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Clastogenic Activity of Pure Chlorophyll and Anticlastogenic Effects of Equivalent Amounts of Crude Extract of Indian Spinach Leaf and Chlorophyllin Following Dietary Supplementation to Mice

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Dietary consumption of green vegetables has been associated with protection against mutagenic and clastogenic activity of genotoxicants. Chlorophyll, being present in all green plants, had earlier been suggested to be the principal factor involved. Mice were administered (i) crude aqueous extract of leaf of Indian spinach, *Beta vulgaris* L. var. *benghalensis* Hort., and equivalent amounts of (ii) chlorophyll extracted from the leaf; (iii) purified chlorophyll, (iv) chlorophyllin, a sodium-copper derivative of chlorophyll; daily for 7 days. On day 7, one set of mice from each treatment was administered potassium dichromate—a known metallic clastogen. The mice were sacrificed after 24 hours. Chromosome preparations were made from bone marrow following the usual colchicine-air dry-Giemsa schedule. The cytogenetic endpoints scored were chromosomal aberrations and damaged cells. Crude leaf extract and chlorophyllin were nonclastogenic and reduced the clastogenic effects of potassium dichromate to the control distilled water level. Chlorophyll alone, whether extracted from the leaf or obtained in commercially purified form, was clastogenic and could reduce the effects of the chromium salt only to its own level. The protective action of the crude leaf extract may be attributed to the total effect of the interaction between the different components within the leaf extract, in which the clastogenicity of chlorophyll had been neutralized. © 1996 Wiley-Liss, Inc.

Key words: chlorophyll, genotoxic effects, chemoprevention, anticlastogenic effects of leaf

INTRODUCTION

The increase in information on the use of plants and plant products in protecting against cancer has led to renewed interest in dietary chemoprevention of genotoxic effects of environmental xenobiotics. The types of food and food components of plant origin now classified as chemopreventors from a large heterogeneous class of chemicals identified in almost all categories of plant parts. Of these, green vegetables form a major proportion. After the initial observation of antimutagenic effects of common vegetables by Kada and coworkers [1978], protective effects were recorded with a large number of leafy vegetables against the mutagenicity of known mutagens, tested mainly in microbial systems [Morita et al., 1978, Shinohara et al., 1988, Nakashima, 1989; reviewed in Sarkar et al., 1996b]. Protection against chromosome breakage (clastogenesis) was observed in mammalian systems in vivo following dietary administration of the plant ingredients [Ito et al., 1986, 1989; Sarkar et al., 1996a].

Since chlorophyll is present in all green plants, the protective activity of green plant parts had been attributed to chlorophyll and to its sodium-copper derivative chlorophyllin [Lai et al., 1980; reviewed in Sarkar et al., 1994]. In a series of experiments carried out by our group, the crude aqueous extract of Indian spinach (*Beta vulgaris* var. *benghalensis* Hort.) leaf was recorded to be a potent anticlastogen. It reduced the frequency of chromosomal abnormalities induced by the known clastogen potassium dichromate to control levels. Chlorophyll extracted from the leaf, however, could not protect against the effects of the chemical.

The present investigation was undertaken to compare the potential of crude aqueous extract of Indian spinach leaf with that of equivalent amounts of chlorophyll extracted from the leaf itself, pure chlorophyll, and chlorophyllin, in protecting against the clastogenic activity of a common metallic clastogen. It was also proposed to find out if the clastogenic effects, if any, were from the protectants themselves.

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MATERIALS AND METHODS

Animals

Swiss albino male mice (*Mus musculus*, $2n = 40$), 8–10 weeks old, 25–30 g, were used in the study. The mice were bred in the departmental animal house, five per cage with husk bedding, and were fed a commercial diet (Hindusthan Lever, India) and water ad libitum. The light cycle was 12 hours light/dark, room temperature 25 ± 2°C, and relative humidity 60%.

Plant Products and Chemicals Tested for Protection Against Clastogenic Effects

1. *Extract of chlorophyll* in acetone was prepared from fresh leaves of Indian spinach (*Beta vulgaris* var. *benghalensis* Hort), according to the method of Arnon [1949] and Coombs et al. [1985]. The amount of chlorophyll present in the leaves was estimated, taking the optical densities at 663 and 645 nm in a spectrophotometer (160A, UV Vis, Shimadzu Corporation, Japan), and using the formula.

mg chlorophyll/g of tissue

$$=[20.2(D_{645}) + 8.02(D_{663})] \times \frac{V}{100 \times W}$$

where D_{645} is the absorbance at 645 nm and D_{663} is the absorbance at 663 nm; V is the volume of 80% acetone extract of chlorophyll; W is the fresh weight in grams of macerated tissue. Extract of chlorophyll was administered at a dose of 1.5 mg/kg body wt. Computing the absorbance values of the 80% acetone extract of chlorophyll in the above formula, it was observed that 1.5 mg of chlorophyll was present in 1 g of fresh spinach leaves. The solution was lyophilized, acetone evaporated, and the aqueous suspension used for the experiments.

2. *Crude aqueous leaf extract of Indian spinach* was prepared from fresh leaves purchased in bulk from the local market. An amount of leaves containing 1.5 mg of chlorophyll, as estimated in step 1 was macerated in distilled water. The crude extract was used for the experiments.

3. *Purified chlorophyll* was purchased from Sigma Chemical Company (St. Louis, MO) and dissolved in pure ethanol. Ethanol was evaporated and the chemical suspended in distilled water before use. The dosage administered was 1.5 mg/kg body wt.

4. *Chlorophyllin*, a synthetic, Na-Cu derivative of chlorophyll (Sigma Chemical Co., St. Louis, MO), was dissolved in glass distilled water to provide a dose of 1.5 mg/kg body wt. At this dose, chlorophyllin has been found to provide total protection against a number of known clastogens [A. Ghosh et al., 1991; A.K. Ghosh et al., 1991a,b; Palit et al., 1991; Sen et al., 1991; Sarkar et al., 1993].

Clastogen

The hexavalent chromium compound, potassium dichromate ($K_2Cr_2O_7$; mol. wt. 294.21; Cr 35.36%; E. Merck, India) was used as the clastogen. It was dissolved in distilled water and administered at a dose of 20 mg/kg body wt. (corresponding to 0.1 LD_{50} of the metallic compound). This dose had earlier been shown to be clastogenic [Sarkar et al., 1996a].

Experimental Protocol

Seven groups of mice were prepared for the experiments as given in Table I. In group I, kept as negative control, mice were given distilled water alone. Group II mice were administered a single dose of the mutagen mitomycin C (Sigma, USA 1.5 mg/kg body wt.) intraperitoneally to serve as positive control. Group III mice were dosed by gavaging the clastogen potassium dichromate at a dose of 20 mg/kg body wt. In group IV, two sets of mice were fed an aqueous solution of chlorophyllin (1.5 mg/kg body wt). After 2 hours, one set, IVA, was administered $K_2Cr_2O_7$, while set IVB was kept as control. Similarly, mice of groups V and VI were administered extracted and purified forms of chlorophyll, respectively, for 7 consecutive days. One group each, VA and VIA, were then given the clastogen $K_2Cr_2O_7$ on day 7, one hour after priming. Crude leaf extract was administered daily, for 7 days, to the mice of group VII. One of the sets of this group was exposed to $K_2Cr_2O_7$, on day 7, while the second set was kept as control. Five mice were used for each experimental set and were sacrificed 24 hours after receiving the final treatment.

Colchicine (0.04%, Sisco Laboratories, India) was injected intraperitoneally to each mouse 90 minutes before sacrifice. Animals were killed by cervical dislocation. Bone marrow cells from both femurs were flushed into 0.075 M KCl, incubated at 37°C for 15 minutes, repelleted and fixed in cold (1:3) acetic acid:ethanol. Slides were prepared by flame-drying and stained in Giemsa (1:20 dilution) [Preston et al., 1987; Sharma and Sharma, 1994].

The slides were coded and scored blind. From each set of five mice, 500 well-spread metaphase plates were scored for chromosomal aberrations. The different types of aberrations were recorded separately according to the categories given by the World Health Organization [1985]. These included chromatid and isochromatid gaps and breaks and rearrangements with centric fission, fusions, and dicentrics. For calculating the chromosomal aberrations per cell (CA/cell) each chromatid break was taken as one break, while each isochromatid break or rearrangement was taken as two breaks. Gaps were not included. In computing the percentage of damaged cells (% DC), all cells with at least one aberration (excluding gaps) were included.

For statistical analysis, Student's t test [Fisher and Yates, 1963] and one-way analysis of variance (ANOVA) [Sokal and Rohlf, 1973] were carried out. When the F statistic in ANOVA was significant, comparison was made using Duncan's multiple range test, to detect significance of differences, if any, among the different experimental sets [Harter, 1960; Kotz and Johnson, 1982].

RESULTS

In Table II, sets I, II, and III give the results of exposure to the negative control (distilled water), positive control (mitomycin C), and clastogen (potassium dichromate), respectively. As compared to distilled water, both mitomycin C and $K_2Cr_2O_7$ were highly clastogenic when observed at 24 hours following single exposure.

Priming with protectants was carried out, and observations were made after 24 hours for chlorophyllin, and after 7 days consecutive gavaging, for the others. Both chlorophyllin (set IVA) and crude leaf extract (set VIIA) were nonclastogenic, as compared with distilled water as negative control. Crude plant extract induced a higher frequency of chromosomal aberrations (CA) but not to a significant extent. Extracted chlorophyll (set VA) and pure chlorophyll (set VIA), however, were strong clastogens, as shown by the highly significant increase in CA induced (0.05 and 0.042 CA/cell respectively) from the control values of 0.02 for distilled water and 0.028 for crude leaf extract, respectively.

After priming with the protectants, mice from the sets IVB, VB, VIB, and VIIB were exposed to a single dose

TABLE I. Experimental Protocol*

	Treatment sets	Chemicals administered	Doses (mg/kg body weight)	Observed after
Control sets	I	Distilled water (negative control)	—	7 d
	II	Mitomycin C (positive control)	1.5	24 hr
Clastogen	III	$K_2Cr_2O_7$	20	24 hr
Plant products tested	IVA	Chlyn	1.5	24 hr
	IVB	Chlyn $\xrightarrow{2hr}$ $K_2Cr_2O_7$	1.5 → 20	24 hr
	VA	Chlyll E	1.5	7 d
	VB	Chlyll E $\xrightarrow{1hr}$ $K_2Cr_2O_7$	1.5 → 20	24 hr
	VIA	Chlyll P	1.5	7 d
	VIB	Chlyll P $\xrightarrow{1hr}$ $K_2Cr_2O_7$	1.5 → 20	24 hr
	VIIA	Ext	—	7 d
	VIIB	Ext $\xrightarrow{1hr}$ $K_2Cr_2O_7$	—	24 hr

*Five mice were used for each experimental set.
$K_2Cr_2O_7$, potassium dichromate; Chlyn, chlorophyllin; Chlyll E, extracted chlorophyll; Chlyll P, purified chlorophyll; Ext, leaf extract.

TABLE II. Data on Chromosomal Aberrations Induced by the Treatment Sets†

Set nos.	Chemicals administered	Total chromosomal aberrations G′	G″	B′	B″	RR	CA/cell (mean ± SD)	% DC (mean ± SD)
I	Distilled water	7	—	4	—	3	0.02 ± 0.0141	1.2 ± 0.894
II	Mitomycin C	29	6	100	3	8	0.244 ± 0.025*	15.4 ± 1.67*
III	$K_2Cr_2O_7$	27	—	18	1	7	0.068 ± 0.0148*	5.2 ± 1.643*
IVA	Chlorophyllin	13	—	4	—	3	0.02 ± 0.0122	1.2 ± 0.441
IVB	Chlyn → $K_2Cr_2O_7$	11	—	9	—	2	0.026 ± 0.0144	2.2 ± 0.836
VA	Extracted chlorophyll	8	—	25	—	—	0.05 ± 0.00894*	4.6 ± 0.8*
VB	Chlyll E → $K_2Cr_2O_7$	9	—	24	1	—	0.052 ± 0.00748	4.8 ± 0.748
VIA	Purified chlorophyll	7	—	21	—	—	0.042 ± 0.004*	4.2 ± 0.4*
VIB	Chlyll P → $K_2Cr_2O_7$	4	—	19	1	—	0.042 ± 0.011	3.8 ± 0.748
VIIA	Leaf extract	14	—	8	—	3	0.028 ± 0.00836	2.2 ± 0.836
VIIB	Ext → $K_2Cr_2O_7$	12	—	5	—	5	0.03 ± 0.00707	2.0 ± 0.

†G′, G″, chromatid and isochromatid gaps; B′, B″, chromatid and isochromatid breaks; RR, rearrangements; CA, chromosomal aberrations; DC, damaged cells. See Table I for other abbreviations.
*$P \leq .001$.

of the clastogen, potassium dichromate, and observed after 24 hours. Both chlorophyllin (set IVA) and crude leaf extract (set VIIB) significantly reduced the clastogenic effects of potassium dichromate. The frequency of CA/cell induced by potassium dichromate (0.068) was reduced to 0.026 by chlorophyllin and 0.03 by the crude extract. Chlorophyll alone, however, whether extracted (set VB) or in the pure from (set VIB), was much less effective, and reduced CA/cell only to their respective levels of clastogenicity.

Table III shows Duncan's multiple range tests. For both endpoints, chromosomal aberrations per cell and percentage of damaged cells, a linear correlation is observed between experimental sets with nonsignificant clastogenic effects (distilled water, chlorophyllin; crude extract plus potassium dichromate; crude extract alone and chlorophyllin plus potassium dichromate). Significant to highly significant effects are observed after exposure to pure chlorophyll (P) alone, pure chlorophyll (P) plus potassium dichromate; extracted chlorophyll alone (E); extracted chlorophyll (E) plus potassium dichromate and potassium dichromate alone in an ascending order.

DISCUSSION

It was observed by our group that a low dose of chlorophyllin (1.5 mg/kg body wt) significantly reduced the clastogenic activity of a variety of chemicals, including inorganic heavy metals in mice in vivo [A. Ghosh et al., 1991; A.K. Ghosh et al., 1991a,b; Palit et al., 1991; Sen et al., 1991; Sarkar et al., 1993].

When mice were fed aqueous crude leaf extract of Indian spinach (*Beta vulgaris* var. *benghalensis* Hort.) for 24 hours to daily for 7 days, the frequency of chromosomal aberrations and damaged cells induced by potassium dichromate was also reduced significantly. An equivalent amount of chlorophyll extracted from the leaf

TABLE III. Duncan's Multiple Range Tests*

For chromosomal aberrations/cell

Experimental sets	Dist. water	Chlyn	Chlyn ↓ $K_2Cr_2O_7$	Ext	Ext ↓ $K_2Cr_2O_7$	Chlyll P	Chlyll P ↓ $K_2Cr_2O_7$	Chlyll E	Chlyll E ↓ $K_2Cr_2O_7$	$K_2Cr_2O_7$
Sample means	0.02	0.02	0.02	0.028	0.03	0.042	0.042	0.05	0.052	0.068

For % damaged cells

Experimental sets	Dist. water	Chlyn	Ext ↓ $K_2Cr_2O_7$	Ext	Chlyn ↓ $K_2Cr_2O_7$	Chlyll P ↓ $K_2Cr_2O_7$	Chlyll P	Chlyll E	Chlyll E ↓ $K_2Cr_2O_7$	$K_2Cr_2O_7$
Sample means	1.2	1.2	2.0	2.2	2.2	3.8	4.2	4.6	4.8	5.2

*The straight lines denote insignificant differences between the means at $P \leq .05$. See Table I for abbreviations.

could not reduce the effects and was clastogenic itself [Sarkar et al., 1995, 1996a].

The work was therefore extended to include pure chlorophyll purchased from Sigma Chemical (USA). The results show that pure chlorophyll also was significantly clastogenic and reduced the effect of the toxicant potassium dichromate only slightly (see Tables II and III).

The antimutagenic and anticlastogenic effects of extracts of green vegetables against several environmental toxicants had been earlier attributed to chlorophyll, because of its presence in all green plant parts [Abraham et al., 1986; Arimoto et al., 1980a,b; Lai et al., 1980; Kimm et al., 1982; Katoh et al., 1983; Terwel and van der Hoeven, 1985; Hayatsu et al., 1988]. These works, however, used chlorophyll as a part of the plant extract. Chlorophyllin, in which the magnesium atom is replaced by copper, was also shown to have similar properties [Ong et al., 1986, 1989; Whong et al., 1988; Renner et al., 1990; Sen et al., 1991; Warner et al., 1991].

The protective action of plant leaf extract and chlorophyllin against genotoxic activity of potassium dichromate has been shown by us to be against other genotoxicants as well [Sarkar et al., 1993; Sarkar et al., 1995]. The clastogenic effects of chlorophyll when given alone to mice in vivo, however, had not been recorded earlier.

Possible Mode of Action

The nonclastogenic and protectant effects of chlorophyllin and crude leaf extract and the clastogenic activities of chlorophyll, both extracted and pure, may be attributed to their structure and mode of action when administered in the diet in vivo.

Chlorophyllin is a synthetic derivative of chlorophyll formed by alkaline hydrolysis, where the phytyl tail and alkaline radicals of carbomethoxy groups of chlorophyll are replaced by sodium. The bound magnesium is very unstable and can be easily replaced by other heavy metals. The protection given by chlorophyllin against the effects of mutagens and clastogens may be attributed to its ability to scavenge free radicals, to bind to the active site of the mutagen and to adsorb or absorb toxic compounds [Hayatsu et al., 1988; Dhir, 1989; Sharma, 1990; Sarkar et al., 1993]. It also has been shown to trap xenobiotics with polycyclic planar structure by complex formation through the planar surfaces of the molecules [Arimoto et al., 1993].

Chlorophylls are porphyrin structures with a core of magnesium and the formation of a fifth ring by the linkage of position 6 to the y-methine bridge. They are relatively unstable and in solution, there is a tendency for oxidation or isomerisation to occur with replacement of H at position 10 by —OH or —OCH_3, leading to the formation of free radical. Chlorophyll molecules are also known to act as both electron acceptors and donors in charge transfer complexes [Katz et al., 1977]. The only earlier record of clastogenic effects of extracted chlorophyll was on *Allium cepa* roots [Sharma and Majumdar 1959, Sharma and Datta, 1962]. No report is yet available of its clastogenic effects on animal systems.

Previous workers had mostly related the inhibitory action of plant extracts against genotoxicants to their chlorophyll content, which was reported as nontoxic [Lai et al., 1980; Ong et al., 1986]. It was also reported to be an antioxidant [Sato et al., 1984]. Presumably the data were based on effects of the extract containing chlorophyll, rather than isolated chlorophyll alone. Sato and coworkers [1990] had suggested that only 20% of the antimutagenic effects of aquatic plant extracts was contributed by chlorophyll. Gentile and Gentile [1991] also observed that the concentration of chlorophyll is a vital factor to be taken into account while testing plants for antimutagenic action. Barale and associates [1983] had observed that lettuce with higher chlorophyll content (5.5 mg/kg body wt) was less effective as an antimutagen in the intragastric system than spinach leaves with less chlorophyll (3.0 mg/kg body wt).

In our experiments, administration of the individual plant products, crude extract, extracted or pure chlorophyll or chlorophyllin as a dietary supplement for 24 hours to 7 days, possibly resulted in binding at the sites for the activity of dichromate or Cr(VI), making them not available.

Our observation here of the strong chromosome-damaging activity of chlorophyll, whether extracted directly from the leaf of Indian spinach or obtained in a commercially purified form, therefore, is a significant deviation from the general reports on chlorophyll as an antimutagenic, anticlastogenic, or anticarcinogenic agent. The protective action of green plant parts against genotoxic effects therefore cannot be attributed to chlorophyll itself, which possibly acts as a free radical. Such protection is afforded by the complex mixture of the crude leaf extract, the ingredients of which include a variety of antioxidants, like vitamins, ascorbic acid, polyphenols, and fibres. These interact and neutralize the genotoxic effects of chlorophyll [Sarkar et al., 1995, 1996b]. The sum total of the interactions between the constituents and with the genotoxicant, leads to the protective effect observed against environmental xenobiotics.

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