Lack of significant genotoxicity of purified soy isoflavones (genistein, daidzein, and glycitein) in 20 patients with prostate cancer1–3

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ABSTRACT

Background: Genistein may be useful in the prevention or treatment of prostate cancer; however, it causes genetic damage in cultured human cells.

Objective: The objective was to assess the potential genotoxicity of a purified soy unconjugated isoflavone mixture in men with prostate cancer.

Design: Twenty patients with prostate cancer were treated with 300 mg genistein/d for 28 d and then with 600 mg/d for another 56 d. In peripheral lymphocytes, DNA strand breaks were assessed as nuclear tail moment, chromosomal damage was assessed as micronucleus frequency (MF), and translocations of the MLL gene (11q23) were assessed by using fluorescence in situ hybridization. Values are also reported for 6 healthy men. The studies were performed under Investigational New Drug application no. 54 137 at a tertiary referral academic medical center.

Results: No changes in group average or individual nuclear tail moment and MF were observed. We observed a single elevated MF value in one subject that exceeded a clinical threshold set before we initiated the study. A significant decrease in average COMET tail moment was observed on day 28 relative to day 0. We detected no genistein-induced rearrangements of the MLL gene in the 3 subjects we studied with this technique. MF increased significantly in lymphocytes exposed in vitro to unconjugated genistein at concentrations ≥ 100 μmol/L. Total genistein never exceeded a peak concentration of 27.1 μmol/L, and unconjugated genistein never exceeded a peak concentration of 0.32 μmol/L.

Conclusion: Although isoflavones are capable of inducing genetic damage in vitro, a similar effect was not observed in subjects treated with a purified soy unconjugated isoflavone mixture. Am J Clin Nutr 2003;77:875–82.

KEY WORDS Genistein, daidzein, glycitein, soy isoflavones, lymphocytes, genotoxicity, prostate cancer

INTRODUCTION

Many people supplement their diets with soy isoflavones because epidemiologic and animal studies suggest that consumption of soybeans and soy-containing foods may lower one’s risk of breast (1–4) and prostate (5–7) cancer. The chemopreventive effects of soybeans and soy-containing foods may be related to their isoflavone content (8–12). Daily intakes of 39–47 mg isoflavones/d have been reported in Asian populations (13, 14). In the United States, the dietary consumption of soy isoflavones in the general population is much less than this amount (<5mg/d), and diet supplements are being used to increase daily intakes. We believe that these isoflavones are relatively safe, but we know that they exert multiple effects, including estrogen receptor activation (15, 16), antiestrogenic actions (17), antioxidant activity (17), inhibition of growth factor receptor signaling via tyrosine kinases (18–21), induction of apoptosis (22–25), induction of cell differentiation (26), and inhibition of angiogenesis (27).

Recent reports suggest that soy isoflavones, particularly genistein, can induce genetic damage. Genistein induces mammalian topoisomerase II (EC 5.99.1.3)–dependent DNA cleavage in purified broken cell preparations (28) and at high doses induces the production of large numbers of micronuclei (a measure of chromosomal damage) in mouse lymphoma cells in culture (30). Other investigators also reported micronucleus formation and DNA strand breaks in cultured Chinese hamster V79 cells (31), in human lymphoblastoid cells exposed to genistein (32), and in human peripheral blood lymphocytes (33) after in vitro exposure to genistein. In contrast, daidzein (another soy isoflavone) did not induce chromosomal aberrations even at high concentrations (33).

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However, when mice were gavaged with 20 mg genistein · kg body wt$^{-1}$· d$^{-1}$ for 5 d (approximately equivalent to the consumption of 2.8 kg soybeans/d by a 70-kg human) there was no observable increase in micronucleus frequency (MF) (29). Genistein in primary cultures of hematopoietic mononuclear cells isolated from umbilical cord blood from healthy adults caused abnormalities in the MLL gene (11q23), including translocation and deletions (34). Changes in the MLL gene in vivo may be associated with acute myelogenous leukemia (35, 36). Thus, there is sufficient reason to suspect that genistein might be genotoxic when given to humans. For this reason, we used blood lymphocytes, collected during a phase I study on potential clinical toxicity, to examine the potential genotoxicity of a purified soy isoflavone mixture (70% unconjugated isoflavones containing genistein, daidzein, and glycine) administered for 84 d to 20 men with prostate cancer.

SUBJECTS AND METHODS

Subjects

The subjects were male volunteers aged >40 y with stages B, C, or D prostate neoplasia and recruited from the local population of the Research Triangle area of North Carolina. The subjects were required to be ≥3 wk postsurgery (major) and 4 wk postsurgery and fully recovered. Concurrent endocrine therapy, other than estrogen therapy, was permitted provided that the subjects were on a stable regimen that was initiated ≥3 mo before dosing. Exclusion criteria included serious intercurrent medical illnesses or history of seizure, significant cardiac disease, abnormalities discovered during a physical examination or biochemical screening that could be metabolically significant, and the presence of an active, acute infection requiring antibiotic therapy. Additional exclusion criteria included a history of another malignancy initially diagnosed within 2 y (other than nonmelanoma skin carcinoma), a history of breast cancer, or a life expectancy of <6 mo. Subjects with a history of substance abuse or addiction, an ethanol intake >2 drinks/d, or a diet containing more than an estimated intake of 20 mg genistein/d were also excluded. Although subjects were advised to limit soy intake to a maximum of 20 mg genistein/d, most chose to abstain from soy products during the study. Before acceptance into the study, the volunteer’s health was verified by medical history (including histologic documentation of adenocarcinoma of the prostate), physical examination by a licensed medical doctor, screening laboratory tests, chest X-ray, and electrocardiogram.

The investigator obtained written informed consent from the subjects, and the study was conducted in accordance with the guidelines of the Institutional Review Boards of the School of Medicine at the University of North Carolina (UNC) and the Research Triangle Institute. The protocol was submitted to the Food and Drug Administration via an Investigation New Drug (IND) application (no. 54 137) and was approved by the National Cancer Institute’s Division of Cancer Prevention. Twenty-one subjects were deemed eligible on the basis of the inclusion and exclusion criteria, agreed to participate, and were enrolled in the study. One enrolled subject was dropped from the study after day 9 because of the advancement of his prostate cancer, which required immediate medical attention.

For the purposes of methods validation, peripheral lymphocytes were collected from 6 untreated healthy men aged 24–46 y recruited from the local population of the Research Triangle area, North Carolina.

Isoflavone formulation and dosage

Protein Technologies International (PTI; St Louis), via the National Cancer Institute, provided the hard-gelatin capsules used (PTI G-2535), which contained ≥70% active substance as total unconjugated isoflavones. Isoflavones were produced under Good Manufacturing Practices guidelines. University Pharmaceuticals of Maryland, Inc (Baltimore), formulated the capsules to contain 150 mg genistein activity. The analytic data for the PTI G-2535 capsules (lot no. UPM 9809-021) used in this clinical study are as follows: 139.5 mg genistein per capsule, 74 mg daidzein per capsule, and 11 mg glycine per capsule. Two laboratories (Ralston Analytic Laboratories, St Louis; Sigma Chemical Laboratories, St Louis) independently analyzed the isoflavone composition and concentrations. The preparation was stable at 40 and 70°C for ≥6 mo and at 25°C for 3 y; the assays were performed at University Pharmaceuticals of Maryland, Inc.

The initial dose of genistein given was 300 mg (≈4 mg/kg body wt) for 28 d. The dose was then escalated to 600 mg (≈8 mg/kg, given as 2 divided doses in the morning and evening) for an additional 56 d. In one subject, the dose was not escalated: after the 300-mg/d phase was completed, the dose was only briefly escalated to 600 mg and then halved to 300 mg/d because the ratio of the lowest (trough) to the highest (peak) genistein concentration exceeded our guidelines. When the trough-peak ratio persisted to be higher than desired, the dose was halved again to 150 mg/d; this subject was retained in the study and the subject’s data were included in the analyses. It was in this subject that we observed a single elevated MF value that exceeded a clinical threshold set before we initiated the study. In another subject (subject 2), twice the planned dose of isoflavone mixture was inadvertently administered on day 84 before the plasma profile study was performed.

Monitoring for general safety

As part of this phase I study, organ function and health were carefully monitored. Tests included a physical examination, a complete blood count with differential (white blood cells, red blood cells, hemoglobin, hematocrit, count, mean cell volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red cell distribution width, neutrophils, lymphocytes, monocytes, eosinophils, and basophils), and measurement of serum sodium, potassium, chloride, carbon dioxide, blood urea nitrogen, creatinine, alkaline phosphatase (EC 3.1.3.1), alanine aminotransferase (EC 2.6.1.2), aspartate aminotransferase (EC 2.6.1.1), lactate dehydrogenase (EC 1.1.1.27), total protein, albumin, uric acid, total bilirubin, calcium, γ-glutamyl transpeptidase (EC 2.3.2.2), prothrombin time, partial thromboplastin time, fasting glucose, cholesterol, triacylglycerols, phosphorous, magnesium, amylase (EC 3.2.1.2), lipase (EC 3.1.1.3), and fibrinogen. The urinalysis included measurements of specific gravity, pH, and protein, glucose, and blood concentrations at The McLendon Clinical Laboratory at the UNC Hospitals. This laboratory is certified on the basis of both the Clinical Laboratory Improvement Act and the College of American Pathologists and maintains quality-assurance logs and standard operating procedures. In addition to these laboratory tests, each subject received a chest X-ray and an electrocardiogram. The National Cancer Institute’s Common Toxicity Criteria for Assessment of the
Toxicity of Chemopreventive Agents (CTC version 2.0, final 30 January 1998) was used to assign a severity grade to adverse events. The general safety and clinical toxicity data obtained from our phase I trial will be published separately from the genotoxicity studies described in this report.

Measurement of genistein concentrations

The plasma profiles of genistein, daidzein, and glycitein were obtained during 2 in-patient stays at the UNC General Clinical Research Center on days 1 and 84 of the study. Blood samples were taken from the antecubital vein for isoflavone measurements before dosing (0 h) and 0.5, 1, 1.5, 3, 4.5, 6, 9, 12, 15, 18, 24, and 32 h postdose. The subjects were discharged after the 24-h plasma and urine samples were collected but returned for the 32-h blood draw. Meals throughout the inpatient study period were free of soy, were isocaloric, and had a standardized macronutrient composition of 55% carbohydrate, 30% fat, and 15% protein. All meals were consumed at specified times during the study period to standardize any effect of food consumption on isoflavone disposition.

Plasma trough concentrations of genistein, daidzein, and glycitein were measured at all follow-up visits 24 h postdose on days 5, 9, 14, 21, and 28 and 12 h postdose on days 31, 35, 42, and 56. The methods used to measure free and total isoflavones in plasma are modifications of the HPLC methods of Supko and Phillips (37) that we previously published (38, 39). In the current study, total daidzein and total genistein are each defined as the amount of unconjugated analyte plus the amount of unconjugated analyte that is released on treatment of the biological matrix with β-glucuronidase (EC 3.2.1.31) and sulfatase (EC 3.1.6.1). The genistin standard was obtained from the INDOFINE Chemical Co (Somerville, NJ). Dimethylsulfoxide, methanol, acetonitrile, and 56.

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Lymphocyte culture

Fresh blood (5–8 mL) was collected by venipuncture with the use of a CPT Vacutainer tube (Becton Dickinson, Franklin Lakes, NJ) with sodium citrate as an anticoagulant. Samples were centrifuged at room temperature (18°C) in a horizontal rotor for 20 min at 1800 × g (relative centrifugal force). After centrifugation, mononuclear cells and platelets were separated and resuspended in the plasma by inverting the unopened Vacutainer CPT tube gently 10 times. The plasma and cells were then transferred to a 15-mL screw-cap vial and centrifuged at 70 × g for 10 min at 10°C. The supernatant fluid was discarded, and the cells were resuspended in 14 mL Hank’s Balanced Salt Solution by gentle inversion. The tubes were centrifuged at 70 × g for 10 min at 10°C. The supernatant fluid was removed and the rinsing was repeated. The cell pellet was then resuspended in 1 mL McCoy’s 5A medium. Cell viability was estimated by using Trypan Blue exclusion. Cell density was estimated by counting cells on a 5 × 5 grid of a hematocytometer. Counts were performed 5 times and averaged. The lymphocytes were cultured in 96-well microtiter plates (Corning Incorporated, Corning, NY) at a density of 1.2 × 10⁶ viable cells/100 μL McCoy’s 5A medium containing 15% (vol:vol) heat-inactivated fetal bovine serum (Gibco BRL, Grand Island, NY) and Pen/Strep and phytohemagglutinin (lectin, 5 μg/mL; Sigma-Aldrich) at 37°C for 24 h in a humidified atmosphere containing 10% CO₂, with added isoflavone mixture (the same preparation used to treat patients) to achieve final unconjugated genistein concentrations of 0, 1, 2, 10, 20, 40, 100, or 200 μmol/L. The medium containing genistein was removed, and the cells were rinsed with phosphate-buffered saline and cultured with fresh medium for micronucleus assay.

Single-cell gel electrophoresis

Single-cell gel electrophoresis (COMET assay) for detection of DNA strand breaks was performed as described previously (40–42). Lymphocytes were sandwiched between 0.5% regular agarose solution ( Fisher, Fair Lawn, NJ) and 0.5% low-melting-point agarose (37°C; Fisher). The resulting slides were placed into cold, freshly made lysis solution [10 mmol tris/L, pH 10; 2.5 mol NaCl/L, 100 mmol EDTA/L, 1% sodium sarcosinate/L, 10% dimethylsulfoxide, and 1% Triton X-100 (Sigma Chemical Laboratories)]. The slides were placed in the refrigerator for ≥ 1 h and then pretreated for 20 min in electrophoresis buffer (300 mmol NaOH/L, 1 mmol EDTA/L; pH 13). Electrophoresis was performed at 25 V and 300 mA for 20 min. After electrophoresis, the slides were incubated 3 times for 5 min in neutralization buffer (0.4 mol/L tris-HCl, pH 7.5), washed with methanol, and stained with ethidium bromide (20 μg/mL; Sigma-Aldrich). For visualization of DNA damage, observations were made at 400X magnification with an Olympus AX70 fluorescent microscope (Olympus Corp, Lake Success, NY). Typically, 100 cells were analyzed per sample point by using cell image analysis software (Scion Corporation, Frederick, MD), and the COMET tail moment was calculated by using a program in the Image Analysis Macro language of the National Institutes of Health (National Technology Information Service, Springfield, VA).

Cytokinesis-block micronucleus assays

Cytokinesis-block micronucleus assays were carried out according to the protocol of Fenech (43, 44). Lymphocytes were cultured in 6-well plates at a density of 1 × 10⁶ cells/mL McCoy’s 5A medium containing 15% (vol:vol) heat-inactivated fetal bovine serum and Pen/Strep. Lymphocytes were stimulated to divide with lectin (5 μg/mL) and incubated at 37°C in a humidified atmosphere containing 10% CO₂. Forty-four hours after lectin stimulation, cytochalasin-B (Sigma-Aldrich) was added to the culture to give a final concentration of 4.5 μg/mL. Twenty-eight hours after the addition of cytochalasin-B, the cells were harvested by transferring them directly to a glass slide with the use of a cytocentrifuge (Cytospin 3; Shandon, Astmoor Runcorn, United Kingdom). Six preparations were made per culture. The slides were air-dried for 10 min, fixed in absolute methanol for 10 min, and stained with Giemsa stain (Sigma-Aldrich) for 45 min. The slides were then air-dried and used for staining.
were examined at 1000× magnification and scored for the number of micronuclei present per 100 cytokinesis-blocked cells.

**Fluorescence in situ hybridization**

Fluorescence in situ hybridization (FISH) was performed on lymphocytes from the last 3 subjects enrolled in the study. Whole blood (500–800 μL) was cultured in 9.5 mL RPMI-1640 with 15% fetal bovine serum, Pen/Strep, L-glutamic acid, and phytohemagglutinin in a vented T25 flask at 37 °C in 5% CO2 for 72 h. KaryoMax Colcemid (0.05 μg/mL; Gibco BRL) was added to cells 45 min before the end of this 72-h period. Cells were collected by centrifugation at 300 × g for 5 min at room temperature and then incubated in 0.075 mol KCl/L for 25 min at room temperature and fixed in 3 changes of methanol/acetic acid (3:1, vol:vol). We used 2 different kits to perform the FISH assays. The initial studies were performed with one probe (catalog no. P5114-DG.5; Oncor Inc, Gaithersburg, MD), but midway through our investigation, unfortunately, the probe stopped production. We then switched to another probe (LSI MLL Dual Color Rearrangement Probe, catalog no. 32-190083; Vysis Inc, Downers Grove, IL). Detection kits were used according to the manufacturers’ instructions, and chromosomes were viewed and photographed with a Zeiss photomicroscope at 60× or 100× magnification.

**Statistical methods**

Linear mixed models (45) were used to assess mean changes in MF and COMET tail moment over time and within-person changes. These analyses were implemented by using the MIXED procedure in SAS (46). The input data for the mixed models were the individual determinations (for MN, n = 1–6 slides; for COMET, n = 8–227 determinations) within each subject and study day. Random effects were formulated as random linear regressions over the study day, each subject having a random intercept and a random slope. Each model included variance terms for subject intercept, subject slope, their covariance, variance about the regression line within subjects, and residual variance (determinations).

For comparison of means, the fixed effects of study day in the model were formulated as one-way analysis of variance effects. The Dunnett-Hsu multiple comparisons procedure for correlated means (47) was used to control the type I error (α = 0.05) for comparisons between study day 0 and days 9, 28, 35, and 84.

To assess within-individual slopes, the fixed effects were formulated as an intercept and slope. Each subject’s slope and P value for testing departure from a slope of zero were estimated from the mixed model. The collection of 20 null hypotheses for the individual slopes was tested for significance by using Fisher’s method for combining independent P values (48). (Although the P values from the model share a common estimate of pooled variance, they should be nearly independent.) A significant result by Fisher’s test implies that at least one of the subjects’ slopes is different from zero but does not identify how many or which subjects. Tests of specific persons’ slopes were conducted at a Bonferroni-corrected level (α = 0.05/20 = 0.0025).

For each outcome, an additional mixed model was used to compare the responses of control subjects against those of treated subjects on days 0, 9, 28, 36, and 85, taking into account the independence of the control and treated subjects and the correlation among study days within treated subjects. The Dunnett-Hsu procedure was used to control for multiple comparisons at α = 0.05.

In vitro genistein concentrations were related to micronucleus formation in incubated peripheral blood lymphocytes from a

<table>
<thead>
<tr>
<th>Subject</th>
<th>Free genistein (μmol/L)</th>
<th>Total genistein (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.04 (11.1)</td>
<td>20.9 (5643)</td>
</tr>
<tr>
<td>2</td>
<td>0.10 (27.9)</td>
<td>16.0 (4309)</td>
</tr>
<tr>
<td>3</td>
<td>0.16 (44.0)</td>
<td>27.1 (7326)</td>
</tr>
<tr>
<td>4, 5</td>
<td>0.13 (34.3)</td>
<td>17.3 (4682)</td>
</tr>
<tr>
<td>6</td>
<td>0.10 (26.0)</td>
<td>10.9 (2934)</td>
</tr>
<tr>
<td>7</td>
<td>0.08 (21.2)</td>
<td>15.8 (4279)</td>
</tr>
<tr>
<td>8</td>
<td>0.12 (33.0)</td>
<td>10.8 (2930)</td>
</tr>
<tr>
<td>9</td>
<td>0.12 (31.5)</td>
<td>9.6 (2591)</td>
</tr>
<tr>
<td>10</td>
<td>0.27 (74.0)</td>
<td>26.7 (7200)</td>
</tr>
<tr>
<td>11</td>
<td>0.29 (79.2)</td>
<td>12.6 (3400)</td>
</tr>
<tr>
<td>12</td>
<td>0.32 (85.5)</td>
<td>18.8 (5069)</td>
</tr>
<tr>
<td>13</td>
<td>0.09 (24.4)</td>
<td>20.8 (5610)</td>
</tr>
<tr>
<td>14</td>
<td>0.09 (24.6)</td>
<td>12.1 (3278)</td>
</tr>
<tr>
<td>15</td>
<td>0.16 (43.7)</td>
<td>9.0 (2425)</td>
</tr>
<tr>
<td>16</td>
<td>0.13 (35.1)</td>
<td>6.1 (1656)</td>
</tr>
<tr>
<td>17</td>
<td>0.07 (18.0)</td>
<td>5.0 (1355)</td>
</tr>
<tr>
<td>18</td>
<td>0.30 (81.5)</td>
<td>5.5 (1494)</td>
</tr>
<tr>
<td>19</td>
<td>0.02 (6.6)</td>
<td>4.1 (1097)</td>
</tr>
<tr>
<td>20</td>
<td>0.06 (14.9)</td>
<td>5.0 (1355)</td>
</tr>
</tbody>
</table>

1 Reported are the highest concentrations of plasma genistein measured on days 1 or at follow-up visits on days 5, 9, 14, 21, 28, 31, 35, 42, and 56 as described in Subjects and Methods. Subject 21 completed only 9 of the treatment protocol and had a peak genistein concentration of 0.19 μmol/L (51 ng/mL) and a peak total genistein concentration of 9.4 μmol/L (2549 ng/mL). Note that 1 μmol/L genistein = 270 ng/mL.

2 Had a high micronucleus frequency value on day 84.

3 Had high trough genistein concentrations; therefore, the 600 mg/d dose of genistein was reduced (see Subjects and Methods).

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single subject with the use of one-way analysis of variance (replications were 3 slides per concentration point). Comparisons with the zero-dose condition were adjusted by using Dunnett’s procedure.

**RESULTS**

Genistein concentrations

Mean (±SE) peak plasma total genistein concentrations during the plasma profile studies were 7.3 ± 0.8 μmol/L on day 1 and 8.1 ± 1.2 μmol/L on day 84 of treatment (after an equivalent 300 mg dose of PTI G-2535). These concentrations were reached 1.5–12 h postdose. Peak plasma free genistein on day 1 was 0.13 ± 0.02 μmol/L and on day 84 was 0.11 ± 0.02 μmol/L. Peak total genistein varied between 2.4 and 2.9 μmol/L when measured 24 h postdose on the days that 300 mg genistein/d was administered and varied between 9.2 and 10.7 μmol/L when measured 12 h postdose on the days when 600 mg/d was administered. The highest individual total genistein concentration measured was 27.1 μmol/L, and the highest individual free genistein concentration measured was 0.32 μmol/L (Table 1). The subject in whom we observed a single elevated MF value that exceeded a clinical threshold set before we initiated the study had a maximum measured plasma total genistein concentration of 17.3 μmol/L and a maximum free genistein concentration of 0.13 μmol/L.
Average peak plasma total daidzein concentrations were 3.9 ± 0.3 \mu mol/L on day 1 and 3.8 ± 0.4 \mu mol/L on day 84 of treatment. Average trough daidzein concentrations were 0.79 ± 0.07 \mu mol/L on days 5–28 and 3.6 ± 0.2 \mu mol/L on days 31–56. Glycitein concentrations were usually below detection limits.

**COMET assay**

The average COMET tail moment in prostate cancer patients did not increase during the 84 d of treatment with the purified soy isoflavones mixture (Figure 1). In fact, each of the treatment means was actually lower than the predose mean, although none of these differences were significant. Moreover, we saw no significant changes in individual COMET values with treatment. Values obtained from 6 healthy untreated subjects were not significantly different from those obtained in the prostate cancer patients. (Note that because all of our isoflavone-treated subjects had prostate cancer, we thought it would be interesting to show values from a younger and healthier group of subjects who did not have cancer and were likely to have minimal genetic damage.)

**Micronucleus assay**

No significant change in average MF (number of micronuclei/100 binucleated cells) was observed after genistein treatment in prostate cancer patients (Figure 2). In addition, we observed no significant changes in individual MF measurements. Values obtained from 6 healthy untreated subjects were not significantly different from those obtained in the prostate cancer patients. Although we observed no significant change in MF in any of the treated subjects, one measurement in one subject (baseline: 5.5; day 84: 10.3) exceeded a clinical threshold set before we initiated the study for our adverse event grading criteria (> 8.07 micronuclei/100 cells and ≥2.5 micronuclei/100 cells greater than the baseline value. This subject presented with an elevated COMET tail moment on this same day, although the value was not significant.

In vitro, using human lymphocytes treated with a range of doses of genistein, daidzein, and glycitein, we observed that genistein concentrations ≤ 20 \mu mol/L did not induce formation of micronuclei, whereas a significant increase in MF was observed at genistein concentrations of 100–200 \mu mol/L (Figure 3).

**FISH analysis**

In the 3 subjects studied, MLL gene rearrangement (deletions and translocations)—as determined by FISH analyses—was not significantly different between days 1 and 84. A representative set of photomicrographs from one subject is presented in Figure 4.

**DISCUSSION**

Because of the widespread use of soy isoflavones as dietary ingredients and supplements, it is imperative that we know whether isoflavones such as genistein induce genetic damage. As discussed earlier, several studies performed in cell cultures showed DNA strand breaks and micronucleus formation after exposure to genistein (29–33). Specific genes may be damaged, including the MLL leukemia gene (34). However,
isoflavones administered to mice in vivo did not cause genetic damage (29). In this study, for the first time, we studied the genotoxicity of unconjugated isoflavones in humans. Although we realize that the concern is greatest for healthy persons who might take isoflavones to prevent cancer, we believed that samples from our ongoing study in men with prostate cancer could contribute valuable insight as to the genotoxicity of isoflavones in humans.

We administered PTI G-2535 (unconjugated isoflavones), a purified genistein-daidzein-glycitein preparation that delivers genistein and daidzein in amounts that greatly exceed dietary intakes from soy, even in Asian populations (13, 14). These amounts equal or exceed doses likely to be self-administered by those using soy dietary supplements. Despite these high doses, our patients’ plasma total genistein concentrations never exceeded measured peak concentrations of 27.1 μmol/L. Total daidzein and glycitein concentrations were lower. Circulating isoflavones were primarily in the conjugated forms as glucuronide and sulfate metabolites. The free forms of the isoflavones were a very small fraction of the total isoflavone circulating in the blood; measured free genistein concentrations never exceeded 0.32 μmol/L.

The COMET assay provides a measure of DNA strand breaks and does not necessarily indicate that chromosomal rearrangements or deletions have occurred (40–42). Genistein induces apoptosis in cells by inhibiting tyrosine kinase growth signaling (25, 32, 49) and should be expected to result in apoptosis-related DNA strand breaks. Also, genistein inhibits topoisomerase II, an enzyme responsible for repairing DNA strand breaks (49, 50). This effect would enhance accumulation of strand breaks. It is surprising, therefore, that we observed no significant increases in strand breaks in any of our subjects and that there were no changes in COMET tail moment (Figure 1). This suggests that, for most patients, the concentrations of genistein achieved even at the 600 mg/d genistein dose were not sufficient to induce DNA strand breakage.

Cytokinesis-block micronucleus assay is a reliable and precise method for assessing chromosome breakage and chromosome loss (44); however, the relative values reported by different laboratories vary because of laboratory protocol and scoring criteria (control mean values in the literature ranged from 0.2 to

**FIGURE 3.** Mean (±SE) number of micronuclei in peripheral lymphocytes isolated from healthy control men. Cytokinesis-block micronucleus (BN) assays were performed as described in Subjects and Methods. The lymphocytes were cultured in media containing the isoflavone mixture used to treat humans (as described in Subjects and Methods), to achieve the final concentrations of unconjugated genistein indicated. n = 3 wells per point. **Significantly different from 0 μmol/L, P < 0.05 (Dunnett-Hsu test).

**FIGURE 4.** Photomicrographs from one subject depicting MLL gene fluorescence before (A; day 1) and after (B; day 84) treatment with an isoflavone preparation that delivered 300 mg genistein/d for 28 d and then 600 mg/d for 56 more days, as indicated in Figure 1. Fluorescence in situ hybridization was performed as described in Subjects and Methods. These data are typical of data obtained from the 3 subjects studied. The MLL gene (11q23) was not damaged in lymphocytes isolated from prostate cancer patients treated with repeated doses of genistein.
Although scientists have speculated that the ingestion of soy foods
may be sensitive to gene damage at lower doses. Our data sug-
gest that gene damage is not likely to occur in humans after the
ingestion of genistein at concentrations ≥100 μmol/L. The 600-mg/d dose of
genistein administered as PTI G-2535 did not result in plasma
considerations of total genistein that were this high, and uncon-
jugated genistein concentrations were <5% of this amount.
Figure 2. In one subject we reported a single time point with
an elevated MF value that exceeded a clinical threshold set before
we initiated the study (rising to 10.3 micronuclei/100 binucleated
cells on day 84 from a baseline value of 5.5). This was associated with
an insignificant increase in the COMET tail moment at the
same time point (0.52 at baseline to 1.54 on day 84); 38 d after
the treatment had been discontinued, the MF and COMET tail moment were
normal. This subject was a 65-y-old man who had not smoked for
the past 20 y and was taking supplemental vitamin E, lycopene,
and selenium. He had been taking a genistein supplement but dis-
continued it 1 wk before our study. He took aspirin or naprosyn as
needed for osteoarthitis pain and was not receiving chemotherapy.
His maximum measured free genistein concentration was
0.13 μmol/L, and his maximum total genistein concentration was
17.3 μmol/L. This subject was unusual in that his interdose con-
centrations of genistein were higher than expected on the basis of
the low dose of isoflavones he was receiving, and his dose was only
b Briefly escalated to 600 mg/d, halved to 300 mg/d, and then halved
gain to 150 mg/d when his trough concentrations were persist-
ently higher than desired. Perhaps this subject metabolized
isoflavones differently than did the rest of the subjects studied.
Perhaps because of the genetic polymorphisms, there may be some
persons who metabolize genistein more slowly, and these individ-
uals may be sensitive to gene damage at lower doses. Our data sug-
gest that gene damage is not likely to occur in humans after the
ingestion of soy foods.

In cell culture we were able to significantly increase genetic
damage (as assessed by micronucleus formation) by exposing
human lymphocytes to ≥100 μmol unconjugated genistein/L
(from PTI G-2535; Figure 3). Therefore, it may be important that
unconjugated genistein concentrations in plasma be kept below
this critical concentration to avoid genetic damage. As noted ear-
lier for humans receiving the 600-mg/d dose, unconjugated and
total genistein concentrations never reached such concentrations.
We do not know whether the conjugated species of genistein have
the same potency for genotoxicity as does the unconjugated form.
Deletions and translocations in the MLL gene, reported after in
vitro exposure of mouse lymphocytes to genistein (34), may be
associated with an increased risk of acute myelogenous leukemia
(35, 36). Although scientists have speculated that the ingestion of
soy foods and formula might provide enough genistein to damage the
MLL gene (35, 36), we did not observe such damage in men
with prostate cancer treated with high doses of PTI G-2535 (Fig-
ure 4). It is possible that rodents and not humans are sensitive to
genistein-induced damage of the MLL gene.

In summary, for the first time, men with prostate cancer were
-treated with large doses of unconjugated purified isoflavones
(including genistein) for ≈3 mo. In the group as a whole, or indi-
vidually, we saw no increases in DNA strand breaks. These
breaks would be expected if genistein activates apoptosis and
inhibits topoisomerase II in lymphocytes. We observed no chro-
mosome deletions and translocations (eg, increased micronu-
cleus formation) caused by these isoflavones in general or by
-genistein in particular. In cell culture studies, we found that
significant chromosomal damage was induced by unconjugated
-genistein at concentrations ≥100 μmol/L. The 600-mg/d dose of
genistein administered as PTI G-2535 did not result in plasma
total genistein concentrations that were this high, and uncon-
jugated concentrations were <5% of this amount. However, it would seem prudent to limit genistein doses to those
that result in plasma total genistein concentrations that are the same as or lower than the highest concentration (27 μmol/L) that
we measured with the 600-mg/d (>8 mg/kg) dose; for compar-
ison, this amount is 3–4-fold greater than that ingested by the
Japanese from soy foods.

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ment, RAJ measured isoflavone concentrations, MAK performed the
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