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Effect of L-Ascorbic Acid on the Mutagenicity of Aflatoxin B_1 in the Salmonella Assay System

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Mutagenic actions of aflatoxin B_1 (AFB_1) in the presence of various concentrations of L-ascorbic acid (AA) in *Salmonella typhimurium* strains TA100 and TA98 were studied. Spontaneous revertants per plate of the tester strains TA100 and TA98 were 121-125 and 25-30 with or without S9 mix, respectively. The negative controls used in the study did not show any mutagenesis in the tester strains. AFB_1 revealed strong mutagenicity at the dose levels of 0.05, 0.1 and 0.25 μg/plate with metabolic activation system in both strains. However, it showed a toxic effect when the levels were more than 0.5 μg/plate. When lower concentrations of AA (5-20 μg/plate) were added to AFB_1 in the Ames assay system with S9 mix the mutagenic action of AFB_1 decreased in both strains. About 70-90% of mutagenicity of AFB_1 disappeared in strain TA100 when 20 μg of AA was added to 0.05 μg of AFB_1. The inhibitory effect was greatly increased by the addition of higher concentrations of AA to AFB_1 in TA100 strain. The mutagenicity of AFB_1 was completely inhibited when 100 μg and 500 μg of AA were added to 0.05 μg and 0.1 μg of AFB_1, respectively. However, this protective effect of AA on AFB_1 mediated mutagenesis was less effective in TA98 strain than that in TA100.

INTRODUCTION

Aflatoxin B_1 (AFB_1) is one of the most potent carcinogens or mutagens and is known to be produced by *Aspergillus flavus* and *Aspergillus parasiticus* as their secondary metabolite when they are contaminated in food or feedstuffs (Park, 1984). It induces liver cancer primarily in several animals including humans (Heathcote and Hibbert, 1978). AFB_1 requires mammalian liver microsomal activation to be an ultimate mutagen, probably to the AFB_1-2,3-oxide which is a very reactive electrophile. The epoxide reacts with nucleophilic sites on DNA bases resulting in altered bases and then may cause mutations to

lead to tumor formation (Singer and Grunberger, 1983). However, the enzymes in the mixed function oxidase (MFO) system in liver microsomes might also metabolize AFB_1 to various non-mutagenic derivatives such as AFB_2 a, AFQ_1 and AFP_1 by hydration, hydroxylation and o-demethylation, respectively, in different dietary conditions (Loveland *et al.*, 1983).

L-ascorbic acid (AA) has been reported to protect against tumor induction and the availability of AA *in vivo* is the determinant factor that regulates various aspects of host resistance to cancer (Cameron *et al.*, 1979). AA not only inhibits the formation of carcinogenic N-nitroso compounds from nitrite and amines (Walters, 1981), but also prevents the mutagenicity of the N-nitroso compound itself (Guttenplan, 1977). It is thus possible that AA might have an effect on liver microsomal enzymes to assist in detoxification or to decrease the mutagenicity of AFB_1. In this study, the effects of AA on the mutagenesis of AFB_1 on *Salmonella typhimurium* strains TA100 and TA98 in the Ames assay system were evaluated.

MATERIALS AND METHODS

Bacterial strains

Salmonella typhimurium strains TA100 and TA98, histidine requiring mutants, were provided by Dr. B.N. Ames, University of California, Berkley, CA, USA and were maintained as described by Maron and Ames (1983). The genotypes of tester strains were checked routinely for their histidine requirements, deep rough *(rfa)* character, UV sensitivity (*uvrB* mutation) and for the presence of R factor.

Chemicals

AFB_1 (from Sigma Chemical Co., St. Louis, MO, USA) was dissolved in spectrophotometric Dimethyl sulfoxide (DMSO) obtained from Aldrich Chemical Co., Milwaukee, WI, USA. N-methyl-N-nitro-N-nitrosoguanidine (MNNG), obtained from Aldrich Chemical Co., was used as a positive control. L-ascorbic acid was purchased from Hoffmann-La Roche, Nutley, NJ, USA. Other chemicals needed for the mutagenicity test were obtained from the companies as indicated by Maron and Ames (1983). These chemicals were sterified through millipore membrane filteration or were autoclaved.

S9 fraction and S9 mix

Sprague-Dawley male rats were injected intraperitoneally with Aroclor 1254 dissolved in corn oil (500 mg/kg of body wt.). Five days after the injections, the rats were sacrified, livers were removed and minced in 0.15 M KCl, and then homogenized with a Potter-Elvehjem apparatus. The homogenates were centrifuged at 9000g for 10 min. in a refrigerated centrifuge and the supernatant (S9 fraction) was distributed in 2-4.5ml portions in Nunc tubes, and stored at -80°C until used for mutagenic studies. In order to prepare S9 mix, S9 fraction was thawed immediately before being used for the preparation of S9 mix following the procedure of Maron and Ames (1983). Ten percent of S9 fraction in S9 mix was used as S9 mix for the experiment.

Mutagenicity test

A modified plate incorporation test in which 30 min. liquid preincubation of the organisms with the test compound was employed (Matsushima *et al.*, 1980). 0.5 ml of S9 mix was distributed in sterile capped tubes in ice bath and then 0.1 ml of testers from overnight

culture ($1\text{-}2\times10^9$ cells/ml) and 0.1ml of test compounds were added. The tubes were vortexed gently and preincubated at 37°C for 30 min. 2 ml of the top agar in each tube kept at 45°C were added and vortexed for 3 seconds. The resulting entire mixture was overlaid on the minimal agar plate. The plates were incubated at 37°C for 48 hrs and then the revertant bacterial colonies on each plate were counted. Appropriate concentrations of AA and DMSO were checked to see whether they showed any mutagenicity on the tester strains. Dose response tests of AFB_1 (0-1 μg/plate) on the tester strains were carried out to determine the regions of revealing mutagenicity and toxicity induced by AFB_1.

RESULTS

As shown in Table 1, spontaneous revertants of tester strains TA100 and TA98 were 121-125 and 25-30 per plate with or without addition of S9 mix, respectively, being in good agreement with other results (Maron and Ames, 1983). AFB_1 occurred the frequencies of revertants with about 10 times and 40 times spontaneous revertants in TA100 and TA98, respectively. MNNG which was also used as positive control showed its mutagenicity on the TA100 strain regardless of the presence of S9 mix, but not on the TA98 strain. The negative controls of AA and DMSO did not reveal any mutagenicity on the tester strains. They were all in the spontaneous revertant number regions.

The dose response curves for AFB_1 illustrate the regions of mutagenic and toxic effect toward TA100 and TA98 (Fig. 1). The revertant numbers increased linearly up to 0.25 μg of AFB_1 per plate and then the toxic effect revealed gradually and the revertant numbers decreased markedly at dose level of 1μg

Table 1. Reversion of *Salmonelda typhimurium* TA100 and TA98 in the presence of several test chemicals.

Test chemicals	+S9 mix				-S9 mix			
	Revertants/plate[a]		Mutagenicity[b]		Revertants/plate		Mutagenicity	
	TA100	TA98	TA100	TA98	TA100	TA98	TA100	TA98
Aflatoxin B_1 (0.1 μg/plate)	1209	1025	+	+	116	23	–	–
MNNG (2μg/plate)	1107	19	+	–	1214	19	+	–
Spontaneous reversion	125	25	–	–	121	30	–	–
L-ascorbic acid[c] (500 μg/plate)	170	45	–	–				
Dimethyl sulfoxide	121	17	–	–				

[a]Mean revertants values of two seperate experiments with triplicate plates.
[b]Based on a yield of two times as many revertant colonies over the spontaneous mutation rate.
[c]Solvent used was sterile distilled water.

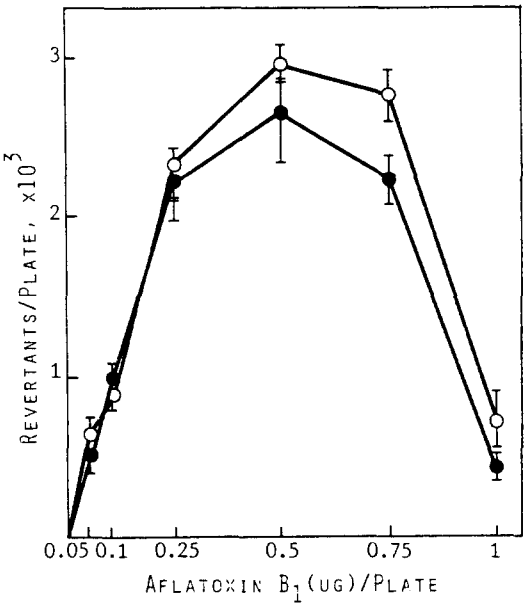

Fig. 1. Dose response effect of aflatoxin B_1 in *Salmonella typhimurium* strains TA100 and TA98.
Symbols: TA100 (●——●), TA98 (○——○)

Table 2. Effect of L-ascorbic acid addition (low concentration: 5-20 μg/plate) on the mutagenic activity of aflatoxin B_1 in TA100.

L-ascorbic acid concentration (μg/plate)	Revertants per plate			
	Aflatoxin B_1 concentration (μg/plate)	0	0.05	0.1	0.25
0		127 ±7*	771 ±83	1444 ±164	2176±452
5		118 ±3	600 ±104	1324 ±118	2225±184
10		130 ±14	488 ±79	1207 ±153	2028±16
15		138 ±18	195 ±18	1096 ±150	1982±92
20		125 ±6	190 ±50	1084 ±158	1901±47

* Mean revertants value ±SD of three determinations.

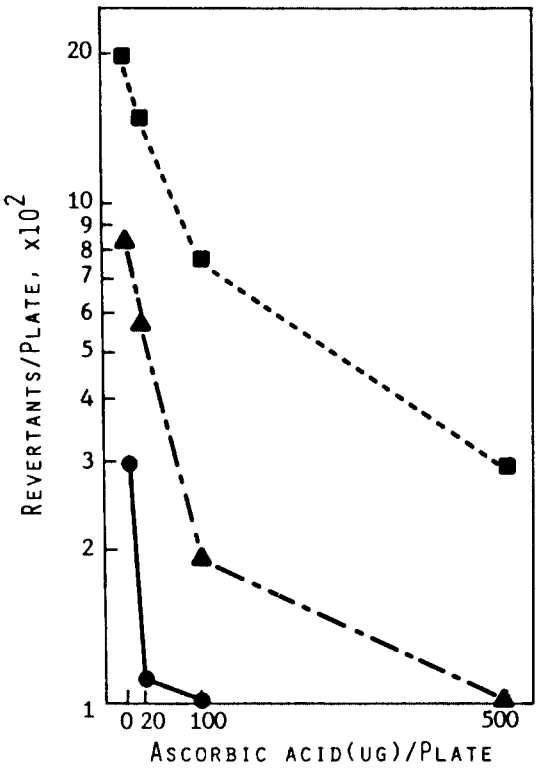

Fig. 2. Effect of ascorbic acid (high concentrations: 20-500 μg/plate) on the mutagenesis of aflatoxin B$_1$ in *Salmonella typhimurium* TA100 strain.
Symbols: 0.05 μg of AFB$_1$/plate (●——●)
0.10 μg of AFB$_1$/plate (▲— —▲)
0.25 μg of AFB$_1$/plate (■– – –■)

per plate. From the results, 0.05, 0.1 and 0.25 μg of AFB$_1$ per plate were employed to evaluate the effect of AA on the mutagenicity of AFB$_1$.

As shown in Table 2, when lower concentrations of AA (5-20 μg/plate) reacted with AFB$_1$ in the Ames mutagenicity test condition, the mutagenic action of AFB$_1$ was greatly reduced to about 70-90% when 0.05 μg of AFB$_1$ reacted with 20 μg of AA in the TA100 strain, while the inhibition rate was decreased in higher concentrations of AFB$_1$ present in the system. However, this effect of reduction in revertant numbers was not much greater in TA98 than as shown in TA100 (Table 3).

Higher concentrations of AA caused greater inhibition of mutagenesis of AFB$_1$. These inhibitory effects on AFB$_1$ mediated mutagenesis in various concentrations of AA are shown in Fig. 2. The mutagenicity of AFB$_1$ in the system was completely inhibited in the TA100 strain when 100μg and 500μg

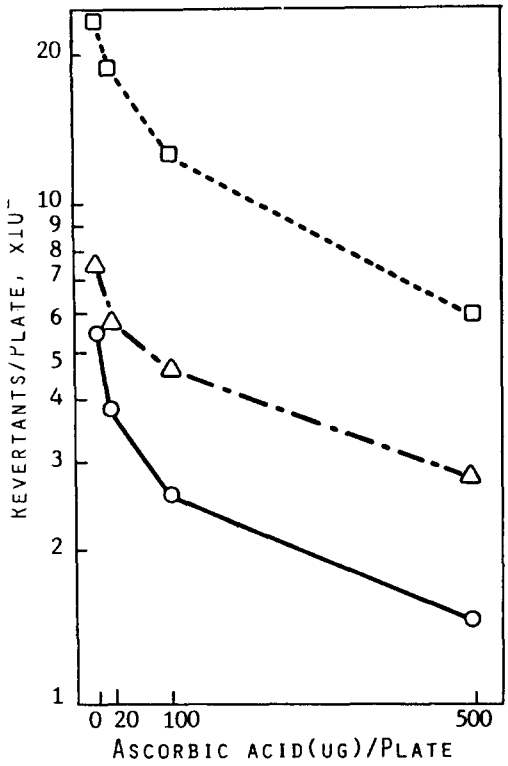

Fig. 3. Effect of ascorbic acid (high concentrations: 20-500 μg/plate) on the mutagenesis of aflatoxin B_1 in *Salmonella typhimurium* TA98 strain.
Symbols: 0.05 μg of AFB_1 /plate (○——○)
0.10 μg of AFB_1 /plate (△— —△)
0.25 μg of AFB_1 /plate (□– – –□)

Table 3. Effect of L-ascorbic acid addition (low concentration: 5-20μg/plate) on the mutagenic activity of aflatoxin B_1 in TA98.

L-ascorbic acid concentration (μg/plate)	Revertants per plate			
	Aflatoxin B_1 concentration (μg/plate)	0	0.05	0.1	0.25
0		24± 1*	524 ±6	954 ±62	1880 ±341
5		23± 1	518 ±10	927 ±38	1815 ±154
10		28± 7	454 ±71	812 ±12	1733 ±56
15		24± 4	417 ±103	831 ±51	1733 ±45
20		20± 2	314 ±116	780 ±152	1661 ±88

* Mean revertants value ± SD of three determinations.

of AA were added to 0.05 μg and 0.1 μg of AFB_1, respectively. When the concentration of AA per plate was increased in the fixed concentration of AFB_1 the inhibition rate was proportionally increased, but as shown in Fig. 3, this inhibition rate by AA in the TA98 strain was not as effective as shown in TA100. The numbers of revertant were higher in TA98 than those that appeared in the TA100 strain with addition of the same concentration of AA to the strains.

DISCUSSION

Although MNNG showed the mutagenicity only in TA100 (base pair substitution mutant) AFB_1 caused back mutation of both strains, TA100 and TA98 (frame shift mutant) only in the presence of the microsomal activation system. Since AFB_1 was shown to be completely dependent upon the enzyme activation to be an ultimate carcinogen, it might be possible to modify the liver activation system to prevent its carcinogenesis *in vivo*. Several studies indicated that many dietary compounds such as some proteins, lipids, vitamins and antioxidents have shown to influence the activities of the liver microsomal enzyme system (Rogers and Newberne, 1971; Chow and Gairola, 1984; Eisele *et al.*, 1983; Domngang and Bassir, 1981).

AA is a well known nutrient that exerts some biochemical actions in the prevention of cancer (Cameron *et al.*, 1979). As shown in this experiment AA resulted in decreasing the induced mutagenicity of AFB_1 efficiently in the Ames assay system. Eisele *et al* (1983) reported that dietary antioxident alters carcinogen activation and the detoxification mechanism in the hepatic microsomes of rainbow trout by decreasing cytochrome p-450 activities. The demethylation *in vitro* of AFB_1 with livers of female rats fed vitamin C was highly increased compared to the controls (Domngang and Bassir, 1981). AA may increase or decrease the activities of enzymes which metabolize AFB_1 to nonmutagenic derivatives. Thus one of possible actions by which AA inhibits mutagenesis of AFB_1 is altering the metabolism of AFB_1 by modifying the enzyme system in the microsomes. Another possible action is that AA may scavenge active electrophilic metabolite thus preventing this compound from reaching critical nucleophilic target sites of DNA and RNA. The active metabolite, AFB_1-2,3-oxide, is capable of covalent binding with DNA, RNA and protein, especially with mitochondrial DNA (Niranjan *et al.*, 1982). Chen *et al* (1982) reported that covalent binding of AFB_1 to liver DNA and RNA in chicks fed diet supplemented with Se, vitamin E, or both, were significantly depressed as compared with the control group. AA might reveal its inhibitory effect by trapping the ultimate mutagen before it interacts with cellular DNA, preventing the formation of AFB_1-DNA adducts.

Thus it can be suggested that if AFB_1 contaminated foods or feeds are consumed by humans or animals, but these concurrently intaking sufficient amounts of AA, the cancer incidences induced by AFB_1 could possibly be prevented. Though this protective effect of AA was correlated with concentrations

of AFB_1 and AA in the reaction system, the tumor induction is usually caused by low concentrations of the carcinogen and AA can be ingested in moderate quantitites without apparent toxicity (Cameron *et al.*, 1979). A sufficient amount of AA present in the system could effectively reduce the AFB_1 induced mutagenesis. The inhibitory effect by AA in TA98 was somewhat lower than in TA100. This is probably due to the fact that TA98 induced a greater frequency of revertants than TA100 when the revertant numbers are compared with spontaneous revertant numbers (Table 1). More studies are needed to better understand the role or mode of action of AA in the prevention of the mutagenesis of AFB_1 in detailed *in vitro* and *in vivo* experiments.

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Salmonella Assay System에 있어서 Aflatoxin B_1의돌연변이 유발성에 미치는 L-Ascorbic Acid의 영향

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Salmonella typhimurium TA100과 TA98에서 여러 농도의 L-ascorbic acid (AA)에 의한 aflatoxin B_1 (AFB_1)의 돌연변이 유발성 저해에 대한 효과를 검토하였다. TA100과 TA98에서 spontaneous revertant의 숫자는 activation system의 유무에 관계없이 Plate당 각각 121~125와 25~30 이었으며 negative control로 사용되었던 AA과 DMSO는 전혀 돌연변이를 유발하지 않았다. AFB_1은 각균주에서 0.05, 0.1, 0.25 μg/plate의 dose level에서 농도에 따라 비례적으로 강한 돌연변이를 유발 하였지만 0.5μg 이상에서는 toxic 효과를 나타내었다. Ames assag system에서 AA의 농도를 5~20 μg/plate로 하였을때 AFB_1에 의해 유도되는 돌연변이성이 감소되기 시작하여 TA100 균주인 경우 20μg의 AA를 0.5μg의 AFB_1에 첨가했을때 약 70~90%의 돌연변이성이 감소하였다. 이 저해 작용은 고농도의 AA를 AFB_1에 첨가했을때 현저하게 나타났는데 TA100에서 100μg의 AA를 0.05μg의 AFB_1, 그리고 500μg의 AA를 0.1μg의 AFB_1에 첨가 했을때 AFB_1에 의한 돌연변이성은 완전히 저해되었다. 그러나 TA98에서의 이 저해 효과는 TA100보다 다소 낮은 것으로 나타났다.