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Red Dye No. 2 and the Red Pigment Carmine Enhance Aryl Hydrocarbon Hydroxylase and Guanylate Cyclase Activities

Marie H. Rochat and David L. Vesely

Department of Medicine
University of Arkansas for Medical Sciences
Little Rock, Arkansas 72205

and

Cell Biology Branch
National Center for Toxicological Research
Jefferson, Arkansas 72079

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SUMMARY: The food coloring amaranth (F, D and C red No. 2) and the red pigment carmine, but not erythrosine (F, D and C red No. 3), caused a twofold enhancement of two enzymes (aryl hydrocarbon hydroxylase and guanylate cyclase) that have been linked with chemical carcinogenesis. Dose response relationships revealed that carmine enhanced both enzymes to near maximal levels at concentrations as low as 1 to 10 nM while amaranth needed a concentration of 100 nM to cause a maximal enhancement. These data suggest that amaranth and carmine mimic the effects of some chemical carcinogens at the cellular level, but erythrosine does not mimic any of these effects.

Amaranth, Food, Drug and Cosmetics (F, D and C) red No. 2 is a food coloring which has been reported to have carcinogenic properties (1,2) and which has been banned in the United States and Russia but not in European Common Market countries, Australia, Canada or Japan. Since there is less than uniform agreement whether F, D and C red No. 2 is unsafe to add to foods, we undertook the following investigation of F, D and C red No. 2 with two enzymes which are thought to be involved in carcinogenesis. Aryl hydrocarbon hydroxylase (E.C.1.14.14.2) is a microsomal monooxygenase that has been implicated in the metabolism of certain chemical carcinogens to highly reactive electrophiles that react with cellular constituents to initiate a carcinogenic event (3). Guanylate cyclase (E.C.4.6.1.2), the enzyme that catalyzes the production of cyclic GMP from guanosine triphosphate, is thought to be important in both normal and abnormal growth (4-6). Both guanylate cyclase and its product

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cyclic GMP have been noted to interact with a wide variety of chemical carcinogens in vitro (7-9). In the present investigation, F, D and C red No. 2 as well as carmine, the pure red pigment from the female insect Coccus cacti, stimulated both enzymes while F, D and C red No. 3 (erythrosine) had no effect on either of these enzymes.

MATERIALS AND METHODS

Aryl Hydrocarbon Hydroxylase Assay: The livers of male Sprague Dawley rats were processed as previously described (8). Amaranth, erythrosine, carmine, new coccine and cochineal were obtained from Aldrich Chemical Company (Milwaukee, WI). The solvent for the food colorings used in the enzyme assays was triple distilled water. Aryl hydrocarbon hydroxylase was measured by the fluorescent assay method of Dehnen et al. (10). In this assay, the reaction mixture consisted of 5 mM sodium isocitrate, 5 mM $MgCl_2$, 0.005 mM $MnCl_2$, 80 μM benzo(a)pyrene, 0.8 units/ml isocitrate dehydrogenase, and 0.3 mg/ml rat liver microsomes in 0.05 M Tris HCl buffer (pH 7.4). These fluorescent assays were initiated by addition of 1 mM NADPH and were terminated by addition of aliquots of the reaction mixture to an equal volume of chilled acetone. The specific enzymatic activity was obtained using the linear portion of several time points between 0 and 5 minutes at 37°C. After centrifugation the supernatant was added to triethylamine. The fluorimetric measurements (λex = 467 nM, λeme = 518 nM) were performed immediately. All experiments were the average of nine microsomal fraction preparations.

Guanylate Cyclase Assay: Rat liver was obtained for the guanylate cyclase assay from the same Sprague Dawley rats utilized for the above two assays. Guanylate cyclase activity was determined as previously described (11,12). Liver was homogenized in cold 0.03 M Tris HCl, pH 7.6, and centrifuged at 37,000 g at 4°C for 15 minutes. The supernatant was then assayed at 37°C for guanylate cyclase activity using a reaction mixture consisting of 20 mM Tris HCl, pH 7.6; 4 mM $MnCl_2$; 2.67 mM cyclic GMP; a GTP regenerating system (5 mM creatine phosphate, 11.25 U creatine phosphokinase, E.C.2.7.3.2); 100 μg bovine serum albumin; 20 mM caffeine; 1.2 mM [α^{32}P] GTP approximately 5 x 10^5 cpm; and the enzyme preparation having 0.1 to 0.2 mg protein. After a 10 minute incubation, the reaction was terminated by the addition of 10 μl of 0.1 M EDTA, pH 7.6, containing about 30,000 cpm of [^{3}H] GMP (to estimate recovery in the subsequent steps) and boiling for three minutes. After cooling in an ice bath, the cyclic [^{32}P]-GMP formed was isolated by sequential chromatography on Dowex-50H$^+$ and alumina using the modification described in detail previously (11). Each of the respective agents was added to the supernatant without any preincubation.

RESULTS

Amaranth (F, D and C red No. 2) enhanced the activity of both aryl hydrocarbon hydroxylase and guanylate cyclase at the 0.1 μM concentration (Table I). This enhancement was approximately twofold for each of the respective enzymes. Cochineal, a red pigment derived from the female insect Coccus cacti, caused a similar maximal stimulation of each of these two enzymes to that observed with amaranth, but did so at a lower concentration of 0.001 μM. Since

Table I. Effect of 5 Red Dyes on Rat Liver Aryl Hydrocarbon Hydroxylase and Guanylate Cyclase Activities In Vitro

Food Coloring+	AHH Activity≠	Percent Change	Guanylate Cyclase*
AMARANTH			
0	0.86 ± 0.15	--	148 ± 8
0.001 µM	0.86 ± 0.18	--	172 ± 21
0.01 µM	0.94 ± 0.10	+ 10	197 ± 18
0.1 µM	2.02 ± 0.25**	+135	292 ± 15**
COCHINEAL			
0	0.95 ± 0.15	--	135 ± 9
0.001 µM	1.29 ± 0.10	+ 36	246 ± 16**
0.01 µM	1.90 ± 0.18**	+ 97	253 ± 20**
0.1 µM	1.95 ± 0.20**	+105	286 ± 22**
CARMINE			
0	0.92 ± 0.18	--	164 ± 10
0.001 µM	1.41 ± 0.20**	+ 53	336 ± 21**
0.01 µM	1.44 ± 0.20**	+ 56	343 ± 23**
0.1 µM	1.45 ± 0.25**	+ 58	329 ± 25**
NEW COCCINE			
0	0.92 ± 0.18	--	156 ± 11
0.001 µm	0.95 ± 0.18	+ 3	152 ± 17
0.01 µM	0.90 ± 0.15	- 2	161 ± 20
0.1 µM	0.92 ± 0.18	--	167 ± 22
ERYTHROSINE			
0	0.82 ± 0.10	--	168 ± 14
0.001 µM	0.56 ± 0.10	-32	196 ± 23
0.01 µM	0.62 ± 0.15	-25	199 ± 25
0.1 µM	0.86 ± 0.18	+ 5	209 ± 27

+ = Each value is the mean ± S.E.M. of triplicate samples on three animals confirmed in three separate experiments (n = 9)
≠ = Aryl hydrocarbon hydrolase activity is in nMoles of 3-OH benzo(a)pyrene formed / min/mg protein.
* = pMoles of cyclic GMP formed / mg protein/10 minute incubation.
** = Significant at $p < 0.001$ compared to control with Student's t test for unpaired values.

the red pigment cochineal is not a pure pigment, the pure red pigment isolated from cochineal, i.e., carmine, was utilized. Carmine likewise enhanced the activity of both enzymes at concentrations as low as 0.001 µM (Table I). New coccine, another cochineal pigment, did not augment either enzyme's activity (Table I). Erythrosine (F, D and C red No. 3) caused no significant enhancement of either enzyme at the same concentrations (Table I).

Dose response relationships on hepatic guanylate cyclase and aryl hydrocarbon hydroxylase revealed that carmine augmented both enzymes' activities to

TABLE II. The Effect of Varying the Manganese Concentration on Amaranth, Carmine and Cochineal's Augmentation of Hepatic Guanylate Cyclase Activity.

	Cyclic GMP (pmol/mg protein / 10 min incubation)*			
Manganese	Control	Amaranth, 100 nM	Cochineal, 100 nM	Carmine, 100 nM
0	40 ± 7	80 ± 8	82 ± 9	90 ± 11
1 mM	139 ± 9	228 ± 12	238 ± 14	249 ± 12
2 mM	143 ± 11	288 ± 16	292 ± 17	306 ± 18
3 mM	159 ± 14	312 ± 13	303 ± 22	319 ± 19
4 mM	163 ± 12	323 ± 15	319 ± 20	338 ± 21

*Each value is the mean ± S.E.M. of triplicate samples on three animals in three separate experiments (n = 9).

near maximal levels at concentrations as low as 1 to 10 nM, while amaranth needed a concentration of 100 nM to cause a maximal stimulation of both enzymes (Table I). There was no further enhancement with amarath, carmine or cochineal upon increasing the concentration of these respective agents to the mM range. Erythrosine and new coccine did not augment hepatic guanylate cyclase or aryl hydrocarbon hydroxylase activities at any concentration (Table I).

It has recently been reported that certain agents have no effect on guanylate cyclase activity under normal circumstances, but only have an effect when Mn^{2+} is absent from the incubation mixture (13). A series of experiments were done to determine whether manganese affected either amaranth, carmine or cochineal's activation of guanylate cyclase activity. In Table II, it is evident that varying the concentration of $MnCl_2$ had no effect on their enhancement of guanylate cyclase activity except that the activity of this enzyme was less when $MnCl_2$ was absent from the incubation mixture. There was no increase in either enzyme's activity with or without $MnCl_2$ present in incubation media containing erythrosine or new coccine (data not shown).

DISCUSSION

The present studies indicate that F, D and C red dye No. 2 (amaranth) does enhance the activity of two enzymes that have been associated with chemical carcinogenesis. Aryl hydrocarbon hydroxylase stimulation has been associated with both the detoxification of certain chemical carcinogens and their metabo-

lism to highly reactive electrophiles that interact with cellular constituents to initiate a carcinogenic event (3). Other studies indicate that the possible target of electrophilic free radicals within the cell is the enzyme guanylate cyclase (13,14). Thus, the electrophiles produced by aryl hydrocarbon hydroxylase stimulation may lead to activation of a final common pathway, i.e., the enzyme guanylate cyclase. The possibility must also be considered that the augmentation of guanylate cyclase activity by the respective agents causes the observed increased activity of aryl hydrocarbon hydroxylase. It is known that cyclic GMP enhances benz(a)anthracene-induced synthesis of aryl hydrocarbon hydroxylase in induction-suppressed confluent monolayers (15). Which enzyme is stimulated first cannot be determined from the present investigation.

If the interaction with guanylate cyclase is important in chemical carcinogenesis (7-9) the data presented indicate red dye No. 2 mimics the effects of carcinogens at the cellular level. Russian studies (1,2) indicate that amaranth may be a carcinogen and amaranth has also been shown to be toxic to the skeleton (16) and gonads (17). The present investigation would indicate that repeat long-term animal feeding studies of amaranth are necessary to determine its carcinogenic potential. It should be pointed out that although there is no direct correlation yet established between the amount of enhancement of guanylate cyclase activity and the potency of a chemical carcinogen, strong carcinogens such as methylnitrosourea enhance guanylate cyclase activity 30 to 40-fold (8). If long term feeding studies prove amaranth to be a carcinogen, one would expect it to be a weak carcinogen since we observed only a twofold augmentation of guanylate cyclase activity by amaranth.

Cochineal also enhanced guanylate cyclase activity in the present investigation and similar results were obtained with the pure red pigment carmine isolated from cochineal. New coccine, another cochineal pigment, was inactive, suggesting that cochineal's enhancement of these two enzymes was due to its pure red pigment carmine. Erythrosine (F, D and C red No. 3) caused no stimu-

lation in either of the enzymes in the present investigation. Thus, at the cellular level there is no biochemical evidence that red No. 3 is a carcinogen.

Finally, with respect to the mechanism by which amaranth, carmine and cochineal interact with the enzyme guanylate cyclase it has been suggested (13) that manganese, the guanylate cyclase cationic co-factor, may play an important role in the enzyme's interaction with various agents since superoxide dismutase had an effect on guanylate cyclase only when the Mn^{2+} was absent from the incubation mixture. However, varying the concentration of Mn^{2+} had no effect on the augmentation of guanylate cyclase activity in the present investigation.

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