025M-dimethylamine	617.4
0.2M-$NaNO_2$ + 0.05M-dimethylamine	2832.2
0.8M-$NaNO_2$ + 0.20M-dimethylamine	11897.7
1.6M-$NaNO_2$ + 0.40M-dimethylamine	22281.3

Table 2. Effects of vitamin C during formation of N-nitrosodimethylamine from nitrite and dimethylamine and their mutagenicity in the presence of hamster S9 in *Salmonella typhimurium* TA100

Treatment	Revertants
$NaNO_2$ + Dimethylamine	275±15
$NaNO_2$ + Dimethylamine + Vitamin C	123±19[1]*
Spontaneous	124±14

[1]The dissolving solvents for vitamin C was distilled water. 5ml of 2M $NaNO_2$, 2.5ml of 1M dimethylamine HCl, 12.5ml of Sorensen's citrate I buffer(pH 3.0) and 5ml of 8M vitamin C were mixed and incubated for 3hr at 37℃. The reactants were then used for the test.
The asterisk beside value is significantly different from the control at the $p<0.05$ level.

$NaNO_2$ and 0.4M of dimethylamine (the concentration in reaction mixture) were reacted, 22280.3 ppm of NDMA was formed.

Table 2 shows the effects of vitamin C during the formation of NDMA from nitrite and dimethylamine and their mutagenicity in the presence of

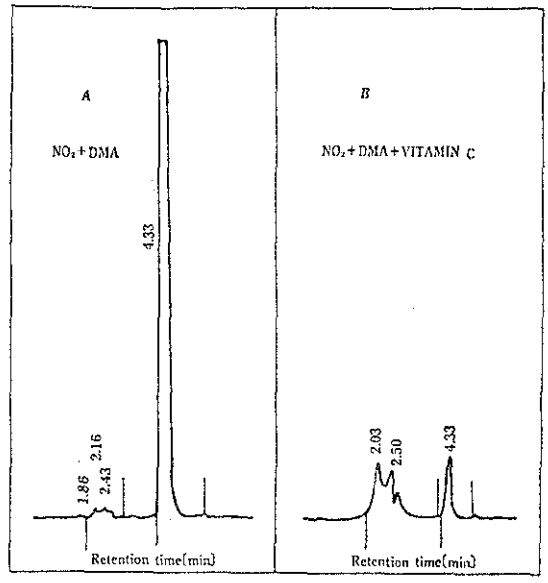

Fig. 4. HPLC chromatograms of N-nitosodimethylamine(NDMA) formed from nitrite and dimethylamine(DMA) in the absence and presence of vitamin C.
Panel A: NDMA formed from 0.8M $NaNO_2$ and 0.2M dimethylamine during incubat-ion at 37 ℃, 3hr and pH 3. Panel B : NDMA formed from 0.8M $NaNO_2$, 0.2M dimethylamine and 0.4M vitamin C during incubation at 37 ℃, 3hr and pH 3.

hamster S9 in *Salmonella typhimurium* TA100. When vitamin C was present in the reaction mixture containing nitrite and dimethylamine, the mutagenicity of the mixture after reaction was decreased significantly ($p < 0.05$) compared to the reaction mixture without vitamin C. The revertant numbers of the vitamin C added sample were similar to those of the spontaneous.

The amounts of formed NDMA from nitrite and dimethylamine during which vitamin C was absent or present in the reaction mixtures was determined by HPLC. As shown in Fig. 4, vitamin C inhibited by about 95% the formation of NDMA from nitrite and dimethylamine.

From these results, it was concluded that vitamin C did not suppress the mutagenicity of the formed NDMA, but it inhibited the formation of NDMA from nitrite and dimethylamine. Vitamin C was reported to suppress the nitrosation of aminopyrine and the mutagenicity of fish treated nitrite.[15,16] Thus it can be suggested in which the presence

of vitamin C in the system that there is a possibility of the formation of NDMA is very important to decrease the level of NDMA, and this reduces the incidence of stomach cancer which might caused by NDMA.

More studies are needed in detail using *in vitro* and *in vivo* experiments to understand the role or mode of action of vitamin C in the prevention of the mutagenesis and carcinogenicity of NDMA.

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Salmonella / Microsome Assay 에서의 N-nitrosodimethylamine의 돌연변이 유발성과 N-nitrosodimethylamine의 생성에 대한 비타민 C의 영향

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요 약

Salmonella/microsome assay system을 이용하여 N-nitrosodimethylamine (NDMA)의 돌연변이유발성을 검토하는 효과적인 방법과 비타민 C가 NDMA자체의 돌연변이유발과 nitrite와 2급아민으로부터의 NDMA 생성에 미치는 영향을 연구하였다. NDMA를 활성화시키기위한 S9중 Aroclor1254로 induce시킨 hamster S9이 가장 효과가 있었으며 Aroclor 1254나 phenobarbital로 induce시킨 rat S9 mix에 의하여서는 활성화가 약하였다. DMSO와 ethanol에 NDMA를 녹였을 때는 돌연변이유발실험에서 저해효과가 나타났으나 phosphate buffer (pH 7.4), 증류수, 95% methanol, Tween 80 + water (1 : 4)는 저해 효과가 없었다. 비타민 C는 이미 생성된 NDMA에 대해서는 돌연변이 유발저해 효과를 나타내지 않았지만 nitrite와 2급 아민 (dimethylamine)으로 부터의 NDMA 생성은 크게 저해하였다. 반응물에 비타민C를 첨가한 경우 *Salmonella typhimurium* TA100의 revertant 수가 spontaneous숫자 수준으로 감소되었으며 HPLC를 이용한 NDMA의 분석에서도 거의 95% 까지의 정량적인 감소를 관찰할 수 있었다.