[CANCER RESEARCH 38, 1410–1413, May 1978]

Inhibition of Polycyclic Aromatic Hydrocarbon-induced Neoplasia by Naturally Occurring Indoles[1]

Lee W. Wattenberg[2] and William D. Loub

Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, Minnesota 55455 [L. W. W.], and Department of Pharmacognosy and Pharmacology, College of Pharmacy, University of Illinois, Chicago, Illinois 60680 [W. D. L.]

ABSTRACT

Indole-3-carbinol, 3,3′-diindolylmethane, and indole-3-acetonitrile, three indoles occurring in edible cruciferous vegetables, have been studied for their effects on 7,12-dimethylbenz(*a*)anthracene-induced mammary tumor formation in female Sprague-Dawley rats and on benzo(*a*)pyrene-induced neoplasia of the forestomach in female ICR/Ha mice. When given by p.o. intubation 20 hr prior to 7,12-dimethylbenz(*a*)anthracene administration, indole-3-carbinol and 3,3′-diindolylmethane had an inhibitory effect on mammary tumor formation, but indole-3-acetonitrile was inactive. Indole-3-carbinol when added to the diet for 8 days prior to challenge with 7,12-dimethylbenz(*a*)anthracene inhibited mammary tumor formation, whereas indole-3-acetonitrile did not. Dietary administration of all three indoles inhibited benzo(*a*)pyrene-induced neoplasia of the forestomach in ICR/Ha mice. The identification of dietary constituents that can inhibit chemical carcinogens ultimately may be of value in understanding the balance of factors that determines the neoplastic response to these cancer-producing agents in the environment.

INTRODUCTION

Studies of factors that can control the activity of the AHH[3] system have shown that naturally occurring inducers of this system are present in certain vegetable species (17–19). In early studies of AHH activity in the small intestine and lung of rodents, substantial levels of AHH activity were observed. It was assumed that these represented normal base-line levels. This assumption proved to be incorrect, and in fact most AHH activity found in these 2 structures is due to exogenous inducers in conventional commercial diets. In contrast, mice and rats that are starved or fed purified diets showed weak or no AHH activity in intestine and lung. Comparable findings have been obtained for the aminoazo dye *N*-demethylase system (1).

Efforts to identify the inducers in commercial diets were initiated with Purina rat chow as a prototype. In this diet the vegetable component, consisting of alfalfa meal, has inducing activity. Comparable findings then were obtained with freshly harvested alfalfa. This information stimulated work on a broad spectrum of common edible plants to determine which would have the same biological action. A number of cruciferous plants including Brussels sprouts, cabbage, cauliflower, and broccoli were found to induce increased AHH activity (18, 19). Subsequently, inducers were identified in Brussels sprouts, cabbage, and cauliflower as indoles, the principle compounds being indole-3-acetonitrile, indole-3-carbinol, and 3,3′-diindolylmethane (11). Indole-3-carbinol is a potent inducer of increased AHH activity, whereas indole-3-acetonitrile is weak in this regard. 3,3′-Diindolylmethane is intermediate in its activity (11).

In previous work it had been found that compounds that induce increased AHH activity can protect against neoplasia induced by PAH. The inducers used were flavones, phenothiazines, and PAH themselves (6, 23–26). Accordingly, work was initiated to determine whether indole-3-acetonitrile, indole-3-carbinol, and 3,3′-diindolylmethane would inhibit these carcinogens. Indole-3-acetaldehyde and its oxime, which also occur in small amounts in cruciferous plants, were studied as well. Two test systems have been used. The first is DMBA-induced mammary tumor formation in Sprague-Dawley rats (5). The second experimental system used is BP-induced neoplasia in the mouse forestomach resulting from p.o. administration of BP.

MATERIALS AND METHODS

Mammary Tumors. The procedure for producing mammary tumors was similar to that described by Huggins *et al.* (5). Female Sprague-Dawley rats from the Holtzman Co., Madison, Wis., were randomized by weight prior to the start of the experiment. When they were 7 weeks of age they were given 12 mg DMBA in 1 ml olive oil by p.o. intubation. Two formats were used for testing compounds for their inhibitory effects against DMBA-induced carcinogenesis. In one, the test compound dissolved in 1 ml dimethyl sulfoxide was administered by p.o. intubation 20 hr prior to DMBA. In the second, the test compound was added to a purified diet, Normal Protein Test Diet, ICN Pharmaceuticals, Inc., Cleveland, Ohio. This diet consists of vitamin-free casein, 27%; starch, 59%; corn oil, 10%; salt mix, 4%; plus a complete vitamin supplement. The diets were fed for 8 days prior to administration of 12 mg DMBA in 1 ml olive oil by p.o. intubation. The day after administration of DMBA, all rats were placed on a diet of Purina rat chow (Ralston Purina Co., St. Louis, Mo.) and remained on this diet for the duration of the experiment. Mammary tumors were counted grossly every 2 weeks starting 10 weeks after administration of DMBA. The diagnosis was checked at autopsy. The tabular data presented for the previous experiments and for the mouse tumor experiments described next were evaluated for significance with Student's *t* test.

[1] Supported by USPHS Grant CA-09599 from the National Cancer Institute.
[2] To whom requests for reprints should be addressed.
[3] The abbreviations used are: AHH, aryl hydrocarbon hydroxylase; PAH, polycyclic aromatic hydrocarbons; DMBA, 7,12-dimethylbenz(*a*)anthracene; BP, benzo(*a*)pyrene; i.g., intragastric.
Received November 2, 1977; accepted February 13, 1978.

Mouse Tumor Experiments. Tumor formation in the forestomach of the mouse was studied by procedures similar to those described previously (20, 21). ICR/Ha mice from ARS/Sprague-Dawley, Madison, Wis., were randomized by weight at 8 weeks of age. At that time they were placed on the Normal Protein Test Diet without any additions for 4 days. They then were fed the test diets containing the compound that was being studied for its inhibitory effect. The mice were maintained on these diets until 3 days after the last dose of carcinogen. At that time, they were placed on Purina rat chow. In experiments in which BP was used, it was dissolved in 0.2 ml corn oil and given by p.o. intubation. The first dose was administered 8 days after the start of the test diets. In 1 experimental format 1 mg of BP was administered 2 times a week for 4 weeks, and the experiment was terminated when the mice were 31 weeks old. A second format entailed administration of 0.3 mg of BP 3 times a week for 8 weeks with the experiment terminating when the mice were 41 weeks old. At autopsy, the stomachs were fixed in an expanded state produced by i.g. injection of formalin. Subsequently, they were split longitudinally. Tumors of the forestomach were counted under a dissecting microscope. Tumors 1 mm or larger were recorded and checked histologically (20, 21).

Chemicals. Indole-3-acetonitrile (Aldrich Chemical Co., Inc., Milwaukee, Wis.) was purified by vacuum distillation at 160° and 0.2 mm Hg to a clear, light yellow liquid (m.p. 35–37°). Indole-3-carbinol (Aldrich Chemical Co., Inc.,) was purified by recrystallization from benzene to yield colorless opalescent flakes (m.p. 96–97°). 3,3′-Diindolylmethane was synthesized according to the method of Leete and Marion (9). The purity of the 3 indoles was further ascertained by comparisons on thin-layer and gas-liquid chromatography with previously prepared authentic standards (11). Indole-3-acetaldehyde was synthesized by the method of Plieninger and Werst (14). Its purity was checked by thin-layer chromatography against an authentic reference standard of this compound. Indole-3-acetaldehyde oxime was prepared by the derivatization of indole-3-acetaldehyde-sodium bisulfite adduct in aqueous solution (pH 8.5) with hydroxylamine hydrochloride. The carcinogens used were DMBA (Eastman Organic Chemicals, Rochester, N. Y.) and BP (Aldrich Chemical Co.).

RESULTS

A single administration of indole-3-carbinol, 3,3′-diindolylmethane, or indole-3-acetaldehyde oxime by p.o. intuba-

Table 1

Effects of indoles on DMBA-induced mammary tumor formation

Experiment	Material administered[a]	Dose (mmol)	Route of indole administration	No. of rats at risk	Wt gain[b] (g)	Mammary tumors[c] % of rats with tumors	Mammary tumors[c] Tumors/rat
1	Dimethyl sulfoxide solvent control		p.o. intubation	11	138	91	1.45 ± 0.28[d]
	Indole-3-carbinol	0.10		14	146	21[e]	0.29 ± 0.16[e]
	3,3′-Diindolylmethane	0.05		11	139	27[e]	0.36 ± 0.20[e]
	Indole-3-acetonitrile	0.10		11	145	73	1.55 ± 0.46
2	Dimethyl sulfoxide solvent control		p.o. intubation	10	149	60	0.90 ± 0.28
	Indole-3-carbinol	0.10		14	142	14[f]	0.21 ± 0.16[f]
	3,3′-Diindolylmethane	0.05		14	175	36	0.57 ± 0.25
	Indole-3-acetonitrile	0.10		14	175	50	0.86 ± 0.29
3	Dimethyl sulfoxide solvent control		p.o. intubation	14	149	64	1.00 ± 0.28
	Indole-3-carbinol	0.10		14	147	21[f]	0.21 ± 0.11[f]
	Indole-3-acetaldehyde	0.10		14	125	36	0.43 ± 0.17
	Indole-3-acetaldehyde oxime	0.10		16	107	19[f]	0.19 ± 0.10[f]
4	None		Addition to the diet	15	169	73	1.20 ± 0.30
	Indole-3-carbinol	0.014/g of diet		15	178	20[e]	0.33 ± 0.19[f]
	Indole-3-acetonitrile	0.030/g of diet		16	173	54	1.00 ± 0.39

[a] Female 7-week-old Sprague-Dawley rats were given by p.o. intubation a single dose of 1 ml dimethyl sulfoxide only or of the indicated indole in dimethyl sulfoxide 20 hr prior to administration of 12 mg DMBA in 1 ml olive oil also by p.o. intubation. In Experiment 4, the indoles were added to the diet, which was given for 8 days prior to administration of 12 mg DMBA by p.o. intubation.

[b] Experiments 1 to 3, from 7 to 28 weeks of age; Experiment 4, from 6 to 28 weeks of age.

[c] Mammary tumors present when rats were 21 weeks old.

[d] Mean ± S.E.

[e] $p < 0.01$.

[f] $p < 0.05$.

Table 2
Effects of indoles on BP-induced gastric tumor formation

Experiment	Additions to the diet[a]	Carcinogen and dose schedule[b]	No. of mice at risk	Diet intake (g/day)	Age at sacrifice (wk)	Wt gains (g)	Tumors of the forestomach: % of mice with tumors	Tumors of the forestomach: Tumors/mouse
1	None	1 mg BP by p.o. intubation 2 times/wk for 4 wk	39	4.2	31	5.9	93	5.0 ± 0.54[d]
	Indole-3-carbinol, 0.03 mmol/g		20	4.2	31	6.6	80	1.9 ± 0.35[e]
	3,3′-Diindolylmethane, 0.02 mmol/g		18	4.2	31	7.8	94	3.2 ± 0.51[f]
	Indole-3-acetonitrile, 0.03 mmol/g		19	4.1	31	6.4	68	1.6 ± 0.43[e]
2	None	0.3 mg BP by p.o. intubation 3 times/wk for 8 wk	33	3.1	41	20.2	100	7.1 ± 0.64
	Indole-3-acetonitrile, 0.03 mmol/g		18	2.8	41	15.8	44[e]	1.1 ± 0.33[e]
3	None	None	19	3.5	41	19.4	0	0
	3,3′-Diindolylmethane, 0.02 mmol/g		17	3.3	41	20.2	0	0
	Indole-3-carbinol, 0.03 mmol/g		20	2.8	41	18.2	0	0
	Indole-3-acetonitrile, 0.03 mmol/g		18	3.5	41	21.9	0	0

[a] Female ICR/Ha mice were fed a semipurified diet, Normal Protein Test Diet ICN Pharmaceuticals, Inc., containing the indicated indole starting when the mice were 63 days old and continuing until they were 98 days old in Experiment 1 or 126 days old in Experiments 2 and 3.
[b] The initial dose of carcinogen was given 8 days after the start of the experimental diets.
[c] Experiment 1, from 8 to 31 weeks of age; Experiments 2 and 3, 41 weeks of age.
[d] Mean ± S.E.
[e] $p < 0.01$.
[f] $p < 0.05$.

tion produced a significant inhibitory effect on the occurrence of DMBA-induced mammary tumors as is evident both from a reduction in the number of animals that have tumors and from the number of tumors per animal (Table 1). In contrast, indole-3-acetonitrile does not suppress neoplasia in this experimental model. In an experiment in which indoles were added to the diet, indole-3-carbinol again inhibited the neoplastic response, whereas indole-3-acetonitrile did not. The results obtained in experiments in which BP was administered by p.o. intubation to mice show an inhibitory effect of indole-3-acetonitrile as well as of indole-3-carbinol and 3,3′-diindolylmethane on the occurrence of neoplasms of the forestomach (Table 2).

DISCUSSION

The naturally occurring indoles have been studied for their capacity to inhibit formation of neoplasms in 2 experimental models. Indole-3-carbinol and 3,3′-diindolylmethane produce an inhibitory effect against DMBA-induced mammary tumor formation in the rat and the occurrence of BP-induced neoplasia of the forestomach in ICR/Ha mice. Indole-3-acetonitrile was effective in suppressing neoplasia of the mouse forestomach but did not inhibit mammary tumor formation. The mechanism or mechanisms by which the indoles inhibit PAH-induced neoplasia is not known. They have been shown to induce increased AHH activity (11). The AHH determination reflects formation of phenolic metabolites of BP. The metabolisms of BP and DMBA are complex. The relative amounts of metabolites of detoxification as compared to those leading to formation of ultimate carcinogenic species would control the response to a particular dose of carcinogen. Pertinent information concerning this balance can be obtained only from detailed studies of the metabolite pattern of the carcinogen and are not provided by levels of AHH activity. At present, detailed information on the effects of indoles on PAH metabolism is lacking.

Indole-3-carbinol, 3,3′-diindolylmethane, and indole-3-acetonitrile occur in vegetables such as Brussels sprouts, cabbage, cauliflower, and broccoli, which are widely consumed (8, 10, 12). Thus alterations in carcinogen metabolism by these vegetables would be of some interest. Animal experiments in which the whole vegetable has been used have been limited thus far to determinations of their effects on the activity of the mixed-function oxidase system towards several substrates, including BP. In all instances induction of increased activity was found (13, 18, 19). The degree to which biological effects of consumption of a whole vegetable can be attributed to specific indoles is difficult to ascertain. Extraction procedures show that the predominant indole in the cruciferous plants studied is indole-3-acetonitrile. However, the origins of the indoles are complex. Indole-3-carbinol, 3,3′-diindolylmethane, and

indole-3-acetonitrile are formed in cruciferous plants from the hydrolysis of a parent compound, indolylmethyl glucosinolate (3, 4, 16). Two pathways of hydrolysis have been described, giving different end products. Thus the ultimate amounts of the 3 indoles will be dependent on multiple factors. The concentration of the parent compound will be one of these and could be influenced by genetic determinants, growing conditions, and the maturity of the plant when harvested. The hydrolytic pathway will be affected by storage, by handling, and possibly by conditions of *in vivo* digestion. Since each indole appears at the expense of another, their exact ratio would be important in the overall effect obtained by administration of crude plant material. Consideration of this nature could be important in any study aimed at evaluating the impact of differing intakes of cruciferous vegetables on incidence of neoplasia.

A number of inhibitors of chemical carcinogenesis have now been found to occur in dietary components. These inhibitors include naturally occurring compounds such as flavones, aromatic isothiocyanates, coumarin, selenium salts, and indoles (2, 7, 15, 21, 22, 25). In addition some synthetic food additives, in particular phenolic antioxidants, inhibit carcinogen-induced neoplasia (20, 22). The identification and evaluation of these naturally occurring and synthetic inhibitors ultimately may be of value in understanding the balance of factors that determines the neoplastic response to environmental chemical carcinogens.

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