Genotoxicity and Toxicity of the Potential Cancer-Preventive Agent Polyphenon E

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The potential health benefits of green tea continue to attract public and scientific interests and are attributed in part to polyphenolic catechin constituents. Polyphenon E (Poly E) is a decaffeinated green tea catechin mixture containing about 50% epigallocatechin gallate and 30% other catechins. We evaluated the toxicity and genotoxicity of Poly E by using two in vitro assays: bacterial mutagenesis in a Salmonella typhimurium–E. coli assay and the L5178Y mouse lymphoma cell thymidine kinase (Tk) gene mutation assay. In addition, we used two in vivo genotoxicity assays: the mouse micronucleus assay and the Big Blue cII transgenic mouse mutation assay. Repeat-dose toxicity evaluations were performed in mice in parallel with the Big Blue transgenic mutation assays. No significant increases in the revertant colonies were found in the bacterial mutagenesis assay, but a significant increase in the mutant frequency (MF) at the Tk locus was observed in the mouse lymphoma test system. We observed toxicity in mice when Poly E was administered at doses of 2,000 mg/kg/day. Lower doses produced no significant increases in micronucleated erythrocytes in the bone marrow of Swiss-Webster mice and no significant increases in cII transgene MF in the liver, lung, or spleen compared with controls. These results indicate that Poly E, although toxic at high doses (2,000 mg/kg/day), poses minimal genotoxic concern. In addition, these studies highlight the importance of using both in vitro and in vivo systems in genetic toxicity screening of pharmaceuticals before they are administered to humans. Environ. Mol. Mutagen. 41:43–54, 2003.© 2003 Wiley-Liss, Inc.

Key words: polyphenon E; Salmonella test; Tk; micronucleus; lacI/cII transgenic; mutation

INTRODUCTION

Regular consumption of tea, from the leaves of Camellia sinensis, may have health benefits. The reported activities of green tea polyphenols (GTP) include, for example, antioxidant and free radical scavenging, cancer prevention, lipid and glucose lowering, antiviral and antibacterial activities, hypotensive activity, and beneficial effects on intestinal flora [Katiyar and Mukhtar, 1997; Bravo, 1998; Anderson, 2001; Ferguson, 2001; Hara, 2001]. Some of the hypothesized mechanisms of action include efficient scavenging of free radicals and reactive oxygen species [Pietta, 2000], cell cycle arrest [Ahmad et al., 2000a, 2000b; Gupta et al., 2000; Liberto and Cobrinik, 2000], AP-1 and NF-κB modulation [Bode and Dong, 2000], and promotion of apoptosis [Isemura et al., 2000; Islam et al., 2000].

Polyphenon E (Poly E) is a well-standardized decaffeinated green tea catechin (GTC) mixture containing five different catechins: epicatechin, galloclatechin gallate, epigallocatechin, epicatechin gallate (ECG), and epigallocatechin gallate (EGCG), with EGCG being the most abundant [Namiki and Osawa, 1986]. ECG and EGCG have demonstrated strong antioxidative activity against the in vitro microsomal lipid peroxidation system and are highly effective as scavengers of superoxide anion radicals.

Studies in laboratory animals have shown cancer-preventive efficacy in models of lung and liver [Cao et al., 1996], skin [Wang et al., 1992], esophagus [von Pressentin et al., 2001], prostate [Gupta et al., 2001], and stomach [Katiyar et al., 1993] cancers, although human nutritional epidemiological studies have been inconclusive. GTC has also been reported to have protective effects on the pathogenesis of
both DL-ethionine–induced [Takabayashi et al., 1995] and cerulin-induced acute pancreatitis [Takabayashi and Harada, 1997] in rats. Hirose et al. [1997] tested the effects of GTC on the progression or late promotion step of mammary gland carcinogenesis in Sprague–Dawley rats pretreated with 7,12-dimethylbenz[a]anthracene and concluded that the catechins exerted only a weak inhibitory effect on the early promotion stage of mammary gland carcinogenesis. In a separate report, polyphenon-60, containing 60% pure catechin, was effective in reducing the incidence of large renal cell tumors in rats, possibly due to antioxidative properties of the catechins [Yoshioka et al., 1999]. Tea consumption by rodents induces adaptive responses affecting blood and tissue levels of tea catechins with time [Kim et al., 2000], and investigation of a similar phenomenon in humans is warranted. A recent study reporting the results of a clinical phase 1 pharmacokinetic study shows that dose-proportional levels of unchanged EGCG and glucuronide/sulfate conjugates of epigallocatechin (EGC) and epicatechin (EC) were found in the plasma and urine samples after Poly E administration [Chow et al., 2001].

Genetic toxicology tests are an important component of the drug development process and are particularly important for a potential cancer-preventive agent where the agent might be administered for long periods of time. The genotoxic potential of Poly E was evaluated using two standard in vitro gene toxicity tests, the Salmonella typhimurium–Escherichia coli/microsome assay [Ames et al., 1975] and the L5178Y thymidine kinase mutation (Tk<sup>−/−</sup>→Tk<sup>−/−</sup>) mouse lymphoma cell assay [Clive et al., 1972, 1979, 1983; Clive and Spector, 1975]. This latter assay has been widely used to detect and quantify the induction of mutations in mammalian cells. In addition, two in vivo assays were performed: the mouse micronucleus assay, which tests the ability of Poly E to induce chromosomal or mitotic spindle abnormalities in bone marrow cells of treated mice, and the cII Big Blue transgenic mouse mutation assay, which tests the ability of Poly E to induce mutations in the Big Blue model system. In parallel with the Big Blue transgenic mouse mutation assay, toxicology evaluations were also performed. All studies were conducted in compliance with Good Laboratory Practice (GLP) regulations.

**MATERIALS AND METHODS**

**Chemicals**

Poly E was manufactured by Mitsui Norin (Fujieda City, Japan) and supplied by McKesson Bioservices (Rockville, MD) through the Division of Cancer Prevention, National Cancer Institute. Formulations were prepared in advance and wrapped in foil to protect them from light and stored refrigerated (4–8°C) until use the following day; formulations for the transgenic mutation study were prepared at 2-week intervals based on the established stability protocol. Stability, homogeneity, and concentration analyses of the test article in the vehicle were performed at SRI using an HPLC method. EGCG was the major catechin (51.4%) found in this green tea extract; four other catechin (per the Certificate of Analysis) HPLC peaks were observed. All the formulations were stable at each testing interval.

The positive controls used in the absence of metabolic activation were sodium azide at 5 µg/plate (for Salmonella strains TA1535, TA100), 9-aminolevulinic hydrochloride at 50 µg/plate (for TA1537), 2-nitrofluorescein at 5 µg/plate (for TA98), and N-ethyl-N-nitrosourea at 5 µg/plate (for E. coli strain WP2uvrA). In the presence of metabolic activation, 2-anthramine (2-aninoanthracene) was used as the positive control at 2 µg/plate (TA98, TA100), 4 µg/plate (TA1535, TA1537), and 20 µg/plate (WP2uvrA).

For the mouse lymphoma assay, methyl methanesulfonate (MMS; Aldrich, Milwaukee, WI) and ethyl methane sulphonate (EMS; Sigma, St. Louis, MO) were used as the positive controls without activation. 3-Methylcholanthrene (MCA; Sigma) was used as the positive control with activation. Sterile water (Baxter Healthcare, Deerfield, IL) was used as the negative control.

For the mouse micronucleus assay, a single dose of 300 mg/kg of urethane (Baxter Healthcare) was used as the positive control. For the Big Blue assay, urethane (Sigma) was administered as a positive control at 50 mg/kg/day for 28 days.

**Bacterial Mutagenesis**

The test procedures were the same as those previously described [Mortelmans and Riccio, 2000; Mortelmans and Zeiger, 2000]. *S. typhimurium* LT2 strains TA1535, TA1537, TA98, and TA100 were obtained from Dr. Bruce Ames (University of California, Berkeley, CA) and *E. coli* strain WP2<sup>(uvrA)</sup> was obtained from the National Collection of Industrial and Marine Bacteria (Aberdeen, Scotland). Salmonella strains were evaluated by methods described in detail by Ames et al. [1975] and revised by Maron and Ames [1983]. All tests were done in the standard plate-incorporation assay on at least two separate days with and without exogenous metabolic activation (MA). Aroclor 1254-induced Sprague–Dawley male rat liver homogenate (S9) mixtures were used for the activation system. The test article was assayed twice using the five tester strains, with three plates per dose level, at doses of 5,000, 2,500, 1,250, 625, 312.5, and 156.2 µg/plate in the presence and absence of rat liver MA. Means and standard deviations were calculated from the individual plate counts, and a Levene’s test [Levene, 1960] was performed to determine if a significant difference existed between treatment variances. The data from the treated groups were compared with those for controls by using a one-tailed Dunnnett’s t-test [Dunnnett, 1980] and dose-relatedness for all treatments was made by regression analysis [Draper and Smith, 1981] of revertant counts versus the log of the concentrations. The significance of the regression was tested using t-statistic.

**Mouse Lymphoma Cell L5178Y Gene Mutation Assay**

Mycoplasma-free L5178Y mouse lymphoma cells (MOLY), clone 3.7.2C, heterozygous at the Tk locus, were originally derived by Dr. D. Clive (previously Burroughs Wellcome Laboratories, Research Triangle Park, NC) and karyotyping in our laboratory confirmed the <sup>Tk<sup>−/−</sup></sup> genotype of these cells [Blazak et al., 1986]. Standard assay conditions and methodology were used [Clive et al., 1972, 1979, 1983; Clive and Spector, 1975].

For mutagenesis, 6 × 10<sup>5</sup> L5178Y cells/culture were exposed for 4 hr to either the solvent, Poly E, or to positive control articles diluted in the appropriate solvent. After the exposure, cells were washed and resuspended in fresh medium. Cell growth was monitored daily over a 2-day period. For selection of TFT-resistant (TFT<sup>+</sup>) cells, approximately 3 × 10<sup>5</sup>
cells were seeded in cloning medium supplemented with TFT and approxi-
mately 600 cells were seeded in nonselective cloning medium to deter-
mine the percentage of viable cells. Cultures were incubated for 11–14
days in a humidified 37°C atmosphere containing 5% CO₂ in air. The
colonies were counted and the number of colonies greater than 0.2 to 0.3
mm in diameter was recorded. TFT colonies in dishes containing TFT
were recounted to determine the relative numbers of large (> 0.6 mm in
diameter) and small (< 0.6 mm in diameter) colonies using a colony
counter with settings that discriminate between the two populations.

Preliminary range-finding cytotoxicity experiments with and without
MA (S9) were conducted to determine the concentrations of Poly E to be
evaluated in the definitive mutagenesis experiments. For each activation
condition, six concentrations of Poly E were selected that caused a pro-
gressive increase in toxicity from little or no effect to approximately 80%
reduction in cell growth during the expression period. Duplicate mutagen-
esis experiments were conducted. Two cultures per dose level were used
for test article-treated cultures and positive controls; three cultures were
used for solvent controls. Cell growth during the expression period (rela-
tive suspension growth, or RSG), relative total growth (RTG), cloning
efficiency (CE), mutant frequency (MF), and numbers of small (< 0.6 mm
in diameter) colonies from TFT cells were recorded for each experiment.

The linear regression trend test of the log-transformed frequency of TFT
cells as a function of Poly E concentrations was performed. The signifi-
cance of the trend test was determined from a two-sided test of the
significance of the slope in the linear regression. In addition, the mean of
the log-transformed TFT cell frequencies of cultures treated with each test
article concentration was compared to the control solvent mean by Dun-
nett’s test, with significance assessed at the 0.05 level. The mean frequency
of TFT cells for positive controls was compared to the control solvent
mean, after log-transformation, by a one-sided t-test with significance
assessed at the P = 0.01 level. A positive response was recorded when a
significant (P < 0.05) dose-related increase in the MF occurred; the mean
MF of a set of duplicate cultures treated with one or more of the three
highest acceptable concentrations of the test article was statistically sig-
nificant (P < 0.05); at least one concentration induced an average absolute
increase in MF (net over mean solvent control MF in this study) greater
than 70 × 10⁻³ ; and the results were reproducible in a second experiment.

Mouse Bone Marrow Micronucleus Assay

Animals

Male and female Swiss-Webster mice were obtained from Charles River
Laboratories (Hollister, CA) and were 5–7 weeks of age at the time of dose
administration. Animals were group-housed in suspended polycarbonate
cages with Sani-Chips hardwood bedding (P.J. Murphy Forest Products,
Montville, NJ) and were provided Purina Certified Rodent Chow 5002
(Richmond, IN) and purified (filtered, deionized, and UV-treated) tap water
ad libitum. The room was maintained on a 12-hr light/12-hr dark cycle at a
temperature of 72–74°F, and humidity ranged from 37.5% to 43%. The
general procedures for animal care and housing were in accordance with
the National Research Council Guide for the Care and Use of Laboratory
Animals [National Research Council, 1996].

Dosing Regimen

An initial dose range-finding experiment used three male and three
female mice per treatment group, with samples taken at 48 hr. Treatment
groups consisted of one vehicle control and five test article groups: 125,
250, 500, 1,000, and 2,000 mg/kg. Single doses of Poly E were adminis-
tered via oral gavage, and the concentration of test article was adjusted
such that the dosing volume was 10 ml/kg per animal. The definitive study
included 10 male and 10 female mice per treatment group, and samples
were taken at 24 and 48 hr after dose administration. Treatment groups
included one vehicle control group and three Poly E-treated groups (dose
levels of 375, 750, and 1,500 mg/kg). In addition, urethane was used as the
positive control, and samples from five urethane-treated male mice were
harvested at 24 hr after treatment.

Micronucleus Assay

Bone marrow cells were flushed from one femur into about 0.5 ml of
fetal bovine serum (Gibco-Invitrogen, Carlsbad, CA). Cells were concen-
trated by centrifugation, spread on ethanol-cleaned microscope slides,
air-dried, and fixed for 5 min in absolute alcohol. One slide from each
animal was stained with acridine orange and evaluated using epifluores-
cence microscopy at a magnification of 630 or 10,000 ×. The criteria used
for identifying micronuclei are those described by Schmid [1976]. After
blinded analysis, the slides were decoded and the data summarized. The
ratio of polychromatic erythrocytes to red blood cells (PCE/RBC) was
evaluated for each experiment, and ≥ 200 RBCs were counted per animal.
To calculate the micronucleus frequency, ≥ 2,000 PCEs were counted per
animal.

Statistical Analysis

The data were analyzed separately for each gender and each harvest
time. The ratio of micronucleated PCEs to total PCEs and the ratio of PCEs
to total erythrocytes (in percentages) were calculated for each animal. The
frequency of micronucleated PCEs was statistically analyzed using the
Cochran-Armitage test for trend in binomial proportions to determine if a
significant dose-response relationship was present, and the normal test for
equality of binomial proportions [Kastenbaum and Bowman, 1970] was
used to determine whether values for individual dose groups were statisti-
cally different from those from vehicle controls. These tests and their
rationale are discussed in the ASTM Standard Guide for the Conduct of
Micronucleus in Mammalian Bone Marrow Erythrocytes and other publi-

Big Blue Mutation Assay

Animals

Male and female B6C3F1 Big Blue lacI-II transgenic mice were pur-
chased from Stratagene Cloning Systems (La Jolla, CA) and were approx-
imately 7 weeks of age at the time of dose administration. Animals were
singly housed in suspended polycarbonate cages with Sani-Chips hard-
wood bedding and were provided Purina Certified Rodent Chow 5002
and purified (filtered, deionized, and UV-treated) tap water ad libitum.
The room was maintained on a 12-hr light/12-hr dark cycle at a tempera-
ture of 74–79°F. Mice were treated for 28 consecutive days with Poly E admin-
istered by oral gavage at dose levels of 500, 1,000, and 2,000 mg/kg/day
or 50 mg/kg/day urethane. Control mice were untreated. Each group con-
tained seven male and seven female mice. Necropsy occurred 28 days after
the final dose was administered.

clII Assay

Mice were euthanized with 100 mg/kg sodium pentobarbital adminis-
tered intraperitoneally on day 56 (28 days posttreatment), and lung, liver,
and spleen tissues were collected. Moribund animals were necropsied out
of sequence as needed. Genomic DNA was prepared from homogenized
tissue samples by using the manufacturer’s recommended procedure for the
RecoverEase DNA Isolation Kit (Stratagene).

The lambda shuttle vector was recovered as viable phage by exposing
the genomic DNA to lambda packaging extracts from the λ Select-clII
Mutation Detection System for Big Blue Rodents (Stratagene) using the
procedure recommended by the manufacturer. Phage particles were as-
sayed for clII mutations by infecting G1250 E. coli (Stratagene) and
screening for mutant plaques on 100 mm TB1 agarose plates. Plating for
clII selection was performed using the method suggested by the manufac-

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turer with the following modifications: 1.8 ml of packaged DNA sample was diluted 1:100. A 20 µl aliquot was added to each of three tubes labeled “titer 20” and a 100 µl aliquot was added to each of three tubes labeled “titer 100.” The remaining undiluted packaging reaction was added to the mutant tube. A minimum of 200,000 plaque-forming units (PFUs) were evaluated for each sample and no more than five packagings were done for each sample to attain the targeted number of PFUs. The MF was calculated by dividing the number of mutant plaques by the total number of plaques screened. Mutation data were evaluated using Students’ t-test and the Cochran-Armitage trend test.

RESULTS

Bacterial Mutagenicity

The range-finding experiment was conducted with strain TA100 at dose levels of 0–5,000 µg/plate of Poly E in sterile water in the presence or absence of MA by 4% S9. No dose-related increases in the number of revertants and no cytotoxicity were observed. Based on these results, the first experiment for mutagenicity was conducted with all five tester strains using the same conditions and the same dose levels. No statistically significant increases in the number of revertants and no cytotoxicity were observed. In a separate experiment, Poly E mutagenicity was evaluated using the same dose levels but higher S9 (10%) was used for MA. The slight increases in the number of revertants seen with WP2uvrA at doses of 312.5 and 1,250 µg/plate in the absence of MA were statistically significant (P < 0.01) by Dunnett’s test. However, the counts were well within the historical range for the strain, were not dose-dependent, and were not observed in the first mutagenicity experiment and thus not reproducible. The statistical analyses for these two experiments are presented in Table I. No cytotoxicity and no consistent significant increases in the number of revertants were observed.

Mouse Lymphoma Assay (MLA)

In the first experiment, a white precipitate was noted at final Poly E concentrations of 375 and 625 µg/ml when the dosing solutions were added to the culture medium, suggesting that the solubility of Poly E is lower in culture medium than in water. The precipitate was also observed in cultures during the expression period, suggesting that the treatment exposure period was longer than the planned experimental design. Dose-related increases in toxicity were observed under both MA conditions, with a pronounced drop in the RSG from 59% at 225 µg/ml to 13% at 625 µg/ml of Poly E without S9 and from 92% at 135 µg/ml to
44% at 375 μg/ml of Poly E with S9. Tables II and III summarize the results of these experiments. In all the experiments, significant increases in MF (P < 0.01 by Students’ t-test) were induced by the positive control chemicals, EMS and MCA.

At the end of the 4-hr exposure period, the color of the

### TABLE II. Mouse Lymphoma Mutagenicity Results: Without S9 Metabolic Activation*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (μg/mL)</th>
<th>Average RSG (%)</th>
<th>Average RCE (%)</th>
<th>Average RTG (%)</th>
<th>MF</th>
</tr>
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<tr>
<td>First experiment</td>
<td></td>
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<td>Sterile water</td>
<td>0</td>
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<td>Polyphenon E</td>
<td>49</td>
<td>92</td>
<td>104</td>
<td>96</td>
<td>53 ± 2</td>
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<td></td>
<td>81</td>
<td>87</td>
<td>107</td>
<td>93</td>
<td>52 ± 23</td>
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<td></td>
<td>135</td>
<td>88</td>
<td>102</td>
<td>90</td>
<td>40 ± 4</td>
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<td>200</td>
<td>65</td>
<td>146</td>
<td>95</td>
<td>251 ± 76c</td>
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<td>40</td>
<td>64</td>
<td>25</td>
<td>68 ± 0c</td>
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<td></td>
<td>4008</td>
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<td>57</td>
<td>13</td>
<td>105 ± 15c</td>
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<td>200</td>
<td>84</td>
<td>72</td>
<td>60</td>
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*Average RSG = average growth of cells during the expression period relative to that of solvent control cells; Average RCE = average cloning efficiency relative to that of solvent control cells; Average RTG = average relative total growth (RSG x RCE/100); MF = mutant (TFT') cell frequency (per 10^6 cells).

aPrecipitate observed upon addition to culture medium.
bRTG < 10%; data not included in statistical analyses.
cP < 0.05 by Dunnett’s analysis.
dP < 0.01 by Students’ t-test.

### TABLE III. Mouse Lymphoma Mutagenicity Results: With S9 Metabolic Activation*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (μg/mL)</th>
<th>Average RSG (%)</th>
<th>Average RCE (%)</th>
<th>Average RTG (%)</th>
<th>MF</th>
</tr>
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<td>152</td>
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<td>18</td>
<td>59</td>
<td>11</td>
<td>190 ± 33b</td>
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</table>

*Avg. RSG = average growth of cells during the expression period relative to that of solvent control cells; Avg. RCE = average cloning efficiency relative to that of solvent control cells; Avg. RTG = average relative total growth (RSG x RCE/100); MF = mutant (TFT') cell frequency (per 10^6 cells); MCA = 3-methylcholanthrene (positive control).

aPrecipitate observed upon addition to culture medium.
bP < 0.05 by Dunnett’s analysis.
cP < 0.01 by Students’ t-test.
culture solutions appeared increasingly brown with increasing concentration, beginning at 135 μg/ml Poly E, and a black residue was observed at 225 μg/ml. In the second experiments without and with S9, the media showed a progressive increase from orange-brown to darker brown with increasing concentration for all concentrations of Poly E tested, and a black residue was observed at 205 μg/ml with and without S9. Although the amount of residual chemical following the rinsing procedure increased with increasing concentration, it did not appear to interfere with the cell density determinations.

In the first mutagenesis experiments, Poly E was slightly more toxic without S9 than with S9. In the second mutagenesis experiments, the Poly E concentration levels tested were adjusted to accommodate the toxicity observed in the initial experiments; Poly E was again slightly more toxic without S9 than with S9. The RTG of cultures exposed to Poly E at ≥ 500 μg/ml without S9 was below the lowest acceptable value of 10% RTG for evaluation of mutagenicity, and therefore these concentrations were not evaluated for mutagenicity. The limit of 10% RTG is widely accepted as the maximum level of cytotoxicity for mutagenicity evaluation to avoid biologically irrelevant effects that might occur in severely stressed cells (e.g., treatments resulting in cytotoxicity > 90%) [Clive et al., 1995].

Statistically significant (P < 0.05 by Dunnett’s analysis) increases in MF were induced at Poly E concentrations of ≥ 164 μg/ml in the second experiment without MA. With S9, statistically significant increases in MF were induced at Poly E concentrations of ≥ 375 μg/ml in the first experiment and ≥ 205 μg/ml in the second experiment. The numbers of both large and small colonies in all acceptable cultures (RTG > 10%) increased with increasing concentration compared to those in the solvent control cultures.

Because significant increases in MF were observed only in one out of two experiments, the results of the mutagenesis experiments testing Poly E without MA were evaluated as equivocal; however, the results of the mutagenesis experiments with MA met the criteria for a positive response.

**Mouse Micronucleus Assay**

In the initial dose range-finding assay, one female and three male mice in the 2,000 mg/kg group and one female in the 1,000 mg/kg group died. Just prior to euthanasia, two male mice in the 2,000 mg/kg dose group had ruffled fur (slight to moderate) and one male mouse in this group was hypoactive. No other adverse clinical observations were noted and no significant suppression of the PCE/RBC ratio was observed in any dose group. Based on these results, a top dose of 1,500 mg/kg was selected for the subsequent definitive micronucleus experiments.

Erythrocytes harvested from bone marrow were examined, and the percentages of PCEs among total RBCs and of micronucleated PCEs among total PCEs were determined. The results are presented in Table IV. A statistically significant increase in micronucleated PCE (MN-PCE) frequencies at 24 hr was seen in male mice treated with 375 and 750 mg/kg Poly E when the results were analyzed using the binomial proportion test; however, using the Cochran-Armitage trend test, no dose-related statistically significant increase was found. The control MN-PCE levels fell within normal historical ranges for our laboratory, indicating that the performance of the test system was consistent with

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**TABLE IV. Micronucleus Frequency in Bone Marrow of Male and Female Mice**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sex</th>
<th>Dose (mg/kg)</th>
<th>Time (hr)</th>
<th>Number of surviving animals</th>
<th>PCE/RBC mean ± SE</th>
<th>Number of PCEs with MN</th>
<th>Number of PCEs with MN</th>
<th>MN (%), mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control/water</td>
<td>Male</td>
<td>0.0</td>
<td>24</td>
<td>5</td>
<td>49.23 ± 4.01</td>
<td>10,043</td>
<td>17</td>
<td>0.17 ± 0.03</td>
</tr>
<tr>
<td>Polyphenon E Male</td>
<td>375.0</td>
<td>24</td>
<td>5</td>
<td>52.19 ± 6.34</td>
<td>10,046</td>
<td>37</td>
<td>0.37 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>Polyphenon E Male</td>
<td>750.0</td>
<td>24</td>
<td>5</td>
<td>46.31 ± 2.78</td>
<td>10,045</td>
<td>37</td>
<td>0.37 ± 0.07*</td>
<td></td>
</tr>
<tr>
<td>Polyphenon E Male</td>
<td>1,500.0</td>
<td>24</td>
<td>5</td>
<td>48.59 ± 4.86</td>
<td>10,075</td>
<td>23</td>
<td>0.23 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>Urethane</td>
<td>Male</td>
<td>300.0</td>
<td>24</td>
<td>66.5 ± 1.15</td>
<td>10,043</td>
<td>238</td>
<td>2.37 ± 0.33*</td>
<td></td>
</tr>
<tr>
<td>Control/water</td>
<td>Female</td>
<td>0.0</td>
<td>24</td>
<td>52.09 ± 3.79</td>
<td>10,153</td>
<td>20</td>
<td>0.20 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>Polyphenon E Female</td>
<td>375.0</td>
<td>24</td>
<td>5</td>
<td>48.98 ± 7.80</td>
<td>10,176</td>
<td>32</td>
<td>0.32 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>Polyphenon E Female</td>
<td>750.0</td>
<td>24</td>
<td>5</td>
<td>48.47 ± 3.28</td>
<td>10,122</td>
<td>15</td>
<td>0.15 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>Polyphenon E Female</td>
<td>1,500.0</td>
<td>24</td>
<td>5</td>
<td>34.79 ± 3.42</td>
<td>10,190</td>
<td>10</td>
<td>0.10 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>Control/water</td>
<td>Male</td>
<td>0.0</td>
<td>48</td>
<td>50.55 ± 3.29</td>
<td>10,065</td>
<td>26</td>
<td>0.26 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Polyphenon E Male</td>
<td>375.0</td>
<td>48</td>
<td>5</td>
<td>49.35 ± 2.23</td>
<td>10,076</td>
<td>33</td>
<td>0.33 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>Polyphenon E Male</td>
<td>750.0</td>
<td>48</td>
<td>5</td>
<td>46.35 ± 3.73</td>
<td>10,066</td>
<td>26</td>
<td>0.26 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>Polyphenon E Male</td>
<td>1,500.0</td>
<td>48</td>
<td>5</td>
<td>49.85 ± 2.69</td>
<td>10,079</td>
<td>290.04</td>
<td>0.29 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>Control/water</td>
<td>Female</td>
<td>0.0</td>
<td>48</td>
<td>34.77 ± 3.93</td>
<td>10,082</td>
<td>16</td>
<td>0.16 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Polyphenon E Female</td>
<td>375.0</td>
<td>48</td>
<td>5</td>
<td>43.37 ± 2.05</td>
<td>10,050</td>
<td>17</td>
<td>0.17 ± 0.01*</td>
<td></td>
</tr>
<tr>
<td>Polyphenon E Female</td>
<td>750.0</td>
<td>48</td>
<td>5</td>
<td>34.05 ± 3.89</td>
<td>10,058</td>
<td>13</td>
<td>0.13 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Polyphenon E Female</td>
<td>1,500.0</td>
<td>48</td>
<td>5</td>
<td>44.94 ± 3.82</td>
<td>10,066</td>
<td>13</td>
<td>0.13 ± 0.03</td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.01 by test for binomial proportions.
acceptable standards. In the urethane-treated mice, a statistically significant elevation in micronucleated cells was observed. These results suggest that single administration of the tested dose levels of Poly E in mice does not induce significant increases in the incidence of micronuclei in this assay.

**Big Blue Transgenic Mouse Mutagenesis Assay**

This assay was conducted as both a 28-day toxicity study and a mutagenesis assay. Toxicity and mutagenesis evaluations are discussed separately.

**Toxicity**

**Mortality and clinical observations.** All mice in the 2,000 mg/kg/day Poly E dose group died early or were euthanized in moribund condition during the first week of the study. In each case, the animal appeared normal on the first 3 days of dosing and then showed adverse clinical signs 1 or 2 days before death or euthanasia, including hypoactivity, ruffled fur, low body temperature, and hunched posture. No animals in the other dose groups showed any adverse signs that were related to treatment with Poly E.

**Body weights and food consumption.** There were no statistically significant differences in group mean body weight or weight changes at any time point when test article-treated mice were compared with controls (data not shown). Male mice in the 1,000 mg/kg/day Poly E group consumed significantly more food than controls \(P < 0.05\) between days 22 and 29 and significantly less food than controls \(P < 0.05\) between days 50 and 56. Females in the 1,000 mg/kg/day Poly E group consumed significantly more food than controls between days 43 and 50 \(P < 0.01\) and between days 50 and 56 \(P < 0.05\). These differences were small, and in the absence of significant body weight changes are considered of minimal biological relevance (data not shown).

**Hematology.** Small, statistically significant changes relative to controls were observed in the 1,000 mg/kg/day dose groups, including reduced \(P < 0.01\) mean corpuscular volume (MCV) in males and increased \(P < 0.01\) platelet counts (PLC) in females; however, these changes fall within expected ranges and are not considered biologically relevant. Hematology data were only available for one mouse in the 2,000 mg/kg/day group, a female euthanized in moribund condition on day 8. Many results from this animal differed greatly from the mean results from the female control group. The PLC was 264 \(\times 10^3/\text{mm}^3\) compared with 918 \(\pm 108 \times 10^3/\text{mm}^3\) in controls; reticulocytes were 0.1% compared with 1.4% \(\pm 0.16\%\) in controls; and lymphocytes were 180/\text{mm}^3 compared with 3,310 \(\pm 1,339/\text{mm}^3\) in controls. Additionally, white blood cells, MCV, and mean corpuscular hemoglobin were low in this mouse compared with the mean control values, and monocytes were 200/\text{mm}^3 compared with 36 \(\pm 54/\text{mm}^3\) in controls.

**Clinical chemistry.** Small, statistically significant changes in clinical chemistry relative to controls were observed in the 1,000 mg/kg/day dose group, including increased \(P < 0.05\) cholesterol (CHO) in males and females, increased \(P < 0.05\) total bilirubin (TBI) in males, and decreased \(P < 0.05\) alkaline phosphatase in females; however, these changes fall within expected ranges and are not considered biologically relevant. Clinical chemistry data were only available from one mouse in the 2,000 mg/kg/day group, a male euthanized in moribund condition on day 7. Many results from this animal differed greatly from the mean results from the male control group. Aspartate aminotransferase was 18,816 IU/L compared with 119 \(\pm 36.9\) IU/L in controls; alanine aminotransferase was 9,132 IU/L compared with 44 \(\pm 18.5\) IU/L in controls; and TBI was 1.2 mg/dL compared with 0.2 \(\pm 0.00\) mg/dL in controls. CHO was 39 mg/dL compared with 131 \(\pm 4.8\) mg/dL in controls, and triglycerides were 39 mg/dL compared with 189 \(\pm 20.4\) mg/dL in controls.

**Organ weights.** In male mice, absolute liver weights and liver-to-body and liver-to-brain weight ratios were significantly lower in the 500 and 1,000 mg/kg Poly E groups. Brain- and heart-to-body weight ratios were significantly elevated in the 1,000 mg/kg dose group, but this is considered due to the slightly lower body weights in this dose group. A significant decrease in spleen-to-brain weight was observed in the 1,000 mg/kg group and decreased testes-to-brain weight was observed in the 500 and 1,000 mg/kg groups, but these changes are likewise considered due to general body weight decreases. No statistically significant changes were observed in female mice.

**Histopathology.** Mice in the 2,000 mg/kg/day dose group that died early had numerous histopathological lesions, including cardiac myofiber degeneration in six of seven (87%) males and four of seven (57%) females, hepatic fatty change in seven of seven (100%) males and five of seven (71%) females, hepatic centrolobular necrosis in seven of seven (100%) males and six of seven (86%) females, mesenteric lymph node necrosis in four of seven (57%) males and three of six (50%) females, splenic lymphoid depletion in six of seven (76%) males and four of six (67%) females, and thymic lymphoid depletion in six of seven (86%) males and four of seven (57%) females.

The cardiac myofiber degeneration was characterized by pale staining and separation of myofibrils within the cardiac muscle fiber bundles (Fig. 1). The change was not associated with myofiber necrosis or leukocyte cell response. Hepatic fatty change was characterized by single or multiple lipid droplet accumulation in the hepatocellular cytoplasm of numerous hepatocytes that appeared viable in the sections. Hepatic centrolobular necrosis was characterized by dissolution of hepatocytes, and the change was associated
with a leukocyte cell response. Mesenteric lymph node necrosis was characterized by dissolution of the lymphocyte population with accumulation of free-floating and phagocy-tosed nuclear debris in the sections. Splenic lymphoid depletion was characterized by an overall paucity of the lymphoid elements within the red and white pulp of the splenic sections, while lymphoid depletion in thymic sections was characterized primarily by decreased lymphoid cells in the thymic cortex.

No microscopic changes were observed in the 1,000 mg/kg/day dose group, and the 500 mg/kg/day dose group was therefore not evaluated microscopically.

**Mutant Frequency**

The MF in liver, lung, and spleen are summarized in Table V. For the majority of the collected liver, lung, and spleen tissues, the frequency of \( cII \) mutants was calculated based on a minimum of 200,000 PFUs. Some tissues were collected from animals found dead or sacrificed in moribund condition in the 2,000 mg Poly E dose groups and analyzed for \( cII \) mutations; however, packaging was generally difficult in these tissues and counts of 200,000 PFUs were not attainable. To validate the \( cII \) mutation analysis procedure, positive control DNA samples from previous experiments conducted in our laboratory were packaged and analyzed with selected sets of packaging reactions. The MF results were comparable to the previously reported MF. In addition, control phage DNA supplied by Stratagene was plated to verify packaging efficiency and, in multiple trials, the MF was shown to be within the expected range specified by the manufacturer.

Students’ t-tests comparing each treated group with the vehicle controls show that there were no significant increases in \( cII \) MFs in the liver of Big Blue (\( lacI/cII \)) mice after the 56-day study period (28 days of oral treatment plus a 28-day expression period) with either 500 or 1,000 mg/kg/day of Poly E or 50 mg/kg/day urethane, and no Poly E dose-related statistically significant increases were found using the Cochran-Armitage trend test.

There were also no significant increases in grouped \( cII \) MF in the lung and spleen of Big Blue (\( lacI/cII \)) mice after
the 56 days with 500 or 1,000 mg/kg/day of Poly-E, and no dose-related statistically significant increases were found using the Cochran-Armitage trend test. Students’ t-test comparing the grouped cIH MF in the lung and spleen of urethane-treated male mice with the vehicle control showed that the MF is significantly different from that of the control animals (P < 0.01). This result is consistent with the potent carcinogenicity of urethane in the lung and spleen in these animals.

Since no significant increases above spontaneous levels of mutations were found in the three tissues analyzed, the sequences of individual mutants were not analyzed. The overall lack of a statistically significant response in the livers of all urethane-treated transgenic mice is not
surprising and is consistent with the tissue-specific nature of mutagenesis and carcinogenicity of this compound. We have previously reported significant increases in MF in livers of urethane-treated cII mice [Hursey et al., 2000]; however, treatment times of 6 to 9 months were required to elicit these responses. Treatment for 28 days with a 28-day expression period may be insufficient to induce a significant increase in MF in the liver. Additional DNA samples included within the packaging and plating phase of the analysis confirmed that the assay was being performed properly.

DISCUSSION

Poly E is a well-defined pharmaceutical-grade mixture of polyphenols, isolated under Good Manufacturing Practices from green tea, containing five catechins, with the potent antioxidant EGCG as the major component. Other minor, uncharacterized peaks observed in the HPLC analysis occur consistently, within product specification limits. In this study, Poly E failed to induce a positive response in bacterial strains but produced a significant increase in mutant colonies in the mouse lymphoma assay. In a separate mouse lymphoma study, we showed that EGCG alone did not elicit a positive response in this system [Riccio et al., 2000]. These findings suggest that other components in the Poly E mixture may have in vitro mutagenic activity that could be detected in this assay.

To address potential mutagenic risk to humans exposed to Poly E, we conducted two in vivo assays to assess clastogenic and mutagenic potential. These in vivo assays provide an assessment of the whole animal uptake, distribution, metabolism, and excretion that is not modeled using purely in vitro systems. Poly E did not elicit any consistent increases in bone marrow micronuclei or any induction of cII mutations in liver, spleen, and lung of mice. Although plasma measurements were not performed, the doses of Poly E in these studies were high and all animals dosed at 2,000 mg/kg body weight died. In a separate toxicokinetic study in wild-type C57BL/6Tac-Trp5 N5 mice, plasma levels of EGCG were proportional to dose and reached an average of 649 ng/ml in mice administered Poly E at 1,000 mg/kg body weight (unpublished observations). Oral bioavailability of EGCG is known to be relatively low in rats [Chen et al., 1997; Nakagawa and Miyazawa, 1997; Zhu et al., 2000] but appears to be more bioavailable in dogs [Swezey et al., 2001]. In phase 1 clinical studies with Poly E, the plasma area under the curve values for EGCG were proportional to dose and the concentration maximum for a 800 mg dose was 377 ± 150 ng/ml; conjugated EGCG, EGC, and EC were also present in plasma in proportion to dose [Chow et al., 2001]. In another study, plasma samples collected at 1 hr from human volunteers after ingestion of 1.2 g of decaffeinated green tea in warm water contained 46–268 ng/ml of EGCG, 82–206 ng/ml of EGC, and 48–80 ng/ml of EC. ECG was not detected in the plasma samples. The maximum urinary excretion of EGC and EC occurred at 3–6 hr [Lee et al., 1995].

Sporadic and non–dose-related but statistically significant MN frequencies in bone marrow were seen in some of the male animals at the early time point, and one female dose group at the later time point. These results suggest that a sex/expression time-specific effect may be present. The positive mouse lymphoma assay (MLA) results obtained in one out of two experiments with an increase in both large and small colonies may suggest that certain components in poly E may have weak clastogenic activities. Given the relatively low oral uptake of catechins, it is possible that Poly E components other than EGCG may have mutagenic potential but are not being detected in in vivo assays because of their low quantity in the mixture and/or poor bioavailability. It is possible that mutagenic activity might be detected at higher doses; however, the upper dose that can be tested is limited by the systemic toxicity of the Poly E mixture. Poly E produced deaths in Swiss-Webster mice following a single administration in the 1,000–2,000 mg/kg dose range, and in B6C3F1 cII transgenic mice within the first week of treatment at 2,000 mg/kg/day. The weak responses in the micronucleus and Big Blue mutation assays might also be due to in vivo metabolism/detoxification that renders constituents nonmutagenic.

While histopathology was not the primary endpoint in this study, among the mice that were found dead or sacrificed moribund, the principal target of Poly E toxicity appeared to be the heart. The primary microscopic changes in mice that died or were sacrificed in moribund condition were myocardial fiber abnormalities. If one assumes that this lesion was a primary effect, damage to myocardial fibers could have caused cardiac functional insufficiency resulting in passive congestion of the liver with further complications of centrilobular necrosis and/or fatty change in that organ. The lymph node necrosis and splenic and thymic lymphoid depletion were considered to have been secondary to the stressful conditions caused by the cardiac and hepatic failures. The heart was not identified as a target organ in 13-week oral toxicity studies of Poly E in rats and dogs [Johnson et al., 1999].

Many food products and extracts demonstrate mutagenic activity in vitro when extracted and purified in such a way that small mutagenic components are present in the extract at a substantial level. The data presented above suggest that such impurities, if indeed present in Poly E, represent a relatively small risk to human volunteers taking Poly E as a potential chemopreventive agent, but one that should be further assessed in human clinical studies.
ACKNOWLEDGMENTS

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REFERENCES


