Nutrition and Cancer

ISSN: 0163-5581 (Print) 1532-7914 (Online) Journal homepage: http://www.tandfonline.com/loi/hnuc20

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To cite this article: Herman A. J. Schut & Ruisheng Yao (2000) Tea as a Potential Chemopreventive Agent in PhIP Carcinogenesis: Effects of Green Tea and Black Tea on PhIP-DNA Adduct Formation in Female F-344 Rats, Nutrition and Cancer, 36:1, 52-58, DOI: 10.1207/S15327914NC3601_8

To link to this article: http://dx.doi.org/10.1207/S15327914NC3601_8

NUTRITION AND CANCER, *36*(1), 52–58

Tea as a Potential Chemopreventive Agent in PhIP Carcinogenesis: Effects of Green Tea and Black Tea on PhIP-DNA Adduct Formation in Female F-344 Rats

Herman A. J. Schut and Ruisheng Yao

Abstract: *The heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is formed during the cooking of proteinaceous animal foods (meat, chicken, and fish). PhIP is a carcinogen in the Fischer 344 (F-344) rat; it induces mammary tumors in female rats and lymphomas and colon and prostate tumors in male rats. In F-344 rats, PhIP forms DNA adducts in various organs, including the target organs. Inhibition of PhIP-DNA adduct formation is likely to lead to inhibition of PhIP tumorigenicity. We have examined the chemopreventive properties of green tea and black tea in PhIP carcinogenesis by evaluating their effects on PhIP-DNA adduct formation in the female F-344 rat. Young adult animals were maintained on powdered AIN-76A diet while receiving regular drinking water or 2% (wt/vol) infusions of green tea or black tea for a total of six weeks. During Weeks 3, 4, and 5, all animals received PhIP by gavage (1 mg/kg/day). Three rats per group were euthanized on Days 1 and 8 after termination of PhIP exposure. DNA was isolated from a number of organs and analyzed for PhIP-DNA adducts by ^{32}P-postlabeling assays. Compared with animals on regular drinking water, PhIP-DNA adduct formation was inhibited in small intestine, colon, liver, and mammary epithelial cells (MECs) of animals receiving green tea or black tea as the sole source of drinking fluid. Green tea inhibited adduct formation in colon, liver, and MECs (33.3–80.0%) on both days, but only on Day 8 (54.4%) in small intestine. Black tea inhibited adduct formation on both days in liver (71.4–80.0%), on Day 1 in colon (40.0%), and on Day 8 in small intestine (81.8%); it had no effect on MEC adducts. Neither green tea nor black tea had an effect on adduct levels in pancreas, lungs, white blood cells, heart, kidneys, spleen, cecum, or stomach. Similarly, these teas did not affect the rate of adduct removal (percent change from Day 1 to Day 8) in any organ. It is concluded that green tea and black tea are potential chemopreventive agents in PhIP-induced tumorigenesis in the F-344 rat.*

Introduction

The worldwide consumption of tea has prompted searches for possible beneficial effects of tea and its components in cancer prevention. Although the results of epidemiological studies are mixed and often inconclusive, the results of studies in rodents have demonstrated significant chemopreventive effects of tea and tea components in a number of experimental models (reviewed in References 1 and 2). These effects have been ascribed to tea's high content of polyphenolic compounds, principally catechins and other flavanols (3). Tea catechins not only suppress the *in vitro* mutagenicity of chemical carcinogens (4) but are also potent inhibitors of the initiation and promotion stages of chemical carcinogenesis (1,2). The anticarcinogenic and chemopreventive properties of tea polyphenols are thought to be due to their effects on carcinogen activation/deactivation enzymes (5–8), their growth-suppressing properties (9,10), and their antioxidant properties (11–13).

Cooking of proteinaceous foods (meat, fish, and chicken) results in the formation of a number of highly mutagenic compounds (14), collectively called heterocyclic amines, most of which belong to the subclass of aminoimidazoazaarenes (AIAs). All the AIAs tested are carcinogenic in animals (reviewed in Reference 15). 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), the most abundant AIA in cooked meat (16), is carcinogenic in rats and mice (15). PhIP induces principally mammary tumors as well as some colon tumors in female Fischer 344 (F-344) rats and lymphomas and prostate and colon tumors in male F-344 rats (15).

Most carcinogens, including PhIP, need to be enzymatically activated to a DNA-interacting species before their carcinogenicity is expressed (17). PhIP is activated in two steps: 1) by cytochrome *P*-450 (CYP) 1A1 or 1A2 to yield *N*-hydroxy-PhIP and 2) by further esterification of *N*-hydroxy-PhIP (acetylation or sulfation) to generate a reactive species, probably the nitrenium ion, which reacts with DNA to form specific adducts (15). PhIP-DNA adducts have been isolated from a number of animal organs and cells after treatment with PhIP (15); the major adduct has been identified as *N*-(deoxyguanosin-8-yl)-PhIP (18–20).

Green tea and black tea, as well as a number of individual tea polyphenols, have been shown to inhibit the bacterial mutagenicity of PhIP (21–24). Although in the male F-344

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rat green tea and black tea have been shown to inhibit DNA adduct formation of PhIP in the colon (25), as well as that of the related AIA, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) in the liver (26), such inhibition has not been tested in the female F-344 rat. PhIP is a mammary carcinogen in female F-344 rats and, since human exposure to PhIP through the diet is well established (27–30), PhIP is a suspect agent in the etiology of human breast cancer. In the present studies, we have, therefore, examined the potential chemopreventive properties of tea in PhIP carcinogenesis by evaluating PhIP-DNA adduct formation in female F-344 rats given prolonged, low oral doses of PhIP while receiving infusions of green tea or black tea as the sole source of drinking fluid.

Materials and Methods

Materials

PhIP (>98% pure) was purchased from Toronto Research Chemicals (North York, ON, Canada). All materials for the ^{32}P-postlabeling assay were from the same sources used in a previous study (31). Green tea and black tea (World Blends) were generously donated by Dr. Douglas A. Balentine (Thomas J. Lipton, Englewood Cliffs, NJ). Infusions of 2% (20 g/l) were prepared in the laboratory by steeping the tea in boiling water for 10 minutes and filtering the cooled product.

Animal Treatment

Eighteen young, adult female F-344 rats (6–7 wk old, 88–105 g) were purchased from Harlan Sprague Dawley (Indianapolis, IN). The animals were kept in polycarbonate cages (3/cage) in a temperature- and humidity-controlled room. Powdered AIN-76A diet was prepared in the laboratory as described previously (32), except the antioxidants were omitted. The experimental design is outlined in Figure 1. Briefly, the animals were maintained on the AIN-76A diet for a total of six weeks and given water, 2% green tea, or 2% black tea (6 animals/group) as drinking fluid during this period. Animals received fresh food twice a week (Tuesday and Friday) and fresh tea three times a week (Monday, Wednesday, and Friday). During Weeks 3, 4, and 5, PhIP was administered daily by gavage (1 mg/kg/day) to all animals, with dimethyl sulfoxide—0.05 M sodium phosphate, pH 4.5 (1:1), as the vehicle (0.5 ml/animal). Animals were weighed once a week, and doses of PhIP were adjusted weekly on the basis of weight gains.

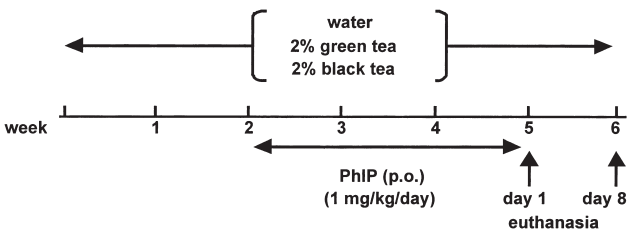

Figure 1. Experimental design. PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine.

On the first day of Week 6 (Day 1), as well as on the first day of Week 7 (Day 8), three animals from each group were euthanized, and blood [for isolation of white blood cells (WBCs)], mammary glands [for isolation of mammary epithelial cells (MECs)], liver, lungs, stomach, small intestine, cecum, colon, kidneys, heart, spleen, and pancreas were collected as described previously (33). Briefly, for the isolation of MECs, pooled mammary glands were added to 10 ml of phosphate-buffered saline, minced into small pieces (<2 mm^3), and then incubated with collagenase type I (0.25%, wt/vol) and collagenase type IV (0.35%, wt/vol) at 37°C for two hours. During this period the mixture was pipetted up and down every 15–20 minutes. At the end of the incubation period, the mixture was filtered through a nylon cell strainer (70 μm, Falcon) and then centrifuged for five minutes at 500 *g*. The pellet (MECs) was dissolved in 2 ml of nuclei lysis buffer [10 mM tris(hydroxymethyl)aminomethane·HCl, 400 mM NaCl, 2 mM Na_4-EDTA, pH 8.2] and stored at –70°C (33). WBCs, isolated as described previously (33), were stored similarly. All organs were added to a small volume of phosphate-buffered saline and then immediately frozen at –70°C until isolation of DNA.

Isolation of DNA and ^{32}P-Postlabeling Analysis

DNA was isolated from organs by a direct salt precipitation method, as described previously (34). PhIP-DNA adducts were isolated and quantitated using the intensification version of the ^{32}P-postlabeling assay, as described elsewhere (34), and the solvents for ion-exchange chromatography, as described by Josyula and co-workers (33). Adduct levels, expressed as relative adduct labeling (RAL) values, represent the sum of the individual PhIP-DNA adducts.

Statistical Analysis

The effect of the type of drinking fluid on adduct formation and rate of adduct removal was analyzed by one-way analysis of variance, followed, in case of a significant effect, by Fisher's least significant difference post hoc test.

Results

Body weight gains were independent of the type of drinking fluid during the entire six-week period of the study ($p > 0.05$ for each of Weeks 1–6, data not shown). During Weeks 1, 2, 3, 4, 5, and 6, body weights of the animals increased (%gain/wk) as follows: 14.6–19.7%, 18.2–23.2%, 7.6–10.1%, 3.9–6.8%, 3.7–6.3%, and 3.2–6.1%, respectively.

The PhIP-DNA adduct pattern was qualitatively similar in all organs/cells and was identical to that observed in our previous PhIP-DNA adduct studies in the F-344 rat (33–36). As described previously (33–36), the major ^{32}P-postlabeled PhIP-DNA adduct was *N*-(deoxyguanosin-8-yl)-PhIP (data not shown).

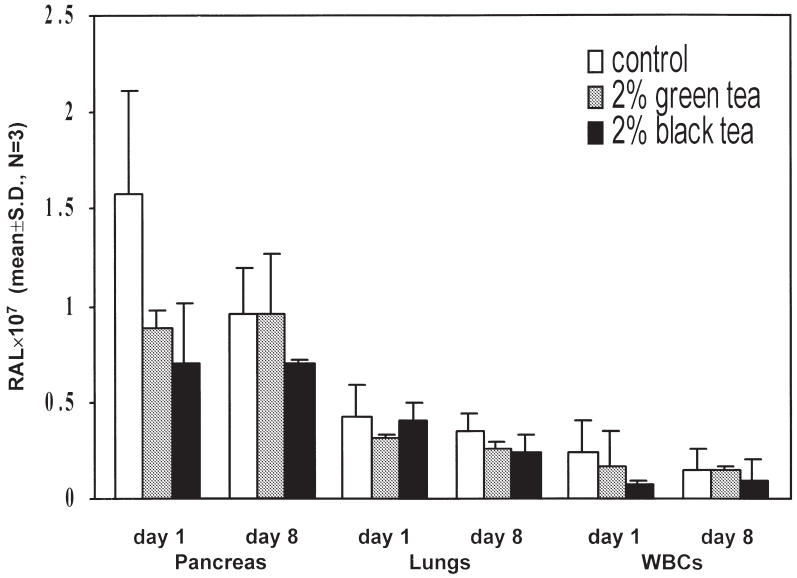

Figure 2. Total PhIP-DNA adducts in pancreas, lungs, and white blood cells (WBCs) of female Fischer 344 (F-344) rats maintained on AIN-76A diet and given water (control), 2% (wt/vol) green tea, or 2% (wt/vol) black tea to drink for 6 wk. All animals were treated with PhIP (1 mg/kg/day) by gavage for 21 days (Weeks 3, 4, and 5). Three animals per time point per group were euthanized on Days 1 and 8 after cessation of PhIP treatment. Adduct levels were independent ($p > 0.05$) of type of drinking fluid. RAL, relative adduct labeling.

Adduct levels in animals receiving regular drinking water were highest in the pancreas (RAL = 1.59×10^{-7}; Figure 2) and lowest in the stomach (RAL = 0.02×10^{-7}; Figure 5). In the colon, adduct formation was inhibited by green tea (40.0% on Day 1 and 75.0% on Day 8) and by black tea (40.0% on Day 1; Figure 3). In the small intestine, green tea and black tea inhibited adduct formation, but only on Day 8 (54.4–81.8%; Figure 3). In the liver, green tea and black tea inhibited adduct formation at both time points (60.0–71.4% inhibition with green tea and 71.4–80.0% inhibition with black tea; Figure 5). Only green tea inhibited adduct formation in MECs (33.3% inhibition on Day 1 and 80.0% inhibition on Day 8; Figure 5). Neither green tea nor black tea had any inhibitory effects on PhIP-DNA adduct formation in the pancreas, lungs, WBCs (Figure 2), heart (Figure 3), kidneys, spleen, cecum (Figure 4), or stomach (Figure 5).

It is possible that tea may affect the rate of adduct removal (repair), and, therefore, adducts were measured immediately after cessation of exposure to PhIP (Day 1), as well as on Day 8, when animals had been on a PhIP-free diet for one week to allow for repair (Figure 1). For all organs and cells, the average change in adduct levels on Day 8 compared with that on Day 1 was –38.7% (range –9.1 to –87.5%) in animals on regular drinking water, –31.5% (range –16.1 to

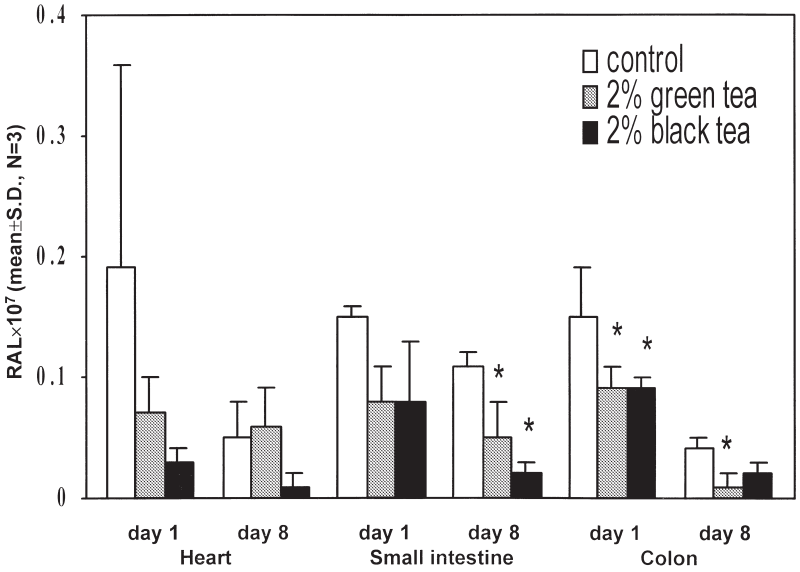

Figure 3. Total PhIP-DNA adducts in heart, small intestine, and colon. Experimental details are as outlined in Figure 2 legend. Adduct levels were dependent ($p < 0.05$) on type of drinking fluid in small intestine (Day 8) and colon (Days 1 and 8) but not in heart ($p > 0.05$). *, Significantly different from corresponding control (water) value.

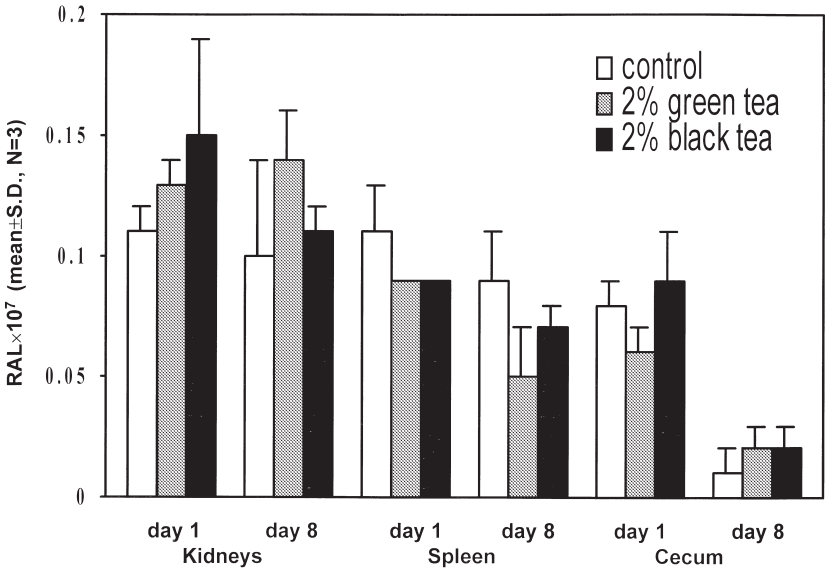

Figure 4. Total PhIP-DNA adducts in kidneys, spleen, and cecum. Experimental details are as outlined in Figure 2 legend. In all 3 organs, adduct levels were independent ($p > 0.05$) of type of drinking fluid.

–88.9%, with 0%, +7.9%, and +7.7% in the liver, pancreas, and kidneys, respectively) in animals on 2% green tea, and –33.2% (range –22.2% to –77.8%, with 0%, +28.6%, and +42.9% in the pancreas, MECs, and WBCs, respectively) in animals on 2% black tea. The overall differences between the three groups were not significant ($p > 0.05$).

Discussion

Interorgan distribution of PhIP-DNA adducts (Figures 2–5) resembled that observed previously in male (37) and female (36) F-344 rats treated with PhIP in the same manner. In these studies, the pancreas, a nontarget organ, has the highest PhIP-DNA adduct levels; the stomach and the liver have the lowest levels. In the female rats, MECs have relatively low adduct levels (36) (Figure 5). Likewise, the colon has relatively low adduct levels in the male rats (37). Therefore, because the mammary gland and the colon are the main target organs in the female and male F-344 rat, respectively (15), these results mean that PhIP-DNA adduct levels in organs/cells are not necessarily correlated with organ sensitivity to tumorigenesis. The results of an immunohistochemical interorgan comparison of PhIP-DNA adducts in F-344 rats (38) also confirm this conclusion. It should be emphasized

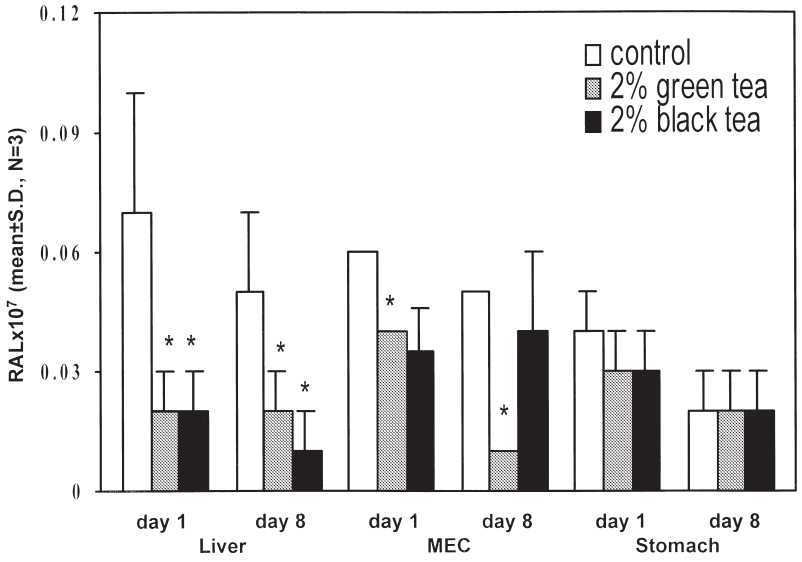

Figure 5. Total PhIP-DNA adducts in liver, mammary epithelial cells (MECs), and stomach. Experimental details are as outlined in Figure 2 legend. For MEC adduct levels, mammary glands from 3 animals were harvested and, to obtain sufficient DNA from isolated MECs, glands from 3rd animal were divided into 2 equal portions and added to that of other 2 animals (thus $n = 2$ for all MEC adduct levels). Where no variability is shown (MEC, control and 2% green tea), SDs were too small to be visualized. Adduct levels were dependent ($p < 0.05$) on type of drinking fluid in liver (Days 1 and 8) and in MECs (Days 1 and 8), but not in stomach ($p > 0.05$). *, Significantly different from corresponding control (water) value.

that interorgan distribution of PhIP-DNA adducts strongly depends on the manner of administration of PhIP, i.e., high, bolus dose or prolonged, low levels in the diet (39).

As we found previously with other potential chemopreventive agents such as conjugated linoleic acid (33), menhaden oil (40), and n–3 fatty acids (41), green tea and black tea inhibit DNA adduct formation only in certain organs and, in some cases, only at certain time points (Figures 2–5). In this regard, these agents are different from indole-3-carbinol, which inhibits AIA-DNA adduct formation in all organs/cells examined (36,42,43). It is likely that inhibition of DNA adduct formation is not only a function of the kinetics of absorption and elimination of the chemopreventive agent but is also related to the enzymatic mechanisms by which the AIA is activated/deactivated in the various organs. For instance, for PhIP it is known that these mechanisms differ, not qualitatively, but quantitatively, in liver, colon, and MECs of the F-344 rat (44,45) and that a compound like indole-3-carbinol may induce phase I and phase II enzymes (36,42).

The inhibition of PhIP-DNA adduct formation in MECs by green tea (Figure 5) would suggest that green tea inhibits initiation of PhIP-induced mammary carcinogenesis. In the few mammary tumorigenesis studies involving chemoprevention by green tea catechins, the principal action of tea was in inhibiting the size, not the incidence or multiplicity, of rat mammary tumors induced by IQ, PhIP, or 7,12-dimethylbenz[*a*]anthracene (46–48), suggesting an effect of tea on promotion rather than initiation. This conclusion was recently affirmed in a study using black tea as the chemopreventive agent in 7,12-dimethylbenz[*a*]anthracene-induced mammary tumors in Sprague-Dawley rats maintained on a high-fat (24.0%) diet (49). Thus, in inhibiting mammary tumor formation, tea may act at the initiation and promotion stages of the process.

In the colon, green tea and black tea inhibited PhIP-DNA adduct formation (Figure 3), confirming a recent finding in male F-344 rats with use of a similar protocol, except PhIP was given as a single dose by gavage (25). Also, green tea and black tea were found to inhibit hepatic IQ-DNA adduct formation as well as colonic aberrant crypt foci in male F-344 rats (26). Thus tea probably offers protection against the initiation phase of colon carcinogenesis induced by PhIP. This interpretation agrees with chemoprevention studies by green tea polyphenols in colon tumor induction by non-AIA carcinogens in rats and mice (50,51). Green tea polyphenols, however, may also inhibit the postinitiation phase of colon carcinogenesis in rats (52). In a complex, multiorgan rat carcinogenesis model, 1% (wt/wt) dietary green tea catechins failed to inhibit colon carcinogenesis (53).

Most studies on the chemopreventive properties of tea and tea polyphenols have been concentrated on experimental skin and lung carcinogenesis in the mouse (1,2). Chemopreventive effects in the liver are not well established (1,2). The observed inhibition of hepatic PhIP-DNA adducts by green tea and black tea (Figure 5) implies a potential protective effect in PhIP-induced liver tumors. PhIP, however, is not a liver carcinogen (15), but its hepatic metabolism, especially that by phase I enzymes, probably plays a major role in determining its carcinogenicity in extrahepatic target organs such as the colon and the mammary gland (44,45,54). In a complex rat liver tumor initiation model, 1% (wt/wt) dietary green tea catechins were found to inhibit hepatic glutathione *S*-transferase placental form (GST-P)-positive foci induced by diethylnitrosamine, followed by a partial hepatectomy and exposure to 0.03% (wt/wt) dietary 2-amino-6-methyldipyrido[1,2-*a*:3',2'-*d*]imidazole (Glu-P-1), a heterocyclic amine structurally related to PhIP (55). When the protocol was modified, however, by replacing the dietary Glu-P-1 exposure with 0.02% (wt/wt) dietary 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline, a dietary AIA and liver carcinogen, the inhibitory effect of the 1% dietary green tea catechins could not be reproduced (56). It is therefore not known whether hepatic AIA-DNA adduct formation is related to induction of GST-P-positive foci, the latter being an excellent preneoplastic marker of the initiation phase of hepatocarcinogenicity (57,58).

Our finding of an inhibitory effect of tea on PhIP-DNA adducts in the small intestine (Figure 3) is in agreement with the inhibitory effect of green tea polyphenols on small intestine tumor formation in a multiorgan carcinogenesis model in the F-344 rat (53).

In inhibiting PhIP-induced carcinogenesis, it appears that tea may act by multiple mechanisms. On the basis of *in vitro* studies (21–24,59), tea polyphenols may inhibit the hepatic microsomal *N*-hydroxylation of PhIP, even though CYP 1A1 and 1A2, which catalyze this reaction, may be induced *in vivo* by green tea and black tea (8,26,60). In view of our present results on the inhibition of PhIP-DNA adducts by tea (Figures 3 and 5), the effects of tea on other carcinogen-metabolizing enzymes, such as induction of phase II detoxifying enzymes (5,7), may predominate *in vivo*. The antioxidant properties of tea polyphenols (11) are expected to exert a principal inhibitory effect on the promotional phase of carcinogenesis (1,2). Several antioxidants also exhibit chemopreventive properties during the initiation phase of IQ liver tumorigenesis, as measured by inhibition of IQ-induced GST-P-positive foci in rat liver (61). Similar evidence for the antioxidant role of green tea polyphenols has been inconsistent (55,56), as discussed above. In an *in vitro* system, however, it has been shown that the production of oxygen free radicals from IQ in the presence of NADPH-cytochrome *P*-450 reductase is quenched by green tea, black tea, and (–)-epigallocatechin gallate (62), the latter being the major catechin in green tea (3). Whether such a mechanism is operative *in vivo* and whether PhIP is also a substrate for this reaction remain to be investigated.

In summary, we have shown that a 2% (wt/wt) infusion of green tea or black tea, when given as the sole source of

drinking fluid to female F-344 rats, inhibits PhIP-DNA adduct formation in the liver, colon, MECs, and small intestine, but not in a number of other organs. The observed inhibitions imply that tea is a potential chemopreventive agent in PhIP-induced mammary and colon tumors in the F-344 rat. The limited data obtained on the protective effects of tea against AIA-induced tumors or preneoplastic lesions (26,47,48), although not uniformly positive (56), support this notion.

Acknowledgments and Notes

These studies were supported by a grant from the American Institute for Cancer Research. Address reprint requests to Herman A. J. Schut, Ph.D., Dept. of Pathology, Health Education Bldg., Rm. 202, Medical College of Ohio, 3055 Arlington Ave., Toledo, OH 43614-5806.

Submitted 17 August 1999; accepted in final form 1 October 1999.

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