6-Thioguanine (6 TG) Resistant Mutation, Chromosomal Aberrations and Sister Chromatid Exchanges (SCE's) in V 79 Cells

I. Induced by Safrole

Ajay K. Jain

I.C.M.R. Centre For Advanced Research in Genetics, Genetics Unit,
Department of Medicine, K. G.'s Medical College,
Lucknow-226 003 (U.P.), India

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It is evident from epidemiological and experimental studies that certain man-made and natural products cause human cancers and inborn diseases (Haenszel 1961, Haenszel and Kurihara 1968, Wynder and Gori 1977, Tomatis *et al.* 1978). Every year a large number of chemicals are introduced into our foods and environment. Therefore, it seems of great importance to identify potential mutagens/carcinogens, since it may enable social, industrial and governmental agencies to take appropriate action to prevent or reduce genetic hazards of environmental mutagens. The present study was undertaken to evaluate genotoxicity of Safrole—a naturally occurring carcinogen in spices (Ames 1983, Parry 1969) in V69 cell line system.

Material and methods

Mutation assay was performed by the method of Kuroda *et al.* 1985.

Cyto-toxicity assay

100 cells in 5 ml MEM (Minimum Essential Medium) in 60 mm petridishes were incubated for 20 hr at 37°C and 5% CO₂ atmosphere. Five petridishes were prepared for each treatment. The cells were treated with different concentrations of Safrole in Hanks balanced salt solution (HBSS) under similar conditions for 3 hr. After treatment the cells were rinsed twice with HBSS and incubated in normal medium for 7 days. The cell colonies formed in petridishes were fixed with ethanol and stained with Giemsa stain. Colonies containing 50 cells or more were scored under a binocular microscope and the colony forming activity was calculated as the average number of colonies with at least 50 cells as a percentage of the number of cells initially inoculated. Cell survival is expressed as fraction of the colony forming activity of untreated control cultures.

Mutagenicity assay

2×10^5 cells in 10 ml of normal medium in 100 mm plastic petridishes were incubated for 20 hr at 37°C and 5% CO₂ atmosphere and then treated with the same concentrations as those used for the cyto-toxicity assay for 3 hr followed by rinsing with HBSS and then incubation in normal medium for mutation expression time of 6 days. The cells were then treated with 0.25% trypsin and 2×10^6 cells were replated each in five 100 mm petridishes in medium containing 6-thioguanine (5 µg/ml). After incubation for 14 days, the cell colonies formed were fixed and stained and the numbers of 6-TG resistant colonies were scored. The observed numbers of 6TG resistant mutant colonies were corrected for the decrease in the colony forming activity of cells in normal medium due to cytotoxic effect. The number of induced mutants was calculated by subtracting the number of colonies in control cultures from

those in treated cultures. The induced mutation frequency was expressed as the number of induced mutants per 10^5 colony forming cells.

Chromosomal assay

2×10^5 cells were implanted in 60 mm petridishes and incubated for 20 hr at 37°C and 5% CO_2 atmosphere. After twice washing with HBSS, cells were treated with different concentrations of Safrole in HBSS for 3 hr followed by washing and incubation in normal medium. The cells were treated with Colcemid ($0.1 \mu\text{g/ml}$) for 3 hr prior to 24 and 48 hr incubation and then harvested by trypsinization. Cells were fixed in 3:1 (methanol:acetic acid) after KCl (0.75 M) treatment for 15 minutes. Slide preparations were made by air dry method. Giemsa

Table 1. Mutagenic activity of Safrole

Dose	Survival fraction	Plating efficiency (%)	Mean no. of mutant colonies	Corrected no. of induced mutants	No. of induced mutant	Mutation frequency/ 10^5 survivors
Control	1.00	90.5	0.2	0.22	0.0	0.0
50 $\mu\text{g/ml}$	0.66	90.8	0.8	0.88	0.66	0.33
100 $\mu\text{g/ml}$	0.54	94.2	0.4	0.42	0.20	0.10
200 $\mu\text{g/ml}$	0.39	81.8	0.4	0.49	0.27	0.13
300 $\mu\text{g/ml}$	0.38	102.2	1.0	0.98	0.76	0.38

Table 2. Chromosomal aberrations (%) induced by Safrole

Dose	Number of cells scored	Aberrant cell (%)	Chromatid gap	Chromatid break	Fragment	Chromosome gap	Exchange	Dicentric
After 24 hr recovery								
Control	100	2	2	0	0	0	0	0
50 $\mu\text{g/ml}$	100	10	5	3	0	1	0	1
100 $\mu\text{g/ml}$	100	11	9	3*	0	1*	0	0
200 $\mu\text{g/ml}$	100	13	12	0	1	0	0	0
300 $\mu\text{g/ml}$	100	9	5	0	2	1	1	0
After 48 hr recovery								
Control	100	1	1	0	0	0	0	0
50 $\mu\text{g/ml}$	100	11	8	0	3	0	0	0
100 $\mu\text{g/ml}$	100	13	9	1	1	0	1	1
200 $\mu\text{g/ml}$	100	15	11	4	0	0	0	0
300 $\mu\text{g/ml}$	100	12	10	0	1	0	0	1

* One cell was showing multiple abnormality.

stained slides were scored blindly. For SCE assay cells were harvested similarly except cells were grown in medium containing 5-bromodeoxyuridine (BrdU) ($10 \mu\text{g/ml}$) and cells were harvested after 30 hr of the treatment. Slides were stained with Hoechst 33258 ($50 \mu\text{g/ml}$) for 10 min and then exposed to near UV light (313 nm) $2 \times 6 \text{ W}$ from a distance of 8.5 cm at 40°C for 20 min and then stained with Giemsa (4%) for 10 min.

Results and discussion

It is evident (Table 1) that cyto-toxicity increased depending upon the dose, but mutagenicity did not show dose dependent effect. The mutation frequency ranged from 0.10–0.38/ 10^5 survivors. Hence Safrole may be classified as weak positive mutagen. The reason

for not strict dose dependency might be either delay in reaching the target cell or non uniform distribution of Safrole in treating solution.

Chromosomal aberrations ranged from 9–13% (after 24 hr recovery) and from 11–15% (after 48 hr recovery) (Table 2), although the effect did not show dose dependent response. It

Figs. 1–4. 1, chromatid break. 2, dicentric chromosome. 3, exchange figure. 4, sister chromatid exchanges.

Table 3. Effect of Safrole on sister chromatid exchanges (SCE's)

Dose	No. of cells scored	Mitotic index	SCE/cell mean $\pm$ S.D.	SCE range	No. of cells in different SCE range					Cell cycle kinetics		
					0–3	4–6	7–9	10–12	>12	1st cycle	2nd cycle	3rd cycle
Control	50	2.53	7.38 $\pm$ 3.08	1–15	4	17	17	9	3	0.0	98.04	1.96
50 μ g/ml	50	2.92	6.60 $\pm$ 2.66	2–15	6	18	21	3	2	5.56	94.4	0.00
100 μ g/ml	50	1.57	7.38 $\pm$ 2.81	3–14	3	20	14	11	2	5.56	94.4	0.00
200 μ g/ml	50	1.31	7.96 $\pm$ 3.13	3–18	2	14	24	7	3	0.0	100.0	0.0
300 μ g/ml	50	1.75	8.02 $\pm$ 3.13	3–20	1	15	21	9	4	5.66	94.34	0.0

suggests to assume that DNA lesions due to Safrole treatment could not eliminate or repaired with recovery time and thus the magnitude of aberrant metaphases may enhance with the increase of recovery period.

The most common abnormalities were chromatid gap, break (Fig. 1), fragment, dicentric chromosome (Fig. 2), exchange figures (Fig. 3). Chromosome type gaps were observed in low magnitude. The occurrence of these chromosomal abnormalities clearly indicates that Safrole is potent to alter the genotype.

Safrole treatment did not significantly enhance the sister chromatid exchanges per cell (Table 3). SCE range varied from 1–20, however, cell cycle kinetic remained unaffected. This suggests to assume that Safrole treatment caused DNA lesions responsible for SCE without affecting the kinetic of DNA synthesis. Hence, it appears that for cyto-toxicity of Safrole besides DNA alteration, other factors like damage of cell membrane and metabolism might be equally significant.

From the present study it can be concluded that Safrole is potentially genetic hazard and being natural product may act as co-factor in enhancing geno-toxicity of other environmental chemicals. Therefore, it seems of utmost importance to have more detailed study to know the interaction effect and also to identify the food products in those it is present and to determine the permissible limit. This may be helpful in minimizing the genetic threat to human population.

Summary

Safrole, a naturally occurring substance in spices, was screened for its genotoxic potentialities in V79 cell line system. It showed weak mutagenic and SCE's enhancing effects. However, the magnitude of aberrant metaphases was considerably high. Chromosomal abnormalities were: gap, break, exchange figures, dicentric chromosome types. The possible genetic hazards have been discussed.

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