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DNA Damage Induced by Red Food Dyes Orally Administered to Pregnant and Male Mice

Shuji Tsuda,*·[1] Makiko Murakami,* Naonori Matsusaka,* Kiyoshi Kano,† Kazuyuki Taniguchi,† and Yu F. Sasaki‡

*Laboratory of Veterinary Public Health, Department of Veterinary Medicine, Faculty of Agriculture, Iwate University, Ueda 3-18-8, Morioka, Iwate 020-8550, Japan; †Laboratory of Veterinary Anatomy, Department of Veterinary Medicine, Faculty of Agriculture, Iwate University, Ueda 3-18-8, Morioka, Iwate 020-8550, Japan; and ‡Laboratory of Genotoxicity, Faculty of Chemical and Biological Engineering, Hachinohe National College of Technology, Tamonoki Uwanotai 16-1, Hachinohe, Aomori 039-1192, Japan

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We determined the genotoxicity of synthetic red tar dyes currently used as food color additives in many countries, including Japan. For the preliminary assessment, we treated groups of 4 pregnant mice (gestational day 11) once orally at the limit dose (2000 mg/kg) of amaranth (food red No. 2), allura red (food red No. 40), or acid red (food red No. 106), and we sampled brain, lung, liver, kidney, glandular stomach, colon, urinary bladder, and embryo 3, 6, and 24 h after treatment. We used the comet (alkaline single cell gel electrophoresis) assay to measure DNA damage. The assay was positive in the colon 3 h after the administration of amaranth and allura red and weakly positive in the lung 6 h after the administration of amaranth. Acid red did not induce DNA damage in any sample at any sampling time. None of the dyes damaged DNA in other organs or the embryo. We then tested male mice with amaranth, allura red, and a related color additive, new coccine (food red No. 18). The 3 dyes induced DNA damage in the colon starting at 10 mg/kg. Twenty ml/kg of soaking liquid from commercial red ginger pickles, which contained 6.5 mg/10 ml of new coccine, induced DNA damage in colon, glandular stomach, and bladder. The potencies were compared to those of other rodent carcinogens. The rodent hepatocarcinogen *p*-dimethylaminoazobenzene induced colon DNA damage at 1 mg/kg, whereas it damaged liver DNA only at 500 mg/kg. Although 1 mg/kg of N-nitrosodimethylamine induced DNA damage in liver and bladder, it did not induce colon DNA damage. N-nitrosodiethylamine at 14 mg/kg did not induce DNA damage in any organs examined. Because the 3 azo additives we examined induced colon DNA damage at a very low dose, more extensive assessment of azo additives is warranted.

Key Words: amaranth; allura red; acid red; new coccine; mouse; colon; embryo; comet; alkaline single cell gel electrophoresis (SCGE) assay; DNA damage.

Synthetic red tar dyes, such as amaranth, allura red, and new coccine are widely used as food colors in many countries, including Japan. Epidemiological studies of food color additives are difficult, because exposure cannot be accurately assessed. Thus, risk assessment largely depends on laboratory toxicity studies.

Amaranth, allura red, and new coccine are azo dyes. Many azo dyes are genotoxic in short-term tests and carcinogenic in laboratory animals (Combes and Haveland-Smith, 1982). The other tar dye, acid red, is not an azo compound, and its use is rather limited to Japan. The only published report on its toxicity showed that it is not mutagenic to Salmonella (Fujita and Sasaki, 1993).

The genotoxicity of amaranth is controversial, it was classified as genotoxic in one review (Combes and Haveland-Smith, 1982) but not in another (Chung and Cerniglia, 1992). The carcinogenicity of amaranth has been tested in mice, rats, and dogs, and those results were also inconclusive. The reasons, according to the Working Group of IARC (1975a), included a high concentration of unspecified impurities, the very unusual absence of spontaneous tumors in controls, the small numbers of animals tested, or inadequate reporting. The IARC reevaluated the carcinogenicity of amaranth in 1987, and again classified the data as inconclusive (IARC, 1987). The embryotoxicity and teratogenicity of amaranth (or its metabolites) were initially reported as positive (Collins and McLaughlin, 1972, 1973), but subsequent studies presented negative results and/or reason to doubt that the observed effects were either biologically significant or reproducible (Collins *et al.*, 1976; Flint *et al.*, 1984; Holson *et al.*, 1976; Larsson, 1975; Piersma *et al.*, 1995). No case reports or epidemiological studies were available to the 1975 IARC (1975a) Working Group. The use of amaranth as a food additive has not been permitted in the United States since 1976 (Prival *et al.*, 1988).

The use of allura red, which is classified as nongenotoxic (Combes and Haveland-Smith, 1982), is permitted in the U.S. The U.S. National Toxicology Program found that allura red is negative to Salmonella (NTP, 2000). Although an impurity in allura red can be reduced to yield an ether-extractable mutagen (Prival *et al.*, 1988), the dye is not carcinogenic in rats (Borzelleca *et al.*, 1989) or mice (Borzelleca *et al.*, 1991). One teratology study with allura red in rats showed negative results (Collins *et al.*, 1989a), while another showed reduced ossifi-

[1] To whom correspondence should be addressed. Tel/Fax: +81-19-621-6981. E-mail: s.tsuda@iwate-u.ac.jp.

cation of the hyoid (Collins *et al.*, 1989b). Allura red produces evidence of both physical and behavioral toxicity in developing rats (Vorhees *et al.*, 1983).

New coccine (Ponceau 4R), which is not permitted as a food color in the U.S., is neither mutagenic in Salmonella (Fujita and Sasaki, 1993), nor teratogenic in mice (Larsson, 1975). A long-term toxicity study of new coccine in rats exposed *in utero* showed no carcinogenicity (Brantom *et al.*, 1988). Water-soluble azo dyes such as amaranth, allura red, and new coccine have highly charged sulfonate groups that preclude significant absorption of ingested dye. When the intact dye reaches the intestine, it can undergo extensive metabolic reduction by intestinal microflora (Levine, 1991), and the reductive cleavage products are rapidly absorbed (Chung *et al.*, 1992). It is also possible for mammalian azoreductase in the intestinal wall or liver to reduce the dye to free aromatic amines (Chung and Cerniglia, 1992; Chung *et al.*, 1992; Prival *et al.*, 1988). In spite of worldwide use of these food additives, we found little information on their genotoxicity, and we were unable to locate recent reports, using a Medline search.

In vivo genotoxicity of test chemicals is often evaluated by their ability to induce micronuclei in mouse bone marrow, but that does not seem to have been done for amaranth, allura red or new coccine. If the aromatic amines released from the azo compounds in liver and intestine were stable, their genotoxicity would be detected in the bone marrow. The aromatic amine moieties of many azo dyes, however, are unstable; this is particularly true when a hydroxyl group is on an aromatic ring adjacent to a free amino group, as is the case with these three azo dyes (Prival and Mitchell, 1982). Therefore, a method is needed to test the *in vivo* genotoxicity of such azo compounds in the target organs. The alkaline comet assay (pH >13) introduced by Singh *et al.* (1988) is a rapid and sensitive procedure for quantitating DNA lesions in mammalian cells. It detects not only strand breaks but also alkali-labile sites, DNA crosslinking, and incomplete excision repair sites (Fairbairn *et al.*, 1995; Singh *et al.*, 1988). We have modified this assay by using isolated nuclei in place of cells obtained by enzyme treatment (Sasaki *et al.*, 1997d). The *in vivo* alkaline comet assay enables the detection of *in vivo* DNA damage in all targets of interest, including embryos, where the DNA damage of embryo cells was detected as a cause of embryotoxicity and teratogenicity of pyrimethamine (Tsuda *et al.*, 1998). Thus, the comet assay applied to the embryo would solve the controversy surrounding the developmental genotoxicity of amaranth and allura red. The purpose of this study was to examine whether the 4 red dyes (3 azo compounds and a synthetic tar dye used specifically in Japan) induced DNA damage in mice, thus suggesting potential carcinogenicity or developmental genotoxicity.

p-Dimethylaminoazobenzene (butter yellow) is mutagenic to Salmonella (Ashby *et al.*, 1982; IARC, 1975b) and a hepatocarcinogen in several animal species (IARC, 1975b). Both N-nitrosodimethylamine and N-nitrosodiethylamine are mutagenic to Salmonella (Gold *et al.*, 1993), and carcinogenic in rodents after their administration by various routes, with the major target organs being the liver, respiratory tract, and kidney (IARC, 1978a,b). Therefore, *p*-dimethylaminoazobenzene, N-nitrosodimethylamine, and N-nitrosodiethylamine were used as reference compounds.

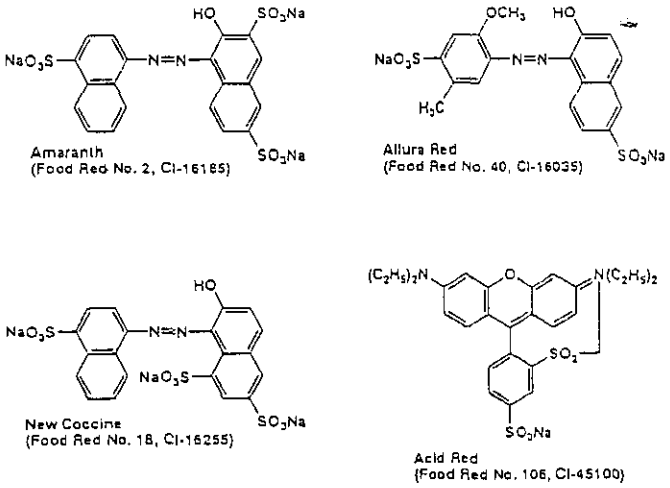

FIG. 1. Structures of amaranth, allura red, new coccine, and acid red.

MATERIALS AND METHODS

Chemicals and animals. All samples of amaranth (Food Red No. 2, CAS 915–67–3), allura red (Food Red No. 40, CAS 25956–17–6), new coccine (Food Red No. 18, CAS 2611–82–7), and acid red (Food Red No. 106, CAS 3520–24–1) were from certified lots approved for food use in Japan and were purchased from Tokyo Kasei Organic Chemicals (Tokyo). Their chemical structures are shown in Figure 1. *p*-Dimethylaminoazobenzene, N-nitrosodimethylamine, and N-nitrosodiethylamine were obtained from Wako Pure Chemicals (Osaka). A package of red ginger pickles was obtained commercially. New coccine concentration in the soaking liquid in the pickles was photometrically determined as 6.5 mg/10 ml. Regular (GP-42) and low melting point (LGT) agarose were obtained from Nacalai Tesque (Kyoto) and diluted respectively to 1% and 2% in physiological saline. CD-1 (ICR) mice were obtained from Charles River Japan, Inc. (Yokohama) at 7 weeks of age and used for the experiments after 1 week of acclimatization. They were fed with commercial pellets MF (Oriental Yeast Industries, Tokyo) and tap water *ad libitum* throughout the acclimatization period and the experiment. The animal room was kept at 22 ± 2°C with a 12-h light-dark cycle; the humidity was 30–50%.

Chemical treatment and observation. For the pregnant mouse study, female mice were mated for a period of one to two days. The morning on which copulation plugs were observed was designated day 0 of gestation. On the morning of day 11, 4 mice were randomly assigned to each treatment group. Chemicals were dissolved in distilled water and administered by gavage at the limit dose of 2000 mg/kg (10 ml/kg). The same volume of distilled water was administered to the control mice at the same time. The limit dose, at which no deaths, morbidity, or distinctive clinical signs were observed, was determined by preliminary acute toxicity tests.

For the male mouse study, chemicals were dissolved in distilled water except for *p*-dimethylaminoazobenzene, which was dissolved in olive oil. The dyes were administered by gavage in a volume of 10 ml/kg. A volume of 20 ml/kg of the red ginger pickle soaking liquid was given by gavage. In previous male mouse comet assays (Sasaki *et al.*, 1997a,b,c,d), we observed no signif-

icant differences in mean migration between vehicle control groups and corresponding untreated groups at any time for any organ. The results enabled us to use untreated (zero time) control animals rather than a larger number of concurrent vehicle control animals.

From shortly after treatment until just before they were sacrificed, the animals were carefully observed for pharmacotoxic signs. They were sacrificed by cervical dislocation 3, 6, or 24 h after treatment, and necropsied; brain, lung, liver, kidney, stomach, colon, bladder, embryos (for pregnant mice), and bone marrow (for male mice) were removed.

All procedures were approved by the Animal Research Committee, Faculty of Agriculture, Iwate University, and were conducted under both Guidelines for Animal Experiment in Iwate University and the "Guiding Principles in the Use of Animals in Toxicology," which were adopted by the Society of Toxicology in 1989.

Comet assay. The assay was conducted as previously described (Sasaki *et al.*, 1997d; Tsuda *et al.*, 1998). The protocol used was in accordance with the recently published comet assay genotoxicity guidelines (Tice *et al.*, 2000). The brain, lung, liver, kidney, and embryos were minced, suspended in chilled homogenizing buffer (pH 7.5) containing 0.075 M NaCl and 0.024 M Na_2EDTA, and then homogenized gently using a Potter type homogenizer. The mucosa of glandular stomach, colon, and bladder was scraped with a paper knife into chilled homogenizing buffer and homogenized in the same manner. To obtain nuclei, the homogenates were centrifuged at 700 × g for 10 min at 0°C, and the precipitates were resuspended in chilled homogenizing buffer. Slides prepared from nuclei isolated by homogenization were placed in a chilled lysing solution (2.5 M NaCl, 100 mM Na_4EDTA, 10 mM Trizma, 1% sarkosyl, 10% DMSO, and 1% Triton X-100, pH 10), and were kept at 0°C in the dark for more than 60 min, then in chilled alkaline solution (300 mM NaOH and 1 mM Na_2EDTA, pH 13) for 10 min in the dark at 0°C. Electrophoresis was conducted at 0°C in the dark for 15 min at 1 V/cm and approximately 250 mA. For the samples obtained 3 h after treatment with 500 mg/kg of *p*-dimethylaminoazobenzene, unwinding and electrophoresis were also conducted in the solution adjusted to pH 12.1 and pH 12.6 by HCl. The slides were neutralized and stained with ethidium bromide (Wako Pure Chemicals, Osaka).

We examined and photographed 50 nuclei per slide at 200× magnification with the aid of a fluorescence microscope. The length of the whole comet ("length") and the diameter of the head ("diameter") were measured for 50 nuclei per organ per mouse. We calculated migration as the difference between length and diameter for each of 50 nuclei. Mean migration of 50 nuclei from each organ was calculated for each individual mouse. The differences between the averages of 4 treated animals and the concurrent control animals were compared with the Dunnett test after one-way ANOVA except for otherwise stated. For each pairwise comparison, Student's *t*-test was used. A *p*-value less than 0.05 was considered statistically significant.

Histopathology. A small portion of each organ was fixed in 10% neutral formalin, dehydrated, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. Histopathological examination was conducted when positive results were obtained in the comet assay. After removal of mucosa, the rest of the glandular stomach and colon tissues were also processed for histopathology to examine if the entire mucosa was removed.

TABLE 1

Migration of Nuclear DNA from Maternal Organs and Embryos of Pregnant Mice Treated with Food Dyes

Sampling time (h)	Migration (μm)							
	Control		Amaranth		Allura red		Acid red	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Stomach								
3	6.39	1.04	14.51	5.06	13.91	2.40	4.40	0.45
6	4.29	0.48	9.94	3.04	10.51	2.40	6.51	0.81
24	6.86	2.35	11.81	1.64	6.41	1.31	6.09	1.07
Colon								
3	5.43	1.27	20.13**	2.49	22.41**	2.89	4.59	0.37
6	5.96	0.75	12.49	4.34	13.66	3.68	7.03	0.72
24	6.39	0.89	11.57	2.62	6.77	1.61	7.51	0.66
Liver								
3	1.48	1.16	2.21	0.62	2.87	0.73	0.56	0.20
6	1.09	0.36	0.67	0.32	0.71	0.11	1.20	0.05
24	0.59	0.35	2.09	0.78	0.27	0.27	0.71	0.44
Kidney								
3	2.62	2.17	2.40	0.81	1.14	0.50	0.17	0.10
6	0.27	0.27	1.21	0.31	0.63	0.23	0.89	0.20
24	0.60	0.22	1.59	0.19	0.63	0.40	0.60	0.20
Bladder								
3	1.21	0.42	2.16	0.47	1.46	0.85	1.50	0.55
6	1.43	0.17	1.07	0.51	2.51	1.26	1.17	0.22
24	1.10	0.19	3.49	1.26	1.29	0.18	1.94	0.32
Lung								
3	2.06	0.32	4.56	1.31	2.84	0.69	1.34	0.28
6	1.07	0.38	3.87*	0.87	2.56	0.39	1.80	0.25
24	1.64	0.41	2.46	0.42	1.79	0.32	2.06	0.35
Brain								
3	0.06	0.06	1.21	1.65	1.00	0.43	0.23	0.13
6	0.53	0.36	0.56	0.06	0.23	0.23	0.00	0.00
24	0.47	0.21	1.01	0.20	0.24	0.14	0.16	0.16
Embryo								
3	0.43	0.26	0.67	0.49	0.20	0.20	0.00	0.00
6	0.30	0.22	0.59	0.40	0.24	0.24	0.13	0.13
24	0.49	0.29	0.73	0.29	0.00	0.00	0.00	0.00

Note. Distilled water (10 ml/kg, for control) or food dyes dissolved in distilled water were administered to mice (4 per group) by gavage at the limit dose of 2000 mg/kg (10 ml/kg) in the morning of day 11 (about 9 A.M.) of pregnancy. Differences between treatments and the control were compared with the Dunnett test after one-way ANOVA; $n = 4$.

*Significant difference between control and treatment group, $p < 0.05$.

**Significant difference between control and treatment group, $p < 0.01$.

RESULTS

No deaths, morbidity, or distinctive clinical signs were observed after any treatment. Necropsy and histopathological examination revealed no treatment effect on any organ examined. Thus, any DNA damage observed was not likely to be due to general cytotoxicity. The histopathology of the glandular stomach and colon tissues left after the scraping showed that the entire mucosa was removed.

An increase in DNA damage was indicated by an increase in migration length of the stained DNA. As shown in Table 1, the assay in the pregnant mice with the limit dose of color additives was positive in the colon 3 h after the administration of amaranth and allura red, and weakly positive in the lung 6 h after amaranth treatment. The biological significance of the slight lung DNA damage was not clear because of the small value of the increase. Acid red did not induce DNA damage in any samples at any sampling time. None of the dyes damaged DNA in the other organs or the embryo.

In male mice, shown in Table 2, amaranth, allura red, and new coccine induced dose-related DNA damage in the colon

TABLE 2
Migration of Nuclear DNA from Organs of Male Mice Treated with Food Dyes

Dose (mg/kg)	Sampling time (h)	Migration							
		Stomach	Colon	Liver	Kidney	Bladder	Lung	Brain	Bone marrow
Amaranth									
0	0	5.91, 0.73	5.60, 0.88	3.07, 0.51	1.16, 0.44	6.01, 0.81	2.20, 0.44	1.06, 0.67	0.90, 0.57
1	3	8.62, 1.52	13.0, 1.99	2.14, 1.03	2.17, 0.35	7.13, 1.64	4.49, 0.71	1.78, 0.76	0.47, 0.47
10	3	8.25, 1.26	25.6, 1.70***	1.06, 0.37	2.43, 0.22	8.42, 0.30	4.08, 0.45	1.46, 0.59	0.85, 0.49
100	3	13.1, 1.22	29.4, 3.15***	1.71, 0.33	4.00, 1.69	6.89, 0.39	3.69, 1.15	2.68, 0.78	0.80, 0.49
1000	3	32.6, 1.22***	34.4, 1.86***	1.09, 0.68	4.67, 0.87	16.8, 1.54*	4.44, 1.27	1.96, 0.67	1.01, 0.34
2000	3	9.27, 1.98	40.4, 3.45***	8.78, 3.20	1.29, 0.49	8.92, 2.52	6.07, 1.28	5.78, 1.61	1.11, 0.64
2000	6	22.0, 2.05***	28.4, 5.98**	13.6, 1.53*	1.32, 0.82	5.06, 2.59	5.40, 1.40	9.61, 2.67*	11.7, 0.59***
2000	24	16.2, 1.13**	10.3, 0.67	10.8, 2.71	5.31, 1.79	15.2, 1.88*	6.47, 1.50	2.79, 1.16	3.44, 0.41
Allura red									
0	0	6.66, 1.19	5.40, 0.99	2.22, 0.59	2.58, 0.54	5.16, 0.40	2.94, 0.42	1.01, 0.73	1.01, 0.43
1	3	6.71, 1.26	8.54, 1.31	1.01, 0.63	2.35, 0.68	7.53, 1.25	2.68, 1.03	2.01, 0.81	1.70, 0.59
10	3	8.41, 1.51	26.6, 5.91*	2.42, 0.53	2.89, 0.81	6.48, 1.34	3.25, 1.07	1.83, 0.78	0.47, 0.47
100	3	20.4, 3.68**	37.5, 3.10***	1.86, 0.76	3.28, 1.23	6.43, 0.45	3.36, 0.79	1.52, 2.00	0.67, 0.40
1000	3	26.2, 1.97***	37.5, 2.00***	1.73, 1.86	3.80, 1.33	9.52, 1.27	8.10, 0.94*	1.57, 0.53	1.24, 0.45
2000	3	27.8, 1.61***	36.1, 3.82***	1.75, 0.60	1.47, 0.55	7.40, 1.42	3.15, 0.97	3.20, 1.36	2.51, 0.71
2000	6	16.7, 2.54*	32.6, 3.05***	1.39, 0.50	2.58, 0.70	11.6, 3.62	5.21, 1.99	3.15, 1.38	2.12, 0.84
2000	24	13.6, 2.31	22.0, 1.69**	1.70, 0.79	4.59, 1.04	8.70, 1.60	4.44, 1.08	0.57, 0.57	1.11, 0.76
New coccine[a]									
0	0	7.21, 0.64	6.32, 0.79	1.67, 0.82	1.93, 0.64	5.89, 0.24	3.47, 0.22	1.36, 0.51	1.29, 0.66
1	3	10.6, 3.21	18.3, 4.25	1.11, 0.62	2.58, 0.45	14.1, 0.31	5.15, 1.21	0.54, 0.32	0.75, 0.75
10	3	6.20, 1.31	20.5, 4.35*	1.63, 0.30	2.35, 0.60	15.7, 3.92	5.55, 1.13	1.94, 0.66	2.27, 0.44
100	3	20.0, 2.61*	33.2, 2.05***	2.63, 1.46	4.21, 0.55	22.1, 5.78**	8.10, 0.80	2.35, 0.97	0.98, 0.71
2000	3	28.8, 4.09**	38.2, 3.18***	6.00, 0.72	4.36, 0.73	18.4, 0.22**	5.83, 1.08	3.67, 1.03	0.72, 0.42
2000	6	12.7, 2.26	14.6, 1.78	3.98, 1.53	7.12, 1.91	19.0, 2.23**	4.34, 0.86	3.10, 0.97	2.50, 1.05
2000	24	15.0, 1.51	15.4, 2.11	12.8, 1.81***	13.0, 2.05**	24.2, 3.08***	13.9, 0.26***	3.15, 0.76	3.59, 1.38
New coccine[b]									
13	3	19.7, 1.36*	30.9, 3.29**	2.14, 1.04	2.63, 0.95	30.2, 6.88*	2.81, 0.38	1.21, 0.41	0.93, 0.36
13	24	13.6, 3.95	35.4, 5.62**	2.12, 0.59	3.80, 0.81	44.7, 3.32***	3.15, 0.83	1.27, 0.83	1.11, 0.52

Note. Food dyes dissolved in distilled water were given to mice (4 mice per group) by gavage with a volume of 10 ml/kg. A volume of 20 ml/kg of soaking liquid from commercial red ginger pickles, which contained 6. 5 mg/10 ml of new coccine, was given to mice by gavage. Differences between treatments and the control were compared with the Dunnett test after one-way ANOVA; $n = 4$. Values (μm) are given as mean, SE.

[a]Given in distilled water.

[b]Given in commercial red ginger pickle soaking solution.

*Significant differences between control and treatment group, $p < 0.05$.

**Significant differences between control and treatment group, $p < 0.01$.

***Significant differences between control and treatment group, $p < 0.001$.

3 h after their administration, when the damage was most prominent. The lowest dose that induced DNA damage in the colon was 10 mg/kg for all 3 dyes. Some other organs also showed DNA damage: stomach, after the treatment with all 3 azo dyes at 100 mg/kg or higher, and bladder mucosal cells after new coccine treatment at the lowest dose of 100 mg/kg. DNA damage in the other organs was sporadic. The commercial red ginger pickle soaking liquid induced DNA damage in colon, stomach, and bladder 3 h after administration, and the damage to the colon and bladder remained for up to 24 h.

The potencies were compared to known rodent carcinogens (Table 3). *p*-Dimethylaminoazobenzene induced colon DNA damage at 1 mg/kg and liver DNA damage only at 500 mg/kg, the highest dose. Although 1 mg/kg of N-nitrosodimethylamine induced DNA damage in liver and bladder, it did not induce colon DNA damage. N-nitrosodiethylamine at 14 mg/kg did not induce DNA damage in any organs examined.

As shown in Table 4, none of the samples obtained 3 h after oral treatment with 500 mg/kg *p*-dimethylaminoazobenzene showed an increase in DNA migration when alkaline electrophoresis was conducted at pH 12.1, whereas DNA of nuclei from colon, liver, and urinary bladder revealed significant increase in migration at pH 12.6.

DISCUSSION

It is consistent with the reported nonteratogenicity of the compounds that embryos did not show chemical-induced DNA damage after the administration of amaranth or allura red

TABLE 3
Migration of Nuclear DNA from Organs of Male Mice Treated with *p*-Dimethylaminoazobenzene, N-Nitrosodimethylamine, and N-Nitrosodiethylamine

Dose (mg/kg)	Sampling time (h)	Migration							
		Stomach	Colon	Liver	Kidney	Bladder	Lung	Brain	Bone marrow
Nontreatment									
0	0	6.37, 0.57	4.78, 0.60	1.94, 0.36	2.54, 0.46	3.00, 0.55	2.89, 0.43	1.26, 0.45	1.14, 0.55
p-Dimethylaminoazobenzene									
1	3	12.6, 1.30	18.8, 3.05**	1.47, 0.55	3.79, 1.12	3.66, 0.42	4.13, 0.77	2.04, 0.51	1.06, 0.67
10	3	19.3, 2.43**	23.2, 1.61***	1.78, 0.81	3.12, 0.57	6.47, 1.62	2.27, 0.23	2.35, 0.61	2.38, 0.44
100	3	12.6, 1.96	24.6, 1.20***	2.74, 1.74	2.94, 1.70	17.8, 1.36***	3.89, 2.27	1.89, 0.23	2.66, 0.47
500	3	5.60, 0.98	10.9, 1.18	14.5, 0.74**	3.41, 1.08	10.1, 1.47*	3.43, 0.80	3.64, 1.64	0.70, 0.41
500	6	6.86, 1.31	23.4, 1.97***	14.9, 2.94**	9.17, 3.17	4.77, 2.13	2.63, 0.57	2.43, 1.03	9.78, 0.93***
500	24	12.7, 0.54**	23.6, 3.03***	5.57, 1.31	1.14, 0.50	9.47, 1.48	6.45, 0.79*	0.90, 0.53	3.72, 0.66
N-Nitrosodimethylamine									
1	3	7.97, 0.88	5.69, 1.84	15.4, 1.61***	9.73, 2.98	7.84, 1.36*	4.85, 1.71	3.31, 1.10	3.82, 0.73
N-Nitrosodiethylamine									
14	3	7.58, 1.04	8.35, 1.51	5.06, 0.39	1.21, 0.08	6.48, 0.55	5.29, 1.3	1.24, 0.51	0.31, 0.31

Note. *p*-Dimethylaminoazobenzene dissolved in olive oil and nitrosoamines dissolved in distilled water were given to mice (4 mice per group) by gavage with a volume of 10 ml/kg. Differences between treatments and the control were compared with the Dunnett test after one-way ANOVA; $n = 4$. Values (μm) are given as mean, SE.

*Significant differences between control and treatment group, $p < 0.05$.

**Significant differences between control and treatment group, $p < 0.01$.

***Significant differences between control and treatment group, $p < 0.001$.

(Collins *et al.*, 1976, 1989a; Flint *et al.*, 1984; Larsson, 1975; Holson *et al.*, 1976; Piersma *et al.*, 1995).

Our results show that amaranth, allura red, and new coccine are genotoxic in mice, with colon epithelium being the most susceptible tissue. The *in vivo* DNA damage caused by amaranth is consistent with one review (Combes and Haveland-Smith, 1982) but not with another (Chung and Cerniglia, 1992). To our knowledge, all the Salmonella mutagenicity tests of allura red or new coccine have been negative (Fujita and Sasaki, 1993; Fujita *et al.*, 1995; Muzzall and Cook, 1979; NTP, 2000; Prival *et al.*, 1988). A number of azo compounds are mutagenic in assays if chemical reduction or microsomal activation, or both, are induced (Chung and Cerniglia, 1992). Orally administered amaranth, allura red, and new coccine are too poorly absorbed to be metabolized, but when they reach the colon, they can undergo extensive reduction by microflora, and the metabolites can be rapidly absorbed (Chung *et al.*, 1992; Levine, 1991). It is also possible for the dyes to be reduced to free aromatic amines by mammalian azo reductase in the intestinal wall or liver (Chung and Cerniglia, 1992; Chung *et al.*, 1992; Prival *et al.*, 1988). Then, the genotoxicity of N-substituted aryl compounds is dependent on their conversion to reactive metabolites, such as the frequently produced N-acetoxyarylamines. The activation is accomplished by acetyltransferases, which are widely distributed in animals (King *et al.*, 1997). Because the activation process of these azo compounds

TABLE 4
Effect of pH on Migration of Nuclear DNA from Organs of Male Mice Treated with *p*-Dimethylaminoazobenzene

pH	Dose (mg/kg)	Sampling time (h)	Migration							
			Stomach	Colon	Liver	Kidney	Bladder	Lung	Brain	Bone marrow
12.1	0	0	2.92, 0.23	3.33, 0.57	0.98, 0.57	0.83, 0.48	2.45, 0.32	0.88, 0.52	0.52, 0.52	0.57, 0.33
	500	3	1.84, 0.53	2.81, 0.57	0.95, 0.43	0.62, 0.41	1.83, 0.87	0.49, 0.34	0.39, 0.25	0.31, 0.31
12.6	0	0	4.00, 0.55	5.52, 0.64	1.55, 0.36	2.45, 0.30	3.69, 0.48	2.37, 0.70	1.11, 1.11	0.31, 0.31
	500	3	3.59, 0.80	12.7, 0.64**	11.6, 0.64**	1.52, 0.28	9.56, 1.67*	3.93, 1.06	1.24, 1.07	1.11, 0.46

Note. *p*-Dimethylaminoazobenzene (DAB) dissolved in olive oil was given to mice by gavage with a volume of 10 ml/kg. Difference between treatment and the control was compared with Student's *t*-test; $n = 4$. Values (μm) are given as mean, SE.

*Significant differences between control and treatment group, $p < 0.05$.

**Significant differences between control and treatment group, $p < 0.001$.

in animals is complex, Salmonella tests with S9 might not efficiently detect mammalian genotoxicity. The *in vivo* comet assay, which has the advantage of reflecting absorption, distribution, and excretion as well as metabolism, would be a more effective tool for detecting the genotoxicity of these food additives.

How do the genotoxic effects shown by the *in vivo* comet assay relate to rodent colon carcinogenicity? If the scraping with a paper knife had selectively removed the terminally differentiated cells and not stem cells from multilayered tissues with many different cell types, as in colon and glandular stomach, the observed DNA damage would have had little if any consequences in terms of carcinogenicity. This possibility may be precluded, because the histopathology of the glandular stomach and colon tissues left after the scraping showed that the entire mucosa was removed. It is interesting to know which cell types of glandular stomach and colon are affected by the dyes, because the mean migration signal might have potentially included the DNA damage of the differentiated cell nuclei.

Consideration might also be given to whether the dyes accelerate a death process that involves nuclease-mediated DNA fragmentation in the epithelial cells. The control migration rates were higher for colon and stomach (5–7 μm vs. 1–2 μm for other tissues), suggesting that the higher background rate of apoptosis, necrosis, and or terminal differentiation in the cells may contribute to the DNA damage signal. If the dyes perturb the kinetics of this process, their effect may also modulate whatever is contributing to the higher control nuclear DNA migration rates in these tissues. However, necropsy and histopathological examination revealed no treatment effect on colon and glandular stomach. Thus, any DNA damage observed was not likely to be due to general cytotoxicity. The death process may also be increased by the accelerated proliferation. The mean turnover time of epithelial cells in the colon of the mouse is 4.85 days (Tsubouchi, 1981). Thus, it seems difficult to ascribe the great DNA damage caused 3 h after the treatment with the dyes (21–41 μm vs. 5–7μm for the control) to the accelerated proliferation process. In addition, alkaline electrophoresis at pH 12.1 did not show an increase of migration in colon DNA 3 h after oral treatment with 500 mg/kg *p*-dimethylaminoazobenzene, an azo compound, whereas at pH 12.6 a significant increase in the migration of DNA was observed. Generally, DNA is denatured and unwound at pH values above 12.0; this is because of the disruption of hydrogen bonds between double-stranded DNA. At pH conditions of 12.6 or higher, alkali-labile sites (e.g., apurinic sites) are quickly transformed to strand breaks (Tice *et al.*, 2000). Thus, the DNA damage induced by food azo dyes may have been due to adduct formation, resulting in alkali-labile sites.

The Comet assay usually detects DNA damage induced by shortly after the administration of relatively high doses, while carcinogenicity assays use long treatment and relatively low doses. That can lead to discordant results. Metabolic processes may become saturated at high tissue concentrations, and the rates and pathways of metabolic activation and detoxication may be different at high single doses and low long-term doses. In the present study, however, the lowest dose that induced DNA damage in the colon was 10 mg/kg for the 3 dyes, which was even lower than the doses used in the carcinogenicity studies. The no-observable-adverse-effect level in the lifetime toxicity/carcinogenicity studies of allura red is 2829 and 901 mg/kg/day for male and female rats, respectively (Borzelleca *et al.*, 1989), and 7300 and 8300 mg/kg /day for male and female mice, respectively (Borzelleca *et al.*, 1991). The no-observable-adverse-effect level was 500 mg/kg/day in the long-term toxicity study of new coccine in rats, using animals exposed *in utero* (Brantom *et al.*, 1987). An alternative explanation may be that the power to detect effects in colon might be lower for rodent carcinogenicity studies than for the comet assay. Hakura *et al.* (1998, 1999) showed that to be the case for benzo-[a]pyrene-induced mutation and carcinogenicity in *lac Z* transgenic mice. Moreover, a compendium of bioassay results organized by target organ for 533 chemicals that are carcinogenic in at least one species shows that none of the 299 chemicals evaluated as carcinogenic in mice and only 15 (4%) of 354 chemicals evaluated as carcinogenic in rats have the large intestine as target organ (Gold *et al.*, 1991).

Human carcinomas of the gastrointestinal tract have high incidence and mortality rates, and colorectal cancer incidence is as much as 10 times higher in industrialized countries than in developing nations, suggesting environmental causes (Goldin-Lang *et al.*, 1996). The 3 azo food dyes induced DNA damage in the colon at as low as 10 mg/kg. Soaking liquid from commercial red ginger pickles that delivered 13 mg/kg of new coccine induced DNA damage in colon, stomach, and bladder. The hepatocarcinogen *p*-dimethylaminoazobenzene damaged colon DNA at 1 mg/kg, but liver DNA only at the highest dose of 500 mg/kg. Although 1 mg/kg N-nitrosodimethylamine induced DNA damage in liver and bladder, it did not do so in colon. N-nitrosodiethylamine, at a dose of 14 mg/kg, did not induce DNA damage in any organ examined. The acceptable daily intake (ADI) levels recommended by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) for amaranth, allura red, and new coccine are 0.5, 7, and 4 mg/kg respectively (Ito, 2000). Because the 3 additives we tested induced DNA damage, mainly in colon, at close to those ADIs, a more extensive safety assessment of azo dyes is warranted.

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