Carcinogenesis vol.17 no.7 pp.1459–1463, 1996

Modulatory influence of chlorophyllin on the mouse skin papillomagenesis and xenobiotic detoxication system

Anjali Singh, Satya P.Singh and Ramesh Bamezai[1]

Human Genetics Laboratory, School of Life Sciences, Jawaharlal Nehru University, New Delhi-110067, India

[1]To whom correspondence should be adressed

The present study evaluates the modulatory potential of chlorophyllin (CHL) on the murine skin papillomagenesis pattern and its influence on the levels of biotransformation system enzymes. Topical application of CHL (100 mg/kg body weight/day) during peri-, post- or peri- and post-initiational stages of 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced papillomagenesis, significantly ($P < 0.01$) reduced the (i) tumor burden to 3.68, 3.56 and 3.33 (positive control value: 5.89); (ii) cumulative number of papillomas to 59, 57 and 60 (positive control value: 112); and (iii) incidence of mice bearing papillomas to 88%, 88% and 90%, respectively (positive control value 100%). CHL treatment alone or during peri-, post-, or peri- and post-initiational stages significantly elevated the glutathione S-transferase (GST) and -SH levels in the liver and skin tissue of the murine system. The potential of CHL in modulating the process of carcinogenesis is suggested by the altered levels of biotransformation system enzymes. The implications of the biochemical changes and inhibition of tumor incidence by CHL are discussed.

Introduction

One approach to cancer chemoprevention involves the administration of natural and synthetic nutrient or non-nutrient compounds in order to examine their potential role in the prevention of initiational and/or promotional stages of carcinogenesis (1). A number of these chemopreventive agents are capable of modulating the biotransformation system enzymes which play an important role in the activation, conjugation and subsequent excretion of xenobiotics including carcinogens (2).

The significant role of chlorophyllin (CHL*), the sodium copper salt of chlorophyll, as a food additive (3) and in the treatment of geriatric patients (4) has been duly recognized. *In vivo* CHL exerts profound action as an anticlastogen (5) and radioprotectant (6) in murine system. Under *in vitro* conditions CHL acts as the modifier of mutagenecity (7). Inhibition of metabolic activation enzymes (8), scavenging of free radicals (9) and complex formation with reactive moieties of promutagens (10) have been suggested as the probable mode(s) of action of CHL. In view of the antimutagenecity and radioprotective ability of CHL, its (i) chemopreventive efficacy on murine skin papillomagenesis model; and (ii) potential role in modulating the biotransformation system enzymes in liver and skin tissue of mice has been undertaken in the present study.

Abbreviations: CHL, chlorophyllin; DMBA, 7,12-dimethylbenz[*a*]-anthracene; BSA, bovine serum albumin; CDNB, 1-chloro-2,4-dinitrobenzene; G-250, coomassie brilliant blue; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GSH, glutathione reduced; NADH, nicotinamide adenine dinucleotide reduced; GST, glutathione S-transferase; Cyt. b_5, cytochrome b_5; Cyt. P-450, cytochrome P-450.

Materials and methods

Animals

Random bred male Swiss albino mice (8 weeks old) obtained from Jawaharlal Nehru University Animal Facility (New Delhi, India) were maintained in an air-conditioned room and provided with standard food pellets (Hindustan Lever Ltd., India) and tap water *ad libitum*.

Chemicals

Bovine serum albumin (BSA), 1-chloro-2,4-dinitrobenzene (CDNB), chlorophyllin (CHL), croton oil, 7,12-dimethylbenz[*a*]anthracene (DMBA), coomassie brilliant blue (G-250), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), glutathione reduced (GSH), nicotinamide adenine dinucleotide reduced (NADH) and 5-sulfosalicylic acid were obtained from Sigma Chemical Co., St Louis, USA. All other chemicals used were of highest available purity grade obtained from local firms.

Experimental design

Experiment 1: Chemopreventive potential of CHL on mouse skin papillomagenesis. Group 1: Animals were topically treated with a single subcarcinogenic dose of DMBA (50 µg/100 µl acetone). Two weeks after the carcinogen treatment, thrice weekly application of croton oil (100 µl of 1% croton oil in acetone) was given for 18 weeks to serve as positive control group.

Group 2: Animals received topical treatment of CHL (100 mg/kg body weight/100 µl acetone/day) 1 week before and 2 weeks after the application of DMBA. Croton oil was applied as for Group 1.

Group 3: The treatment pattern of DMBA and croton oil simulated Group 1. Topical application of CHL was given during promotional stage which initiated from the time of croton oil treatment.

Group 4: Animals were topically treated with CHL throughout the experimental period including 1 week before and 2 weeks after DMBA application followed by the application of croton oil in the promotion stage.

Group 5: Animals received CHL (100 mg/kg body weight/100 µl acetone/day) treatment alone during the experimental period. The animals were not treated with DMBA and croton oil protocol for tumor induction, in order to serve as the negative control group.

Three days before the commencement of the experiment, the dorsal skin in the interscapular area was shaved and animals showing no hair regrowth were used in the experiment. The body weight of each animal and the papillomas appearing on the shaved area of the skin were recorded at weekly intervals. Animals were sacrificed 21 weeks after the commencement of the treatments.

To evaluate the statistical significance of the data, in terms of tumor burden, cumulative number of papillomas induced and percentage of mice with papillomas between control and experimental groups, ANOVA was performed.

Experiment 2: Modulatory influence of CHL on mouse liver and skin detoxication system. The animals were sacrificed by cervical dislocation and their liver as well as skin tissue was excised, after thorough perfusion with chilled normal saline (0.9%), and 10% (w/v) homogenate of each tissue per animal was obtained using buffer containing 0.154 M KCl and 50 mM Tris–HCl (pH 7.4). A part of the homogenate was used for -SH group assay and the rest was centrifuged at 10 086 *g* for 20 min. The resultant supernatant was further centrifuged at 105 907 *g* for 60 min. The supernatant obtained through this second centrifugation formed the cytosolic (soluble) fraction and was used for the glutathione S-transferase (GST) assay. The pellet formed in the differential centrifugation (microsomal fraction) was resuspended in the homogenizing buffer to be used for assays of cytochrome b_5 (Cyt. b_5) and cytochrome P-450 (Cyt. P-450). All the steps were carried out at 0–4°C. Protein contents in cytosolic and microsomal fractions of each sample were determined by the Bradford (11) method using BSA as standard. The cytosolic GST activity was determined spectrophotometrically following the procedure described by Habig *et al.* (12). The activities of Cyt. P-450 and Cyt. b_5 were measured spectrophotometrically according to the procedure of Omura and Sato (13) in the microsomal suspension of 0.5–1.0 mg protein/ml. The

Table I. Effect of CHL on the mouse skin tumorigenesis model system[a]

Group	Treatment			Animal no		Body weight (g) mean ± SE		Tumor size (mm)					
	Modulator	Initiator	Promoter	Initial	Final	Initial	Final	1	2	3	4	5	6
1	Nil	DMBA	Croton oil	20	19	25.24 ± 0.41	30.60 ± 0.18	20	32	40	6	10	4
2	CHL[a]	DMBA	Croton oil	20	18	24.86 ± 0.32	31.20 ± 0.22	17	16	18	2	4	2
3	CHL[b]	DMBA	Croton oil	20	18	25.12 ± 0.36	30.80 ± 0.26	12	23	14	2	2	4
4	CHL[c]	DMBA	Croton oil	20	20	26.62 ± 0.38	31.00 ± 0.42	10	14	20	11	5	0
5	CHL	Nil	Nil	20	19	26.38 ± 0.47	32.22 ± 0.38	0	0	0	0	0	0

[a]Peri-initiational
[b]Post-initiational
[c]Peri- and post-initiational
*Treatment schedule of the groups is specified in Materials and methods

Table II. Effect of CHL on the mouse hepatic and skin levels of Cyt b_5 and Cyt P-450

Group	Treatment			Liver		Skin	
	Modulator	Initiator	Promoter	Cytochrome b_5 (nmol/mg protein)	Cytochrome P-450 (nmol/mg protein)	Cytochrome b_5 (nmol/mg protein)	Cytochrome P-450 (nmol/mg protein)
1	Nil	DMBA	Croton oil	0.44 ± 0.02	0.57 ± 0.03	0.21 ± 0.02	0.28 ± 0.02
2	CHL[a]	DMBA	Croton oil	0.45 ± 0.01	0.56 ± 0.01	0.19 ± 0.03	0.29 ± 0.02
3	CHL[b]	DMBA	Croton oil	0.47 ± 0.02	0.59 ± 0.02	0.23 ± 0.01	0.31 ± 0.02
4	CHL[c]	DMBA	Croton oil	0.47 ± 0.01	0.61 ± 0.02	0.23 ± 0.03	0.33 ± 0.03
5	CHL	Nil	Nil	0.48 ± 0.02	0.61 ± 0.01	0.26 ± 0.02	0.33 ± 0.02

[a]Peri-initiational
[b]Post-initiational
[c]Peri- and post-initiational
*Treatment schedule of the groups is specified in Materials and methods
Values are expressed as mean ± SE; animals per point (*n*) = six

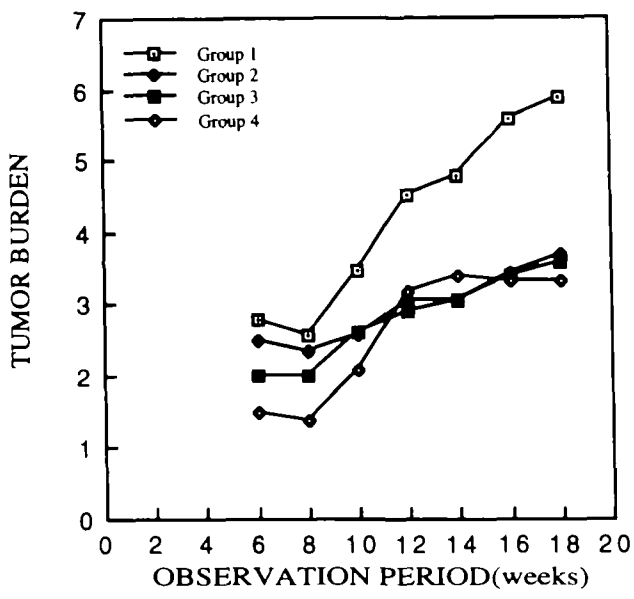

Fig. 1. Tumor burden (average number of tumors per tumor bearing mouse) documented during the observation period. Treatment schedule of the groups is specified in Materials and methods.

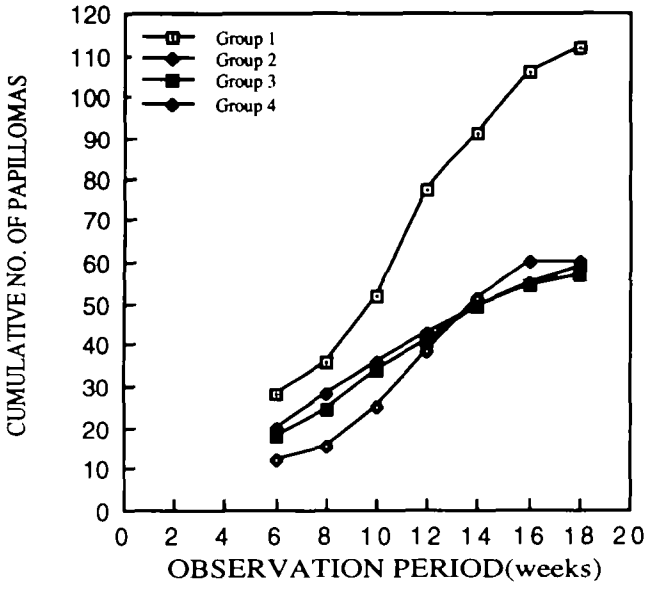

Fig. 2. Cumulative number of papillomas documented during the observation period. Treatment schedule of the groups is specified in Materials and methods.

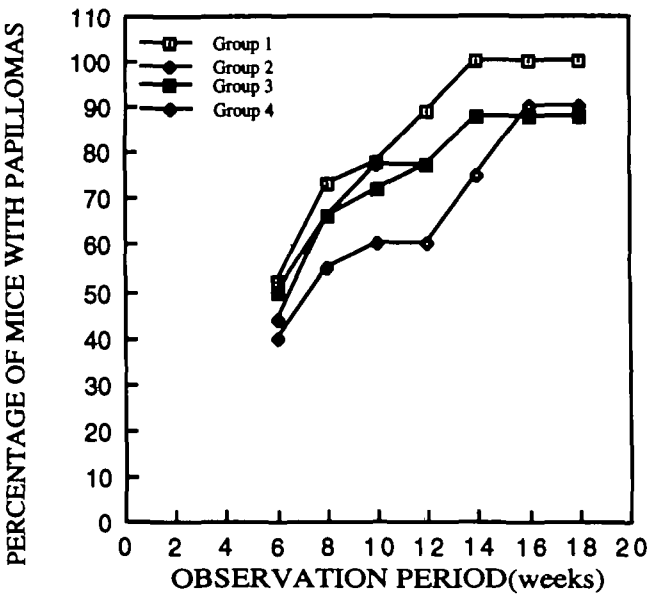

Fig. 3. Percentage of mice with papillomas documented during the observation period. Treatment schedule of the groups is specified in Materials and methods.

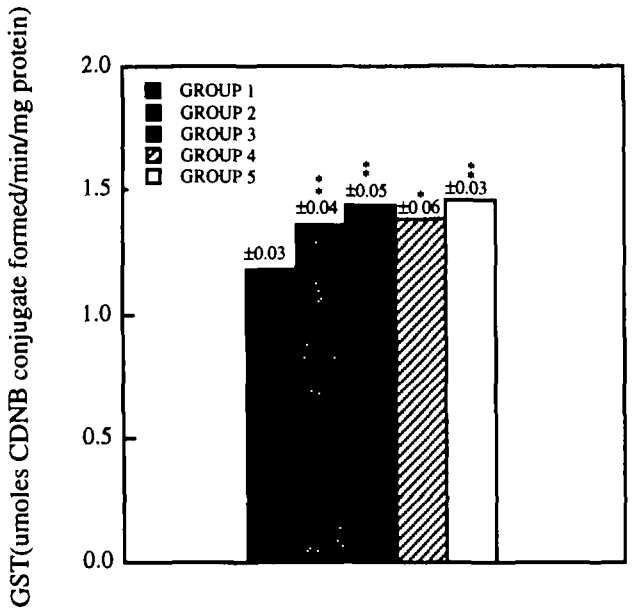

Fig. 4. Modulation of hepatic GST level (mean ± SE) by CHL in mice. Treatment schedule of the groups is specified in Materials and methods. $n = 6$; $^{*}P < 0.05$; $^{**}P < 0.01$.

estimation of -SH content was done by Ellman's (14) method as modified by Sparnins *et al.* (15).

Results

Experiment 1: skin papillomagenesis study

The different treatment schedules of CHL did not appreciably alter the mortality rate and the average body weight gain in the murine system (Table I).

The tumor burden (Figure 1), cumulative number of papillomas induced (Figure 2) and the percentage of mice with papillomas (Figure 3) during the observation period have been depicted to compare the CHL-induced alterations on the pattern and extent of murine skin papillomagenesis.

Topical application of CHL during peri- (Group 2), post-

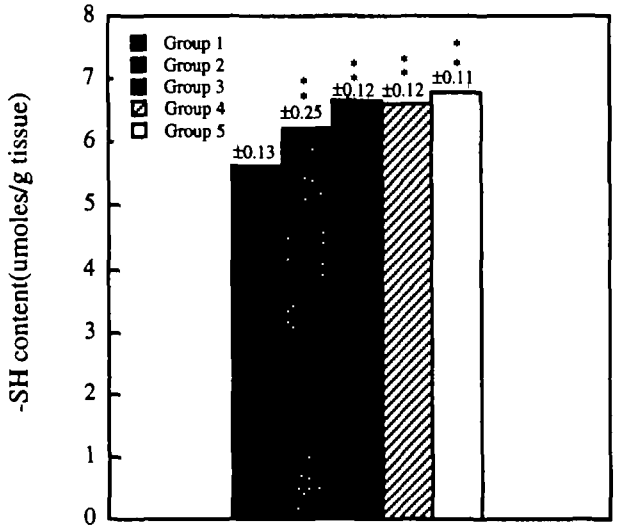

Fig. 5. Modulation of hepatic -SH level (mean ± SE) by CHL in mice. Treatment schedule of the groups is specified in Materials and methods. $n = 6$; $^{**}P < 0.01$.

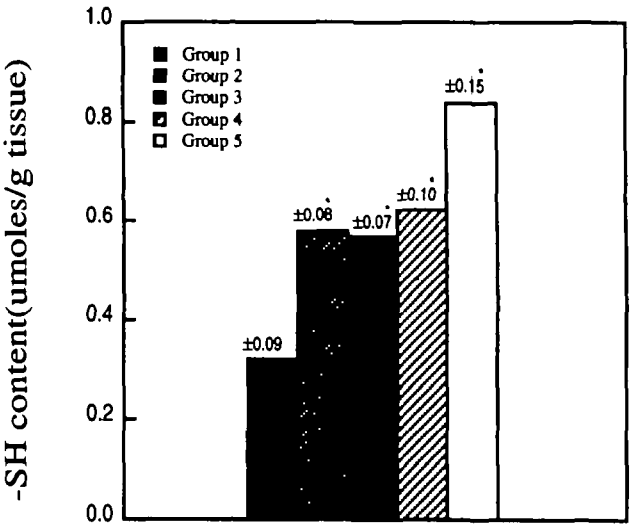

Fig. 6. Modulation of -SH level (mean ± SE) by CHL in skin tissue of mice. Treatment schedule of the groups is specified in Materials and methods. $n = 6$; $^{*}P < 0.05$.

(Group 3) or peri- and post-(Group 4) initiational stages of DMBA-induced papillomagenesis, significantly ($P < 0.01$) reduced the tumor burden to 3.68, 3.56 and 3.33 respectively (positive control value 5.89) while the cumulative numbers of papillomas were reduced to 59, 57 and 60 respectively (positive control value 112). However, the papilloma size between the positive control (Group 1) and experimental groups (Groups 2–4) did not vary significantly (Table I). The mice assorted in Groups 2–4 and given two stage protocol for tumor induction revealed 88% (16 mice out of 18; Group 2), 88% (16 mice out of 18; Group 3) and 90% (18 mice out of 20; Group 4) skin papillomas while the respective figure for positive control (19 mice out of 19; Group 1) was 100%.

No skin papillomas appeared in the animals topically treated only with CHL (Group 5) during the observation period.

Experiment 2: biochemical studies

CHL treatment alone (Group 5) or during peri- (Group 2), post- (Group 3) or peri- and post- (Group 4) initiational stages

significantly ($P < 0.01$) elevated the hepatic GST (Figure 4) and -SH (Figure 5) levels when compared with positive control (Group 1) in murine system. Even in skin tissue of mouse, the topical treatment of CHL (Groups 2–5 versus Group 1) could appreciably ($P < 0.05$) enhance the -SH content (Figure 6). However, the selected dose of CHL could not modulate the skin and hepatic levels of Cyt. b_5 and Cyt. P-450 in the experimental groups (Groups 2–5 versus Group 1, Table II).

Discussion

Epidemiological, laboratory and clinical investigations have suggested the role of dietary compounds as important modifiers in the process of chemical carcinogenesis (2,16,17). Our previous studies have also supported the crucial role of minor dietary constituents including mace (18), black mustard (19), garlic (20) and curcumin (21) in modulating the chemical carcinogenesis in murine systems. A number of these dietary compounds have been observed to influence skin carcinogenesis in experimental animal models or in humans (2).

Chemical carcinogenesis in murine skin has been conceptualized as a stepwise process, consisting of initiation, promotion and progression (22). Biotransformation of most of the chemical initiators to reactive electrophilic species and the generation of free radicals during inflammatory response of tumor promoters, assigns a clear role to modulators of biotransformation pathways in the process of chemical carcinogenesis (2).

The findings of the present study suggest the potential of CHL, an anticlastogen (5) and a radioprotectant (6), to act as a blocking agent by modulating the pattern of murine skin papillomagenesis and its probable correlation with the altered levels of biotransformation system enzymes. Inhibitory effects of CHL on PhIP-induced mammary carcinogenesis (23), 2-amino-3-methylimidazo[4,5-*f*]quinoline-induced tumorigenesis (24) and aflatoxin B_1-induced hepatocarcinogenesis (25) have also been observed in experimental animals.

It was interesting to observe the ability of CHL modulator to result in the absence of skin papillomas in 10–12% mice during peri- (Group 2), post- (Group 3) or peri- and post- (Group 4) initiational stages of carcinogenesis. This observation was in contrast to the positive control (Group 1) where none of the mice was found without skin papillomas. The chemopreventive role of CHL was also substantiated via appreciable decrease in the tumor burden by 56–62% and cumulative number of papillomas by 50–53% in the experimental Groups 2–4 as compared to the positive control, Group 1.

As modulation of phase I and phase II enzymes of biotransformation system projects it to be a critical determinant of chemical carcinogenesis (2), the observed augmentation in the levels of GST and -SH in murine liver and skin tissue of experimental animals (Groups 2–4) as compared to positive control (Group 1) potentially may offer chemoprotection at the (i) initiation level via scavenging the reactive moieties of tumor initiator(s) which in turn could bind covalently to cellular macromolecules (26); and (ii) promotion level by enhancing the availability of non-critical nucleophiles for inactivation of electrophiles and reactive oxygen species generated by the phorbol ester present in croton oil (2). Furthermore, the compounds possessing antioxidant or anti-inflammatory activity including butylated hydroxyanisole (27), α-tocopherol (28) and ascorbic acid (29) have also been reported to inhibit the events associated with tumor promotion. The modulatory efficacy of CHL on the mouse skin papillomagenesis and xenobiotic detoxication system was observed to be similar in the respective groups of experimental design (Groups 2–4 versus Group 1) which in turn suggests its chemoprotection ability during both initiation and promotional stages of carcinogenesis.

The unaltered levels of Cyt. b_5 and Cyt. P-450 on topical application of CHL further support the chemoprotective role of CHL by non-elevation of bioactivated electrophiles of potential toxins in cellular milieu (30). Antimutagenic (5), radioprotective (6) and antioxidant (9) potential of CHL against environmental toxicants, its divergent mechanisms of action (8,10) and absence of noticeable toxic effects either in humans or animal species suggest it to be a potential chemopreventive agent. Further studies evaluating the pre- and post-natal modulatory efficacy of CHL on biochemical events involved in initiation and promotion of tumorigenesis in murine system are in progress.

Acknowledgements

Financial assistance by the Council for Scientific and Industrial Research, India to Anjali Singh and by the University Grants Commission to Satya P.Singh as Research Associates is gratefully acknowledged.

References

1 Boone,C.W., Kelloff,G.J. and Malone,W.E. (1990) Identification of candidate cancer chemopreventive agents and their evaluation in animal models and human clinical trials: A Review. *Cancer Res.*, **50**, 2–9.
2 Wattenberg,L.W. (1992) Inhibition of carcinogenesis by minor dietary constituents. *Cancer Res.*, **52s**, 2085–2091.
3 Negishi,T., Arimoto,S., Nishizaki,C. and Hayatsu,H. (1989) Inhibitory effect of chlorophyll on the genotoxicity of 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole (Trp-P-2). *Carcinogenesis*, **10**, 145–149.
4 Tawashi,R., Cousineau,M. and Denis,G. (1982) Crystallization of calcium oxalate dihydrate in normal urine in the presence of sodium copper chlorophyllin. *Urol. Res.*, **10**, 173–176.
5 Sarkar,D., Sharma,A. and Talukder,G. (1993) Differential protection of chlorophyllin against clastogenic effects of chromium and chlordane in mouse bone marrow *in vivo*. *Mutat. Res.*, **301**, 33–38.
6 Abraham,S.K., Sharma,L. and Kesavan,P.C. (1994) Role of chlorophyllin as an *in vivo* anticlastogen: protection against gamma-radiation and chemical clastogen. *Mutat. Res.*, **322**, 209–212.
7 Romert,L., Curvall,M. and Jenssen,D. (1992) Chlorophyllin is both a positive and negative modifier of mutagenicity. *Mutagenesis*, **7**, 341–355.
8 Tachino,N., Guo,D., Dashwood,W.M., Yamane,S., Larsen,R. and Dashwood,R. (1994) Mechanisms of the *in vitro* antimutagenic action of chlorophyllin against benzo[*a*]pyrene: studies of enzyme inhibition, molecular complex formation and degradation of the ultimate carcinogen. *Mutat. Res.*, **308**, 191–203.
9 Sato,M., Imai,K., Kimura,R. and Murata,T. (1984) Effect of sodium copper chlorophyllin on lipid peroxidation. VI. Effect of its administration on mitochondria and microsomal lipid peroxidation in rat liver. *Chem. Pharm. Bull.*, **32**, 715–722.
10 Dashwood,R.H. and Guo,D. (1993) Antimutagenic potency of chlorophyllin in the *Salmonella* assay and its correlation with binding constants of mutagen-inhibitor complexes. *Environ. Mol. Mutagen.*, **22**, 164–171.
11 Bradford,M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.
12 Habig,W.H., Pabst,M.J. and Jakoby,W.B. (1974) Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *J. Biol. Chem.*, **249**, 7130–7139.
13 Omura,T. and Sato,R. (1964) The carbon monoxide binding pigment of liver microsomes. *J. Biol. Chem.*, **239**, 2370–2378.
14 Ellmann,G.L. (1959) Tissue sulfhydryl groups. *Arch. Biochem. Biophys.*, **82**, 70–77.
15 Sparnins,V.L., Venegas,P.L. and Wattenberg,L.W. (1982) Glutathione S-transferase activity: enhancement by compounds inhibiting chemical carcinogenesis and by dietary constituents. *J. Natl. Cancer Inst.*, **68**, 493–496.
16 Sharma,S., Stutzman,J.D., Kelloff,G.J. and Steele,V.E. (1994) Screening

of potential chemopreventive agents using biochemical markers of carcinogenesis. *Cancer Res.*, **54**, 5848–5855.
17. Singh,A., Singh,S.P. and Bamezai, R. (1995) Postnatal modulation of hepatic biotransformation system enzymes via translactational exposure of F_1 mouse pups to turmeric and curcumin. *Cancer Lett.*, **96**, 87–93.
18. Singh,A. and Rao,A.R. (1993) Modulatory effect of areca nut on the action of mace (*Myristica fragrans*, Houtt) on the hepatic detoxification system in mice. *Food Chem. Toxicol.*, **31**, 517–521.
19. Singh,A. and Rao,A.R (1993) Effect of arecanut on the black mustard (*Brassica niger*, L.)-modulated detoxication enzymes and sulfhydryl content in the liver of mice. *Cancer Lett.*, **72**, 45–51.
20. Singh,A. and Rao,A.R. (1995) Evalution of the modifying influence of arecanut on the garlic-modulated hepatic detoxication system enzymes,sulhydryl content, and lipid peroxidation in mice. *Teratogen. Carcinogen. Mutagen.*, **15**, 127–134.
21. Singh,A., Singh,S.P. and Bamezai,R. (1996) Effect of arecoline on the curcumin-modulated hepatic biotransformation system enzymes in lactating mice and translactationally exposed F_1 pups. *Nutr.Cancer*, **25**, 101–110.
22. Agarwal,R. and Mukhtar,H. (1988) Cutaneous chemical carcinogenesis. In Mukhtar,H. (ed.), *Pharmacology of the Skin*. CRC Press, Boca Raton, FL, pp. 371–387.
23. Hasegawa,R., Hirose,M., Kato,T., Hagiwara,A., Boonyaphiphat,P., Nagao,M., Ito,N. and Shirai,T. (1995) Inhibitory effect of chlorophyllin on PhIP-induced mammary carcinogenesis in female F344 rats. *Carcinogenesis*, **16**, 2243–2246.
24. Guo,D.X., Horio,D.T., Grove,J.S. and Dashwood,R.H. (1995) Inhibition by chlorophyllin of 2-amino-3-methylimidazo[4,5-*f*]quinoline-induced tumorigenesis in the male F344 rat. *Cancer Lett.*, **95**, 161–166.
25. Breinholt,V., Hendricks,J., Pereira,C., Arbogast,D. and Bailey,G. (1995) Dietary chlorophyllin is a potent inhibitor of aflatoxin B_1 hepatocarcinogenesis in rainbow trout. *Cancer Res.*, **55**, 57–62.
26. Rotstein,J.B. and Slaga,T.J.(1988) Anticarcinogenesis mechanisms as evaluated in the multistage mouse skin model. *Mutat Res.*, **202**, 421–427.
27. Copeland,E.S. (1983) A national institute of health workshop report. Free radicals in promotion—a chemical pathology study section workshop. *Cancer Res.*, **43**, 5631–5637.
28. Perchellet,J.-P., Owen,M.D., Posey,T.D., Orten,D.K. and Schneider,B.A. (1985) Inhibitory effects of glutathione level-raising agents and D−α-tocopherol on ornithine decarboxylase induction and mouse skin tumor promotion by 12-*O*-tetradecanoylphorbol-13-acetate. *Carcinogenesis*, **6**, 567–573.
29. Smart,R.C., Huang,M-T., Han,Z.T., Kaplan,M.C., Focella,A and Conney,A.H. (1987) Inhibition of ornithine decarboxylase activity, DNA synthesis, and tumor promotion in mouse skin by ascorbic acid and ascorbyl palmitate. *Cancer Res.*, **47**, 6633–6638.
30. Yang,C.S., Smith,T.J. and Hong,J.-Y. (1994) Cytochrome P-450 enzymes as targets for chemoprevention against chemical carcinogenesis and toxicity: opportunities and limitations. *Cancer Res.*, **54s**, 1982–1986.

Received on January 30, 1996; revised on March 29, 1996; accepted on April 3, 1996