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Subchronic Exposure to Ellagic Acid Impairs Cytotoxic T-Cell Function and Suppresses Humoral Immunity in Mice

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ABSTRACT

Ellagic acid (EA) is present in a variety of foods such as grapes, strawberries, raspberries, and nuts. It is a dietary plant phenol that has been shown to inhibit oxidative stress and chemical carcinogenesis. Although several studies have examined the protective mechanisms of dietary EA including the induction of detoxifying enzymes, regulation of cell cycle, chelation of nickel, and prevention of DNA methylation, none have addressed the role of EA in immunological surveillance. This study investigates the status of immune function in B6C3F1 mice exposed continuously to EA in drinking water at 0.5, 1.0, or 2.0 mg/kg/day for 28 days. Although this range of exposure is above the estimated human daily intake (≈940 μg/day for 70 kg person or 13.4 μg/kg/day), these levels would not be unreasonable if EA were used as a dietary supplement or as a chemotherapeutic agent. Previous reports have demonstrated the anticarcinogenic effects of EA at levels 10- to 250-fold greater than those applied in this study. Immunological parameters

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assessed included natural killer (NK) cell activity, cytotoxic T lymphocyte (CTL) activity, IgM antibody plaque forming cell (PFC) response, thymus, spleen, kidney, and liver mass, and total cellularity for the thymus and spleen. Subchronic exposure to EA for 28 days in drinking water caused significant suppression of specific IgM antibody responses in the 2.0 mg/kg EA treatment group and suppressed cytotoxic T-cell function in the 0.5 and 1.0 mg/kg EA treatment groups. All other immunological parameters were within normal ranges. Kidney and liver mass were not altered after treatment with EA. The results from this study indicate that EA suppressed both IgM antibody responses and CTLs. These observations suggest important implications on human health should EA be prescribed as a chemotherapeutic agent or a preventative dietary supplement for cancer.

Key Words: Ellagic acid; Polyphenol; Immunological function.

INTRODUCTION

Diets rich in fruits and vegetables are known to decrease risk of cancer development. This is attributed to a variety of dietary compounds with known anticarcinogenic activity such as indoles, flavones, isothiocyanates, vitamins, and phenols. Ellagic acid (EA) is a naturally occurring plant polyphenol produced from the hydrolysis of ellagitannins and is found in a variety of foods including grapes, blackberries, cranberries, strawberries, raspberries, walnuts, and pecans.[1] The total annual per capita consumption of ellagitannin containing foods is estimated at 2.5 kg. This corresponds to an annual consumption of 343 mg or a daily intake of nearly 1 mg of EA (13.4 μg EA/kg/day per 70 kg adult).[1,2]

Ellagic acid is a complex planar molecule with four hydroxyl groups and two attached lactone groups (Fig. 1) that has been effective in impeding tumorigenesis by polyaromatic hydrocarbons such as benzo[a]pyrene (BaP), 3-methylchloanthrene (3-MC) and 9,10-dimethy-l,2-benz-anthracene (DMBA).[3–7] Several mechanisms have been described that contribute to the anticarcinogenic and antimutagenic properties of EA. Ellagic acid alters phase I and II metabolic enzymes as demonstrated in several studies with liver,[5,8–10] skin,[7] esophageal,[8] or intestinal tissue.[11] Decreases in phase I CYP450 enzyme activity were observed in rodents fed EA for limited durations ranging from 23 to 28 days.[8,9]

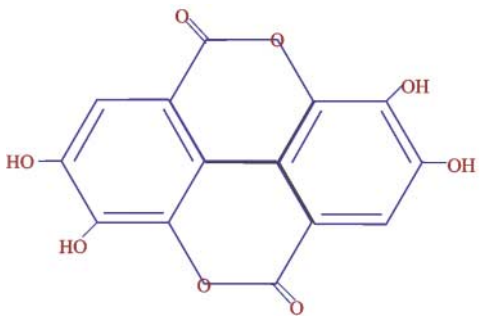

Figure 1. Chemical structure of EA.

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Specifically, CYP2E1 was significantly reduced while CYP450 1A1, 2B1, and 3A1/2 activities were not affected.[8] There is, however, some variability with respect to the profile of P450 isoform activity after EA exposure that may be attributed to strain and species differences. For instance, Sprague–Dawley rats and several mouse strains demonstrate inhibition of hepatic, lung, and epidermal CYP1A1, while CYP1A1 was not inhibited in Fisher 344 rats.[4,7,8] Effects on CYP2B1 and 2E1 also vary between studies and are likely due to similar reasons.[5,8] Ellagic acid most consistently upregulates activity of phase II enzymes including glutathione-*S*-transferase (GST), NAD(P)H : quinone reductase, and UDP glucuronosyltransferase.[8,10,11] The decreased metabolic activation of carcinogens by phase I enzymes coupled with subsequent enhancement of phase II enzymes allows effective elimination of carcinogenic xenobiotics.

Besides enhancing the detoxification processes, EA can prevent carcinogenesis by reducing DNA adduct formation. Specifically, EA binds to the diol-epoxide of benzo[a]pyrene and inhibits the formation of DNA adducts.[3,4] Furthermore, EA may also protectively bind to DNA to prevent O^6-methylguanine formation from methylating carcinogens.[4,6,12,13] Other studies with EA have explored additional mechanisms of action or related effects. For example, EA causes G1 arrest and induces apoptosis in a cervical carcinoma cell line,[14] reduces hepatocellular neoplasms induced by aflatoxin;[15] inhibits superoxide radical formation,[16] inhibits hemorrhage in rodents by activation of intrinsic blood coagulation,[17] is a potent tyrosine kinase inhibitor;[18] and effectively chelates nickel thereby inhibiting hepatic and nephrotoxicity.[19]

Based on a series of convincing reports regarding EAs anticarcinogenic properties, it was considered that EA might also enhance immunity to aid in the prevention of cancer. Clearly, the immune system plays an important role in cancer surveillance and the effect of EA on immunological status should be explored as a potential mechanism of action since other polyphenols are reported to have immunostimulatory actions.[20,21] Accordingly, this study was designed to evaluate various nonspecific, cell mediated, and humoral immune responses in mice exposed to EA for 28 days. Immunological evaluation included gross toxicological evaluations of organs, total cellularity of immunological organs, specific IgM responses to a T-cell dependent antigen, and functional assessment of specific cell types such as natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) that are directly involved with tumor surveillance and killing.

MATERIALS AND METHODS

Chemicals

Ellagic acid was purchased from Aldrich Chemical Co. (Milwaukee, WI). RPMI-1640, phosphate buffered solution (PBS), hank's balanced salt solution (HBSS), fetal calf serum, penicillin (pen), and streptomycin (strep) were obtained from Cellgro, Mediatech (Herndon, VA). Lyophilized guinea pig complement (LGPC), 100X non essential amino acids, and 100X sodium pyruvate were obtained from Gibco Laboratories (Grand Island, NY). Sheep red blood cells were purchased from BioWhittaker, Inc. (Walkersville, MD). Triton X was obtained from Fisher (Pittsburgh, PA). HEPES was purchased from Sigma (St. Louis, MO). ^{51}Cr sodium chromate was obtained from ICN Biomedicals

Inc. (Irvine, California). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was purchased from Radian International (Austin, TX).

Animal Care

Female B6C3F1 mice (7–8 weeks old) were obtained from Harlan Sprague–Dawley (Indianapolis, Indiana). Female DBA/2 mice (7–8 weeks old) were used to passage P815 tumors cells for the cytotoxic T-cell assay and these animals were also obtained from Harlan Sprague–Dawley (Indianapolis, Indiana). Mice were maintained in an AAALAC accredited animal facility and housed 2–3 per plastic cage with filter bonnets and absorbent corncob bedding. Mice received water (with or without EA) and standard rodent chow (Harlan-Teklad, Madison, WI) ad libitum. The B6C3F1 mice were acclimated to the conditions of the treatment room (12 hour light/dark cycle, $22^{\circ}C \pm 2^{\circ}C$, 65% relative humidity) for at least 4 days before EA was administered. Mice were weighed at the start of the experiment and weekly thereafter to monitor for overt toxicity. Water consumption was measured every 3 or 4 days during the study to calculate actual daily intake. All immunological parameters were performed in the same sets of mice, with the exception of the CTL challenge experiments. Additionally, positive control mice were incorporated in many of the studies and were administered TCDD at levels of 15 μg/kg/day for 4 days by daily intraperitoneal injection. This protocol was approved and performed in accordance with the Medical University of South Carolina Animal Care and Use Committee.

Exposure to Ellagic Acid

The exposure range was based on a study by Mukhtar et al.[7] that reported chronic administration of trace amounts (18 μg/mouse/day or 0.8 mg/kg/day) of EA via drinking water inhibited 3-methylcholanthrene-induced skin tumorigenicity in mice. Also considered was the fact that intravenous injections of 0.2 mg/kg were well-tolerated in humans.[22] Based on these reports, this study utilized EA exposure levels of 0.5, 1.0, or 2.0 mg of EA/kg/day while control animals received water only, for a duration of 28 days. Every 3 or 4 days during the study, concentration of EA in water was adjusted according to body weight and water consumption per mouse. Our data demonstrated that the mice drank on the average of 4 mL water/day. The drinking water concentration of EA was approximately 2.5, 5, or 10 μg/mL.

Preparation of Ellagic Acid in Drinking Water

Ellagic acid was prepared in water based on a study reported by Mukhtar et al.[7] However, slight modifications, as suggested by the manufacturer, were incorporated in this preparation to increase the solubility of EA. Three milliliter of 1 N NaOH was added to 32 mg of EA and slightly heated and stirred until dissolved. This solution was then brought to a final volume of 1600 mL with double distilled water and the pH adjusted to 7.5. An aliquot of 800 mL was used to prepare the appropriate dilutions for the drinking water while the second aliquot was frozen at $-20^{\circ}C$ and later thawed in warm water to

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prepare the dilutions for the next water bottle change. Dilutions prepared were 2.5, 5, and 10 μg/mL of EA in water. Water was changed twice per week based on stability information of EA.

Body Mass, Organ Mass, and Cellularity

At the end of 4 weeks (28 days) of exposure to EA, the mice were euthanized via CO_2 asphyxiation and the spleen, thymus, liver, and kidneys were collected. All balances were calibrated prior to each use. For each individual animal, organ mass was normalized for body mass and reported as a somatic index [(organ mass/body mass) × 100]. Over the course of the study, body mass was monitored weekly. Final changes in body mass were determined by subtracting the mass obtained on day 1 from that measured on day 28. To obtain cell suspensions for thymus and spleen, the organs were placed in 3 mL of sterile RPMI 1640 with 10% fetal calf serum and "mashed" using two sterile frosted slides. Cell counts were obtained using a Coulter counter (Model ZM particle counter, Coulter Electronics, Hileah, FL) that was calibrated using cell suspensions whose cell concentrations were determined using a hemocytometer.

Natural Killer Cell Assay

Natural killer cell activity was assessed as a modification of Duke et al.[23] Briefly, YAC-1 (ATCC, Manassas, VA) cells were used as the target and were labeled with ^{51}Cr. Spleens were processed, counted, and diluted to 1×10^7 cells/mL in Complete RPMI-1640 (RPMI-1640, 10% fetal bovine serum, and 1% pen-strep). The ratios of spleen cells to YAC-1 cells used were 100 : 1, 50 : 1, and 25 : 1. These were plated in triplicate for each sample in a 96-well microtiter plate. At least five wells of a negative control (spontaneous release) or a positive control (maximum release) were prepared. Maximum release was determined by adding ^{51}Cr-labeled Yac-1 cells to TritonX to lyse tumor cells. Spontaneous release was determined by incubating Yac-1 tumor cells only, in complete media. Plates were incubated for 4 hours at 37°C and 5% CO_2. After the incubation, plates were centrifuged and 25 μL of supernatant fluid was transferred to a LumaPlate™ (Packard, Meridian, CT) containing solid scintillant. Plates were dried overnight in a biosafety hood. ^{51}Cr counts were measured using a Packard Top Count™-NXT scintillation counter and data were reported as Mean Lytic Units (MLU)/10^7 cells.[24]

Cytotoxic T-Cell Activity

Cytotoxic T lymphocyte activity was determined using a standard 4 hour ^{51}Cr-release assay with P815 (ATCC, Manassas, VA) target cells.[25] On day 18 of exposure, mice were immunized with 1×10^7 P815 tumor cells by intraperitoneal injection. These tumor cells were freshly passaged 1 week prior in DBA mice, and harvested on the day of tumor challenge in the B6C3F1. Ten days after P815 challenge (day 28), the mice were sacrificed and their spleens processed as described above. The splenocytes were incubated for 4 hours with ^{51}Cr-labeled P815 tumor cells at effector to target ratios of 100 : 1, 30 : 1, 10 : 1, and 3 : 1 in 96-well microtiter plates. After the 4 hour incubation, plates were centrifuged and

25 μL of supernatant fluid was transferred to a LumaPlate™ (Packard, Meriden, CT) containing solid scintillant. Plates were air dried overnight in a biosafety hood and ^{51}Cr counts were assessed using a Packard Top Count™-NXT scintillation counter. Data were reported as Mean Lytic Units (MLU)/10^7 cells.[24]

Antibody Plaque-Forming Cell Assay

The number of plaque forming cells (PFCs) was determined using the Cunningham modification of the Jerne plaque assay.[26,27] Four days prior to euthanasia, mice were administered 0.1 mL of a 25% sheep red blood cell (sRBC) suspension in PBS via intraperitoneal injection. All sRBCs for the experiments were drawn from a single donor animal. Following euthanasia, murine spleens were processed, cells were counted, and suspensions diluted to 2.0×10^6 cells/mL in supplemented RPMI-1640 (RPMI-1640, 10% fetal bovine serum, 1% nonessential amino acids, 1% sodium pyruvate, 10 mM HEPES, 1% pen-strep, and 10 μM 2-mercapto-ethanol, pH 7.4). Aliquots of the cell suspensions were added to eppendorf tubes containing supplemented RPMI-1640 and sRBCs. Fifty microliter of reconstituted LGPC (diluted 1 : 2, v/v) was then added on a tube by tube basis. Aliquots of the solution were placed in Cunningham chamber slides, sealed with melted wax, and incubated at 37°C and 5% CO_2 for 1 hour. Results were reported as PFCs/million cells.

Statistics

All experiments were repeated twice. Data were tested for normality (Shapiro-Wilks) and homogeneity (Bartlett's test for unequal variances). A one-way ANOVA was used to determine differences among treatment groups for each endpoint using JMP 4.0.2 (SAS Inst. Inc., Cary, NC). When significant differences were detected by ANOVA ($P \leq 0.05$), Dunnett's *t*-test was used to compare treatment groups to control.

RESULTS

Body and Organ Mass

No overt toxicity was observed in mice treated with EA for 28 days via drinking water. Furthermore, there were no significant differences in body mass gain following EA treatment as compared to control mice (Table 1). An increasing trend in liver mass was observed following EA intake, but this was not statistically significant ($P < 0.05$) when compared to control animals (Table 1). Spleen, thymus, and kidney mass were not altered by EA treatment (Figs. 2 and 3, Table 1).

Organ Cellularity

No significant changes were observed in splenic or thymic total cellularity (Figs. 2 and 3) after exposure to EA for 28 days.

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Table 1. Change in body mass and normalized liver and kidney mass in adult female B6C3F1 mice following a 28-day exposure to EA in drinking water.

Ellagic acid (mg/kg/day)	Body mass change[a] (g)	Normalized liver mass[b] (g)	Normalized kidney mass[b] (g)
0.0	3.33 ± 0.64	5.59 ± 0.12	1.30 ± 0.03
0.5	3.25 ± 0.21	5.56 ± 0.05	1.28 ± 0.02
1.0	3.00 ± 0.25	5.69 ± 0.15	1.28 ± 0.03
2.0	3.5 ± 0.26	5.87 ± 0.10	1.36 ± 0.05

Note: Data are presented as mean ± SEM. Sample size is six for treatment and control groups. Data are representative of two trials ($P \leq 0.05$).
[a](Final mass − start mass).
[b]Normalized mass was calculated as the somatic index [organ mass (g)/body mass (g)] × 100.

Natural Killer Cell and Cytotoxic T-Cell Activity

Natural killer cell responses in EA-treated mice were not significantly changed from control animals (Fig. 4). However, the ability of CTLs to lyse tumor cells was significantly suppressed at the 0.5 and 1.0 mg/kg/day EA exposure levels, but not the 2.0 mg/kg/day levels (Fig. 5). Figure 5 represents two repeated experiments that were combined as there was no statistical interaction between studies. The CTL activity for the 0.5, 1.0, and 2.0 mg/kg/day EA treatment levels were suppressed by 57%, 50%, and 8%, respectively.

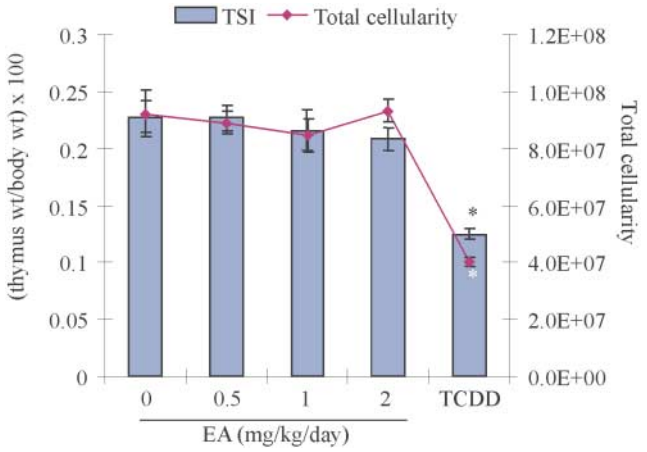

Figure 2. Thymus mass expressed as the somatic index [TSI = (thymus mass/body mass) ∗ 100] and total thymus cellularity (secondary axis) in female B6C3F1 mice following a 28-day exposure to EA in drinking water. Data are presented as mean ± SEM. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (15 μg/kg i.p. for 4 days) was used as a positive control ($n = 4$). Sample size is six for treatment and control groups. Data are representative of two trials. Treatment groups significantly different ($P \leq 0.05$) from control are noted by an asterisk (∗).

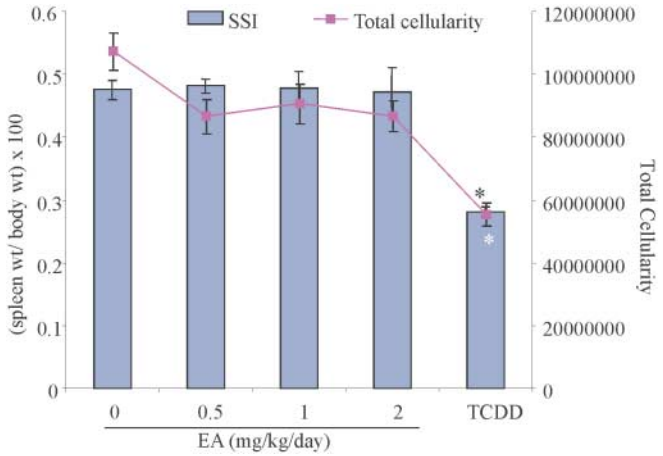

Figure 3. Splenic mass expressed as the somatic index [SSI = (spleen mass/body mass) * 100] and total splenic cellularity (secondary axis) in female B6C3F1 mice following a 28-day exposure to EA in drinking water. Data are presented as mean ± SEM. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (15 μg/kg i.p. for 4 days) was used as a positive control ($n = 4$). Sample size is six for treatment and control groups. Data are representative of one trial. Treatment groups significantly different ($P \leq 0.05$) from control are noted by an asterisk (*).

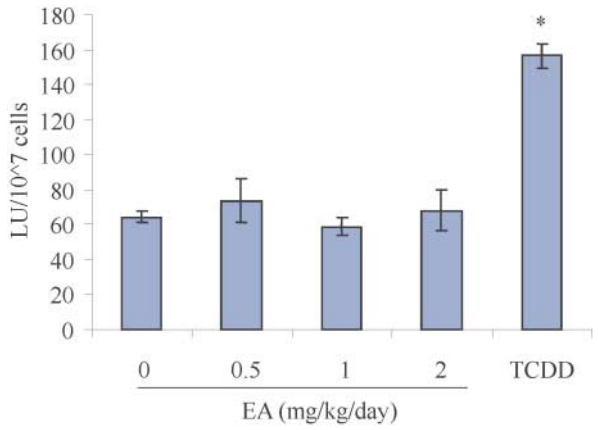

Figure 4. Natural killer cell activity in female B6C3F1 mice following a 28-day exposure to EA in drinking water. Data are presented as mean ± SEM. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (15 μg/kg i.p. for 4 days) was used as a positive control ($n = 4$). Sample size is six for treatment and control groups. Data are representative of two trials. Treatment groups significantly different ($P \leq 0.05$) from control are noted by an asterisk (*).

Antibody Plaque-Forming Cell Response

Treatment with EA resulted in a dose-dependent suppression of the IgM antibody PFC response (10% and 42% for 1.0 and 2.0 mg/kg/day, respectively; Fig. 6).

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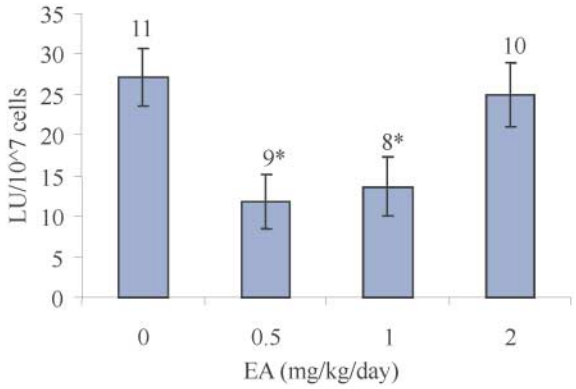

Figure 5. Cytotoxic T lymphocyte activity in female B6C3F1 mice following a 28-day exposure to EA in drinking water. Data are combined from two experiments and presented as mean ± SEM. Experiments were tested for statistical interaction before being combined. Numbers above SEM bars indicate sample size. Treatment groups significantly different ($P \leq 0.05$) from control are noted by an asterisk (∗).

DISCUSSION

Ellagic acid is a naturally occurring polyphenolic antioxidant with verified chemopreventive properties.[8] It has been demonstrated both in vivo and in vitro that EA can inhibit several carcinogens through a variety of mechanisms;[1,3,4,7,14–16,28,29] however, no studies, to date, have assessed its effects on immune function. Other polyphenols, such as those found in green tea, have been reported to possess immunomodulatory characteristics.[20,21] Thus, it seemed rational to hypothesize that EA might also have immunoenhancing properties which could contribute to its reported anticarcinogenic effects.

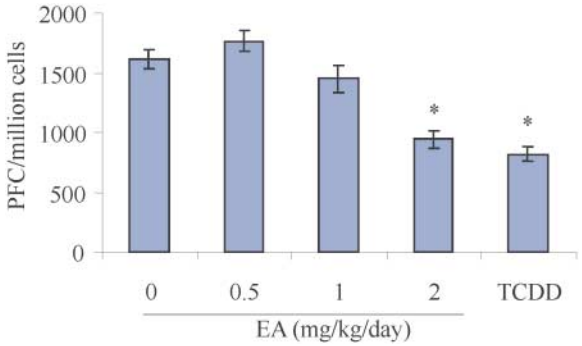

Figure 6. Plaque forming cell activity in female B6C3F1 mice following a 28-day exposure to EA in drinking water. Data are presented as mean ± SEM. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (15 μg/kg i.p. for 4 days) was used as a positive control ($n = 4$). Sample size is six for treatment and control groups. Data are representative of two trials. Treatment groups significantly different ($P \leq 0.05$) from control are noted by an asterisk (∗).

A diverse range of exposure levels have been utilized to establish the anticarcinogenic effects of EA. Typically, EA was incorporated into rodent chow at levels ranging from 0.1 to 10 g/kg and fed for durations of 3 to 20 weeks[8–11,15,30–32] or as a supplement in drinking water for 120 days at levels of 18 μg EA/mouse/day (0.8 mg/kg/day).[7] To place in context the EA levels administered in this study with those previously reported, the exposures were extrapolated into milligram of EA per kilogram of body mass. As body mass and daily consumption data were not available for previously reported studies,[9–11,15,30,32,33] it was assumed that the average daily consumption of food per 100 g body mass was 5 g in the rat studies[33] and 1.5 g per 10 g of body mass in the mouse studies. Consequently, the calculated exposures from previous studies ranged from 20 to 500 mg of EA/kg/day with the exception of the study by Mukhtar and colleagues[7] who reported 0.8 mg of EA/kg/day in mice. Based on these ranges, the present study was conducted with comparatively low-level exposures ranging between 0.5 and 2.0 mg/kg/day. Although the range of exposure used in this study is above the estimated average human daily dietary intake of 0.013 mg/kg, it has been demonstrated that intravenous injections of 0.2 mg/kg were well-tolerated in humans.[22] Thus, if EA were to be utilized as a dietary supplement or chemotherapeutic agent, the exposures examined in this study would seem reasonable.

This study confirmed that low doses of EA can modulate immunological function, however, the observed effects were unexpected in light of EAs anticarcinogenic effects. While gross immune endpoints were not altered, functional cell-mediated immunity was impaired. MHC class I-restricted cytotoxic T-cells were significantly suppressed at the lower exposure levels, while recovery of function was achieved at the highest exposure level (2.0 mg/kg/day) of EA. The loss of inhibitory activity of EA on CTL function at the higher dose is not explained by the typical hormetic dose–response relationship that is defined as stimulation of physiological responses at low doses with suppression of responses at higher doses. However, broader definitions of hormesis include overcompensation stimulation after an initial disruption in homeostasis.[34] This may, in part, explain deficits in CTL function at low exposure levels with compensation at the highest EA dose. Expanding the dose-range in future studies would offer additional understanding of this effect on CTL function.

This study evaluated EA at estimated levels 10- to 250-fold less than those used in previous studies.[9–12,16,30–32] It is not known how CTL function might be affected at higher exposure levels, but it is troublesome that these cytotoxic T cells involved in tumor surveillance[35,36] are suppressed at relatively-low exposure levels. Cell-mediated cytotoxicity also plays a substantial role in the clearance of noncytopathic viruses from infected tissues[37] and can also be effective in systemic protection from listerial infections.[38] Moreover, NK cell activity was not affected by EA treatment. These nonspecific lymphocytes are also important in tumor surveillance and viral immunity and may extend immunological protection when CTL activity is suppressed. Ellagic acid studies utilizing host resistance models would offer insight to the overall impact of CTL functional deficits on host defenses.

Ellagic acid also unexpectedly suppressed humoral immunity by decreasing specific IgM antibody production in response to the T-cell dependent antigen sRBC. These observations may be problematic as reduced proficiency in IgM responses may compromise immunological protection from viral and other infectious agents.[39–42] Moreover, when antibody responses are suppressed in conjunction with CTL function, an increased susceptibility to parasitic infections may occur.[43]

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Several studies have overwhelmingly demonstrated the chemopreventive benefits of EA, yet the present study demonstrated that consumption of relatively low levels of EA for a month suppress immune function without signs of overt toxicity. The production of specific B-cell antibodies was reduced at the highest dose which could potentially lead to impaired resistance to infection. Additionally, at the low and middle doses, CTL activity was suppressed which could lead to reduction in tumor lysis and viral protection. The battery of immunological assays performed in this study needs to be expanded to fully understand the scope of the immunomodulatory effects of EA. Future studies should include host resistance models that evaluate responses after infectious or tumor challenge *in vivo* and evaluation of alterations in splenic immunophenotypes. Based on these results, a comprehensive immunotoxicological evaluation of EA is suggested before this dietary compound can be considered safe as a cancer preventive supplement or chemotherapeutic agent. Accordingly, careful progression from the laboratory to clinical studies would be warranted.

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REFERENCES

1. Daniel, E.M.; Ratnayake, S.; Kinstle, T.; Stoner, G.D. The effects of pH and rat intestinal contents on the liberation of ellagic acid from purified and crude ellagitannins. J. Natr. Prod. **1991**, *54* (4), 946–952.
2. USDA Economic Research Service. *Fruits and Tree Nuts: Situation and Outlook Yearbook*; U.S. Department of Agriculture: Hyattsville, MD, 1988.
3. Shugart, L.; Kao, J. Effect of ellagic and caffeic acids on covalent binding of benzo[a]pyrene to epidermal DNA of mouse skin in organ culture. Int. J. Biochem. **1984**, *16* (5), 571–573.
4. Barch, D.H.; Rundhaugen, L.M.; Stoner, G.D.; Pillay, N.S.; Rosche, W.A. Structure–function relationships of the dietary anticarcinogen ellagic acid. Carcinogen **1996**, *17* (2), 265–269.
5. Castonguay, A.; Boukharta, M.; Teel, R. Biodistribution of, antimutagenic efficacies in *Salmonella typhimurium* of, and inhibition of P450 activities by ellagic acid and one analogue. Chem. Res. Toxicol. **1998**, *11*, 1258–1264.
6. Teel, R.W. Ellagic acid binding to DNA as a possible mechanism for its antimutagenic and anticarcinogenic action. Cancer Lett. **1986**, *30*, 329–336.
7. Mukhtar, H.; Mukul, D.; Bickers, D.R. Inhibition of 3-methylcholanthrene-induced skin tumorigenicity in BALB/c mice by chronic oral feeding of trace amounts of ellagic acid in drinking water. Cancer Res. **1986**, *46*, 2262–2265.

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8. Ahn, D.; Putt, D.; Kresty, L.; Stoner, G.D.; Fromm, D.; Hollenberg, P.F. The effects of dietary ellagic acid on rat hepatic and esophageal mucosal cytochromes P450 and phase II enzymes. Carcinogen **1996**, *17* (4), 821–828.
9. Barch, D.H.; Rundhaugen, L.M.; Thomas, P.E.; Kardos, P. Dietary ellagic acid inhibits the enzymatic activity of CYP1A1 without altering hepatic concentrations of CYP1A1 or CYP1A1 mRNA. Biochem. and Biophys. Res. Commun. **1994**, *201* (3), 1477–1482.
10. Barch, D.H.; Rundhaugen, L.M. Ellagic acid induces NAD(P)H : quinone reductase gene. Carcinogen **1994**, *15* (9), 2065–2068.
11. van Lieshout, E.M.M.; Bedaf, M.M.G.; Pieter, M.; Ekkel, C.; Nijoff, W.A.; Peters, W.H.M. Effects of dietary anticarcinogens on rat gastrointestinal glutathione *S*-transferase theta 1-1 levels. Carcinogen **1998**, *19* (11), 2055–2057.
12. Dixit, R.; Gold, B. Inhibition of *N*-methyl-*N*-nitrosourea-induced mutagenicity and DNA methylation by ellagic acid. Proc. Natl. Acad. Sci. USA **1986**, *83*, 8039–8043.
13. Barch, D.H.; Fox, C.C. Selective inhibition of methylbenzylnitrosamine-induced formation of esophageal O^6-methylguanine by dietary ellagic acid in rats. Cancer Res. **1988**, *48*, 7088–7092.
14. Narayanan, B.A.; Geoffroy, O.; Willingham, M.C.; Re, G.G.; Nixon, D.W. P53/p21 (WAF1/CIP1) expression and its possible role in G1 arrest and apoptosis in ellagic acid treated cancer cells. Cancer Lett. **1999**, *136*, 215–221.
15. Soni, K.B.; Lahiri, M.; Chackradio, P.; Bhide, S.V.; Kuttan, R. Protective effect of food additives on aflatoxin-induced mutagenicity and hepatocarcinogenicity. Cancer Lett. **1997**, *115*, 129–133.
16. Kaul, A.; Khanduja, K.L. Plant polyphenols inhibit benzoyl peroxide-induced superoxide anion radical production and diacylglyceride formation I murine peritoneal macrophages. Nutr. Cancer. **1999**, *35* (2), 207–211.
17. Bock, P.E.; Srinivasan, K.R.; Shore, J.D. Activation of intrinsic blood coagulation by ellagic acid: insoluble ellagic acid-metal ion complexes are the activating species. Biochemistry **1981**, *20*, 7258–7263.
18. Dow, R.L.; Chou, T.T.; Bechle, B.M.; Goddard, C.; Larson, E.R. Identification of tricyclic analogs related to ellagic acid as potent/selective tyrosine protein kinase inhibitors. J. Med. Chem. **1994**, *37*, 2224–2231.
19. Ahmed, S.; Rahman, A.; Saleem, M.; Athar, M.; Sultana, S. Ellagic acid ameliorates nickel induced biochemical alterations: diminution of oxidative stress. Human Exp. Toxicol. **1999**, *18*, 691–698.
20. Katiyar, S.K.; Challa, A.; McCormick, T.S.; Cooper, K.D.; Mukhtar, H. Prevention of UVB-induced immunosuppression in mice by the green tea polyphenol (−)-epigallocatechin-3-gallate may be associated with alterations in IL-10 and IL-12 production. Carcinogen **1999**, *20* (11), 2117–2124.
21. Katiyar, S.K.; Elmets, C.A.; Agarwal, R.; Mukhtar, H. Protection against ultraviolet-B radiation-induced local and systemic suppression of contact hypersensitivity and edema responses in C3H/HeN mice by green tea polyphenols. Photochem. Photobiol. **1995**, *62* (5), 855–861.
22. Bhargava, U.C.; Westfall, B.A.; Siehr, D.J. Preliminary pharmacology of ellagic acid from *Juglans nigra* (black walnut). J. Pharm. Sci. **1968**, *57*, 1728–1732.

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23. Duke, S.S.; Schook, L.B.; Holsapple, M.P. Effects of *N*-nitrosodimethylamine on tumor susceptibility. J. Leuk. Biol. **1985**, *37*, 383–394.
24. Bryant, J.; Day, R.; Whiteside, T.L.; Herberman, R.B. Calculation of lytic units for the expression of cell-mediated cytotoxicity. J. of Immunol. Methods **1992**, *146*, 91–103.
25. Murray, M.J; Lauer, L.D.; Luster, M.I.; Luebke, R.W.; Adams, D.O.; Dean, J.H. Correlation of murine susceptibility to tumor, parasite, and bacterial challenge with altered cell-mediated immunity following systemic exposure to the tumor promoter phorbol myristate acetate. Int. J. Immunopharmacol. **1985**, *7*, 491–500.
26. Jerne, N.K.; Nordin, A.A. Plaque formation in agar by single antibody producing cells. Science **1963**, *140*, 405.
27. Cunningham, A.J.; Szenberg, A. Further improvements in the plaque forming technique for detecting single antibody forming cells. Immunol. **1968**, *14*, 599–601.
28. Mukhtar, H.; Das, M.; Del Tito, B.J., Jr.; Bick; Bickers, D.R. Protection against 3-methylcholanthrene-induced skin tumorigenesis in BALB/C mice by ellagic acid. Biochem. and Biophys. Res. Commun. **1984**, *119* (2), 751–757.
29. Wargovich, M.J. Experimental evidence for cancer preventive elements in foods. Cancer Lett. **1997**, *114*, 11–17.
30. Mandal, S.; Stoner, G.D. Inhibition of *N*-nitrosobenzylmethylamine-induced esophageal tumorigenesis in rats by ellagic acid. Carcinogen **1990**, *11* (1), 55–61.
31. Stoner, G.D.; Morse, M.A. Isothiocyanates and plant polyphenols as inhibitors of lung and esophageal cancer. Cancer Lett. **1997**, *114*, 113–119.
32. Doyle, B.; Griffiths, L.A. The metabolism of ellagic acid in the rat. Xenobiotica **1980**, *10* (4), 247–256.
33. Lawson, P.T. Ed. Rats. *Assistant Laboratory Animal Technician Training Manual*; American Association for Laboratory Animal Science: Chelsea, MI, 1998; 112.
34. Calabrese E.J. Overcompensation stimulation: a mechanism for hormetic effects. Crit. Rev. Toxicol. **2001**, *31* (4–5), 425–470.
35. Rook, A.H.; Zaki, M.H.; Wysocka, M.; Wood, G.S; Duvic, M.; Showe, L.C.; Foss, F.; Shapiro, M.; Kuzel, T.M.; Olsen E.A.; Vonderheid, E.C.; Laliberte, R.; Sherman, M.L. The role for interleukin-12 therapy of cutaneous T cell lymphoma. Ann. N.Y. Acad. Sci. **2001**, *941*, 177–184.
36. Imai, N.; Harashima, N.; Ito, M.; Miyagi, Y.; Harada, M.; Yamada, A.; Itoh, K. Identification of Lck-derived peptides capable of inducing HLA-A2-restricted and tumor-specific CTLs in cancer patients with distant metastases. Int. J. Cancer **2001**, *94* (2), 237–242.
37. Barouch, D.H.; Letvin, N.L. CD8+ cytotoxic T lymphocyte responses to lentiviruses and herpesviruses. Curr. Opin. Immunol. **2001**, *13* (4), 479–482.
38. Baldridge, J.R.; Barry, R.A.; Hinrichs, D.J. Expression of systemic protection and delayed-type hypersensitivity to Listeria monocytogenes is mediated by different T-cell subsets. Infect. Immun. **1990**, *58*, 654–658.
39. Boes, M. Role of natural and immune IgM antibodies in immune responses. [Review] Mol. Immunol. **2000**, *37* (18), 1141–1149.
40. Bachmann, M.F.; Kopf, M. The role of B cells in acute and chronic infections. Cur. Opin. Immunol. **1999**, *11* (3), 332–339.
41. Klasse, P.J.; Sattentau, Q.J. Mechanisms of virus neutralization by antibody. Curr. Top. Microbiol. Immunol. **2001**, *260*, 87–108.

42. Blackwell, N.M.; Else, K.J. B cells and antibodies are required for resistance to the parasitic gastrointestinal nematode Trichuris muris. Infection & Immunity **2001**, *69* (6), 3860–3868.
43. Planelles, L.; Thomas, M.C.; Alonso, C.; Lopez, M.C. DNA immunization with Trypanosoma cruzi HSP70 fused to the KMP11 protein elicits a cytotoxic and humoral immune response against the antigen and leads to protection. Infect. Immun. **2001**, *69* (10), 6558–6563.

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