Daidzein and Genistein Glucuronides In Vitro Are Weakly Estrogenic and Activate Human Natural Killer Cells at Nutritionally Relevant Concentrations1,2,3

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ABSTRACT Daidzein and genistein glucuronides (DG and GG), major isoflavone metabolites, may be partly responsible for biological effects of isoflavones, such as estrogen receptor binding and natural killer cell (NK) activation or inhibition. DG and GG were synthesized using 3-methylcholanthrene-induced rat liver microsomes. The Km and Vmax for daidzein and genistein were 9.0 and 7.7 μmol/L, and 0.7 and 1.6 μmol/(mg protein · min), respectively. The absence of ultraviolet absorbance maxima shifts in the presence of sodium acetate confirmed that the synthesized products were 7-O-glucuronides. DG and GG were further purified by a Sephadex LH-20 column. DG and GG competed with the binding of 17β-(3H) estradiol to estrogen receptors of B6D2F1 mouse uterine cytosol. The concentrations required for 50% displacement of 17β-(3H) estradiol (CB50) were: 17β-estradiol, 1.34 nmol/L; diethylstilbestrol, 1.46 nmol/L; daidzein, 1.6 μmol/L; DG, 14.7 μmol/L; genistein, 0.154 μmol/L; GG, 7.27 μmol/L. In human peripheral blood NK cells, genistein inhibited cytotoxicity at 0.5 μmol/L and DG and GG at 0.1–10 μmol/L enhanced NK cell-mediated K562 cancer cell killing significantly (P < 0.05). At > 0.5 μmol/L, genistein inhibited NK cytotoxicity significantly (P < 0.05). The glucuronides only inhibited NK cytotoxicity at 50 μmol/L. Isoflavones, and especially the isoflavone glucuronides, enhanced activation of NK cells by interleukin-2 (IL-2), additively. At physiological concentrations, DG and GG were weakly estrogenic, and they activated human NK cells in nutritionally relevant concentrations in vitro, probably at a site different from IL-2 action. J. Nutr. 129: 399–405, 1999.

KEY WORDS: • isoflavones • glucuronides • estrogen • human natural killer cell

Epidemiological studies suggest that soybean consumption is one of several factors that may contribute to the lower rates of breast, colon and sex-hormone-dependent prostate cancers in Asian countries (Messina et al. 1994). Urinary excretion of isoflavones and their metabolites was 20–30 times higher among Japanese women and men consuming a traditional diet than in people living in Boston and Helsinki (Adlercreutz et al. 1982). Compared with vegetarian and omnivorous healthy controls, breast cancer patients excreted about 21% less of the isoflavone metabolite equol in urine (Adlercreutz et al. 1982).

When rats consumed a soybean-based diet, they developed fewer mammary tumors following the administration of N-methylnitrosourea and 7,12-dimethylben[a]anthracene than rats did on isonitrogenous and isocaloric diet without soybeans (Barnes et al. 1990). Genistein and daidzein inhibited the growth of both estrogen receptor-positive (MCF-7) and negative (MDA-468) human breast cancer cell lines with 50% inhibition concentrations (IC50)5 of 24–44 and 79–134 μmol/L, respectively (Peterson and Barnes 1991). However, some experiments showed that isoflavones might suppress or enhance tumor risk depending on dose, feeding period or organ. By measuring hepatic glutathione peroxidase activity and quantifying liver γ-glutamyltransferase-positive (GGT+) and placental glutathione transferase-positive altered hepatic altered foci; PTK, protein tyrosine kinase; UDPAG, uridine 5′-diphosphoglucuronic acid; UV, ultraviolet.

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4 Abbreviations used: CB50, concentrations required for 50% displacement of 17β-(3H) estradiol; dein, daidzein; DES, diethylstilbestrol; DG, daidzein glucuronide; ESTR, 17β-estradiol; FBS, fetal bovine serum; gen, genistein; GG, genistein glucuronide; GGT+, γ-glutamyltransferase-positive; HBSS, Hank’s balanced saline solution; HPLC, high performance liquid chromatography; IC50, concentrations causing 50% inhibition; IL-2, interleukin-2; LSD, least significant difference; NK cell, natural killer cell; PGST-1–AHF, placental glutathione transferase-positive altered hepatic foci; PTK, protein tyrosine kinase; UDPAG, uridine 5′-diphospho-N-acetylglucosamine; UDPGA, uridine 5′-diphosphoglucuronic acid; UV, ultraviolet.
foci (PGST<sup>+</sup>-AHF), Lee et al. (1995) found that after 3 mo of feeding, 920 or 1840 μmol total isoflavones/kg body weight soybean extract suppressed hepatocarcinogenesis promoted by phenobarbital in diethylnitrosamine-initiated rats. But when rats were fed 1840 μmol total isoflavones/kg body for 11 mo, there was a greater development of GGT<sup>+</sup> and PGST<sup>+</sup>-AHF than in the group fed the basal diet alone. In azoxymethane-induced rats fed with 910 μmol/L genistein, Rao et al. (1997) found that genistein enhanced the formation of noninvasive adenocarcinomas in colon. These epidemiological studies and experimental results suggested that soybean isoflavones may have cancer-protective effects, but the safety margin may be relatively narrow, and they may have organ specificity.

The main forms of isoflavones found in soybean are daidzin, genistin, glycitin, their malonyl- and acetyl-glucosides and the corresponding aglycones daidzein, genistein and glycitein. The content and forms of the isoflavones differ among soy foods (Wang and Murphy 1994). The bioavailability and biological activities of different isoflavones also differ to some extent. For example, daidzein is excreted in urine to a greater extent (Xu et al. 1995) and is more effective in potentiation of splenocyte proliferation than is genistein (Wang et al. 1997). Before consumers are advised to increase consumption of foods high in isoflavones, it is important to determine how isoflavones are utilized by the body.

After absorption, except for a small amount of aglycone isoflavones and equal, over 95% of the isoflavones appeared as conjugated forms in the circulation, mainly as glucuronides and less as sulfates (Lundh 1990). Classically, conjugation has been considered to be a detoxification step. But in some cases, after conjugation, the biological activities of the conjugates were enhanced. For example, the formation of aromatic amine glucuronides causes bladder cancer (Thorgeirsson et al. 1983). Retinol β-glucuronide and retinyl β-glucuronide are more active than their parent compounds in inhibiting prolactin secretion and less as sulfates (Lundh 1990). Classically, conjugation has been utilized by the body.

In isoflavones, it is important to determine how isoflavones are metabolized in the body. After absorption, except for a small amount of aglycone isoflavones and equal, over 95% of the isoflavones appeared as conjugated forms in the circulation, mainly as glucuronides and less as sulfates (Lundh 1990). Classically, conjugation has been considered to be a detoxification step. But in some cases, after conjugation, the biological activities of the conjugates were enhanced. For example, the formation of aromatic amine glucuronides causes bladder cancer (Thorgeirsson et al. 1983). Retinol β-glucuronide and retinyl β-glucuronide are more active than their parent compounds in inhibiting prolactin secretion and less as sulfates (Lundh 1990). Classically, conjugation has been utilized by the body.

**Materials and Methods**

**Chemicals.** Daidzein and genistein were chemically synthesized in the laboratory of Dr. Patricia Murphy, Iowa State University. Urindine 5'-diphosphoglucuronic acid (trisodium salt) (UDPGA), uridine 5'-diphospho-N-acetylglucosamine (sodium salt) (UDPAG), d-saccharic acid-1,4-lactone, diethylstilbestrol (DES), Tween 80, 17β-(2,4'-H) estradiol (85 Bq), 17β-estradiol (ESTR), dextran-coated charcoal, bovine serum albumin, interleukin-2 (IL-2) and other chemicals were all obtained from Sigma Chemical Co. (St. Louis, MO). Scintiverse fluid was purchased from Fisher Scientific Co. (Pittsburgh, PA). Hank's balanced saline solution (HBSS), RPMI-1640, HEPES, l-glutamine, gentamicin and heat-inactivated fetal bovine serum (FBS) were all from Life Technologies (Gaithersburg, MD).

**Rat hepatic microsomal preparation and isoflavone glucuronide synthesis.** All the animals used in this experiment were purchased through laboratory animal resources, Iowa State University (ISU) (Ames, Iowa). All animal use was approved by the ISU Animal Care Committee. Sprague-Dawley female rats (190–200 g) were gavaged each day for four consecutive days with 3-methylcholanthrene in corn oil (40 mg/kg body weight) before killing. Rat livers were washed with ice-cold 0.25 mol/L sucrose and 50 mmol/L Tris-HCl buffer (pH 7.4), and homogenized in a Kinematika-Gmbh homogenizer (Luzern, Switzerland). The homogenate was adjusted to 100 g/L with buffer and centrifuged at 10,000×g for 10 min. The supernatant fraction was centrifuged at 105,000×g for 60 min, and the resulting microsomal pellet was washed with sucrose-Tris buffer and rehomogenized. Protein was measured by the Lowry procedure (Lowry et al. 1951) with bovine serum albumin as the reference standard.

The final standard incubation mixture contained 100 μmol/L daidzein or genistein, usually added in 50% ethanol. The final concentration of ethanol was 5%. Other reagents were 15 mmol/L UDPGA, 50 mmol/L Tris-HCl buffer, 0.1 mmol/L MgCl₂, 0.25 mmol/L UDPAG and 10 mmol/L saccharic acid 1,4-lactone. The reaction mixture was prewarmed for 20 s before adding 5 g of microsomal protein/L to initiate the reaction. Duplicate reaction mixtures were then incubated for 60 min at 37°C. The reaction was stopped by adding 0.3 mL of methanol and 0.2 mL of 0.4 mol/L trichloroacetic acid–0.6 mol/L glycine buffer (pH 2.0). The mixture was centrifuged and the supernatant was concentrated by rotary evaporation (R-114; BUCHI, Flawil, Switzerland). Concentrated reaction mixture (10 mL) was overlaid on a Sephadex LH-20 column with 40% ethanol as the eluant. The separated isoflavone aglycone and its glucuronide were detected by a single-path monitor ultraviolet (UV) detector (Pharmacia, Uppsala, Sweden) and collected by an Ultrorac fraction collector (LKB-7000 Ultrorac, LKB-producer, Bromma, Sweden).

The glucuronide fraction was lyophilized and stored at −20°C for further use. UDP-glucuronosyltransferase-mediated DG and GG formation kinetics were studied at a wide range of daidzein and genistein substrate concentrations (1.56–400 μmol/L). Incubation conditions were the same as stated above.

The purity and structure identification of DG and GG were determined by high performance liquid chromatography (HPLC) and UV spectral analysis. UV spectral analysis was performed on a Hewlett-Packard 8452A diode array spectrophotometry (Palo Alto, CA). UV spectral shifts of the glucuronides were determined in 100% methanol and ethanol, or acidified to pH 6.0 with glacial acetic acid. The effects of AlCl₃-HCl, sodium methoxide (NaOMe), sodium acetate (NaOAc) and boric acid (HBO₃) in methanol on the UV absorption maxima were determined according to Mabry et al. (1970).

**Quantitation of isoflavones and isoflavone glucuronides.** Sample (20 μL) was injected onto a reversed-phase 5-μm C18 AM 303 column (250 × 4.6 mm μmol) (YMCCo. Ltd., Wilmington, NC). A linear HPLC elution gradient was composed of (A) 0.1% glacial acetic acid in water and (B) 0.1% glacial acetic acid in acetonitrile. Following sample injection, solvent B was kept at 20% for 5 min, then increased to 30% in 25 min, after that increased to 50% in 15 min and decreased to 20% at the end of 45 min. The solvent flow rate was 0.8 mL/min at the first 5 min, then increased to 1 mL/min and kept there for 40 min. The HPLC system was a Hewlett-Packard 1050 series. Wavelengths used to detect isoflavone compounds and their conjugate products were 254 nm. UV absorbance spectra were recorded, and area responses were integrated by Chem station software (Hewlett-Packard Company, Scientific Instruments Division) to identify and quantify isoflavones and their conjugates. Purified aglycones and glucuronides were used as external standards to calibrate the results.

**Cytosol preparation and estrogen receptor assay.** Female B6D2F1 mice (4 wk of age) were obtained from Harlan Sprague-Dawley (Madison, WI). Their uteri were homogenized in Tris-EDTA (10 mmol/L Tris and 1 mmol/L EDTA, pH 7.4) buffer (1:10 wt/vol). The diluted cytosolic fraction was obtained by centrifugation of the
homogenate at 10,000×g for 1 h. Total binding was determined by adding 0.2 mL of Tris-EDTA buffer, 0.2 mL of 3.2 mmol L⁻¹ estradiol/L and 0.2 mL of cytosol fraction. Nonspecific binding was determined by replacing the Tris-EDTA buffer with 0.2 mL of 17β-estradiol (3.2 μmol/L) in the pH 7.4 buffer. Blanks contained 0.4 mL of buffer and 0.2 mL of (³H) estradiol. Competitive binding was determined by replacing the 0.2 mL of buffer with 0.2 mL of the following solutions: 0.32–32 nmol mm⁻¹ ESTR; 0.56–56 nmol mm⁻¹ of diethylstilbestrol; 0.6–60 μmol/L of daidzein or genistein; and 0.35–175 μmol/L of DG or GG at pH 7.4. Duplicates were assayed for each tested compound at different concentrations. Mixtures were incubated at room temperature (23°C) for 2 h. Half mL dextran-coated charcoal (2.5% in pH 7.4 buffer) was added to each mixture and incubated for another 15 min. After 10 min centrifugation at 1,000 × g, the supernatants were decanted into scintillation vials containing 10 mL of scintillation fluid. The samples were counted for 10 min on a Packard liquid scintillation analyzer model 1900TR (Packard Instrument Co., Downers Grove, IL). The relative molar mouse uterine estrogen receptor binding affinity of mice was calculated by dividing the CB₅₀ of unlabeled ESTR by the CB₅₀ of competitor and then multiplying by 100.

To assay the potential hydrolysis of DG and GG by cytosolic enzymes, 0.2 mL of DG or GG was incubated with 0.4 mL Tris-buffer as blank, or with 0.2 mL of tris buffer and 0.2 mL of cytosol. Final concentration of DG and GG was 29.2 and 22.9 μmol/L, respectively. Pure HCl (10 μL) and 0.4 mL methanol were used to terminate the reaction. Final pH of the sample after termination was 3. One set of duplicate samples of cytosol mixtures was terminated immediately after addition of cytosol, another set was incubated for 2.5 hours at room temperature. Samples were centrifuged and filtered. The concentrations of DG and GG were measured by HPLC under the same conditions described above.

**Natural killer activity assay.** Six healthy human subjects, three female and three male, between 18 and 29 y old, participated in this experiment. Subjects were screened according to answers to a health history questionnaire. The use of human subjects was approved by the ISU Human Subjects Committee. Informed consent of subjects was obtained in writing. Peripheral blood samples (20 mL) were collected from each subject in heparinized vacutainer tubes by a licensed medical technologist under stringent aseptic conditions. Mononuclear cells were isolated from diluted blood [1:1 with Hank’s balanced saline solution (HBSS)] using density gradient media (Ficol-hypaque, Pharmacia, Uppsala, Sweden). Cells collected from the interface were washed and diluted with complete medium (RPMI-1640 with 25 mmol/L of L-glutamine, 50 mg/L gentamicin and 10% heat-inactivated FBS) to 2.5 × 10⁵ cells/L before plating. The target K562 cancer cells (ATCC) were maintained in complete medium. K562 cells in log growth phase (7 × 10⁵) were labeled with 700 μBq of ⁵¹Cr for 70 min. The targets were washed three times with complete medium prior to dilution and reactions containing 2.0 × 10⁴ cells/well were prepared. Five concentrations: 0.1, 0.5, 5, 10 and 50 μmol/L of DG, GG and genistein (dissolved in DMSO; 0.0825% of DMSO in total incubation volume) with and without IL-2 (0.31 ng/well) were incubated with 2.5 × 10⁴ isolated peripheral blood lymphocyte in 96-well plates (Model 3595; Costar, Cambridge, MA) in triplicate for 30 min. After enriched chromium targets (1 × 10⁵ cells/well) were added to each well, the plates were incubated for 4.5 h in a humidified CO₂ incubator (5% CO₂) (Fisher Scientific, Pittsburgh, PA), and centrifuged for 5 min at the end of incubation. Supernatant (100 μL) was collected to determine the amount of ⁵¹Cr released by dying cells using a Gamma Trac 1191 counter (TM Instrument Co., Downers Grove, IL). The relative molar mouse uterine estrogen receptor binding affinity of mice was calculated by dividing the CB₅₀ of unlabeled ESTR by the CB₅₀ of competitor and then multiplying by 100.

To determine whether DG and GG were hydrolyzed to aglycones during the NK assay, peripheral blood was obtained from one female (18 y) and one male (27 y) subject; 2.5 × 10⁵ NK cells/L were prepared. DG or GG (5 μmol/L) was incubated with 1.0 × 10⁵ cancer cells/L (without ⁵¹Cr), or 2.5 × 10⁵ NK cell, or with both cancer and NK cells separately. One set of duplicate samples was terminated immediately with 1 mL of methanol; another set of duplicate was incubated for 4.5 hours and terminated by methanol. DG and GG concentrations were measured by HPLC.

**Statistical analysis.** Statistical analyses were conducted using SAS (Statistical analysis system, Gary, NC). A three-way ANOVA was used to compare three isoflavones across five concentrations with and without IL-2. Differences between DG and GG formation kinetics were also analyzed by ANOVA. Individual means were compared using least significant differences (LSD). A P value of < 0.05 was considered significant.

**RESULTS**

**Isoflavone glucuronides synthesis and identification, HPLC analysis.** A typical chromatogram of a reaction mixture of daidzein, genistein and their glucuronides is shown in Figure 1. Isoflavone conjugates were more hydrophilic and appeared earlier than their aglycone counterparts. The retention times were: DG at 15.5 min, GG at 22.1 min, daidzein at 30 min and genistein at 37.7 min. After LH-20 separation, the purity of the isoflavone glucuronides was evaluated by peak spectral analysis. No isoflavone aglycone peak was observed in purified isoflavone glucuronide chromatograms, and the purity of each isoflavone glucuronide was over 95%.

**Maximal UV absorption of glucuronides.** When dissolved in different solvents, the maximal UV absorption of the glucuronides differed. As shown in Table 1, in 30% acetonitrile, which was the same solvent used in our HPLC system, the maximal absorption was at 250 nm for DG and 262 nm for GG. In 100% methanol or ethanol, the maximal absorption was 262 nm for both DG and GG. After acidification to pH 6.0, the maximal absorption of DG shifted to 250 nm and there was no change for GG. There was a large bathochromic

**FIGURE 1** Representation high-performance liquid chromatography chromatogram of enzymatic synthesis of daidzein and genistein glucuronides (Dein = daidzein; Gein = genistein; DG = daidzein glucuronide; GG = genistein glucuronide). A YMC C₅₆ reversed phase column was used to separate the aglycones and their glucuronide products. The elution and gradient conditions were as described in the method.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>DG</th>
<th>Gein</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>40</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>60</td>
<td>30</td>
<td>40</td>
</tr>
</tbody>
</table>
shift in both DG and GG when sodium methoxide was added, which indicated that there was a free 4'-OH in their structure. When the weaker base of sodium acetate was used, a modest bathochromic shift indicated that the 7-position was occupied by another group instead of the hydroxyl group. The bathochromic shift of GG in the presence of AlCl3 and AlCl3/HCl was due to the presence of a 5-OH group, which was absent from DG. Both of the DG and GG bathochromic shift patterns were similar to daidzin (daidzein 7-O-glucoside) and genistin (genistein 7-O-glucoside) (Mabry et al. 1970), respectively. These results indicated that our synthesized compounds were 7-O-glucuronides.

**Enzymatic conjugation of isoflavone aglycones.** With increasing concentration of daidzein and genistein, the rates of formation of both DG and GG increased progressively and approached saturation. The Lineweaver-Burk plot (Fig. 2) exhibited Michaelis-Menten kinetics. However, the calculated apparent affinity constants (Km) and maximal velocities (Vmax) for daidzein and genistein were significantly different (Table 2). Compared to GG, DG synthesis was a higher capacity, lower affinity reaction with rat microsomal UDP glucuronosyltransferase.

**In vitro estrogenicity of isoflavone glucuronides.** No hydrolysis of DG and GG was found by mouse uterine cytosol within a 2.5 h incubation (data not shown). DG and GG had weak affinity for the mouse uterine cytosol estrogen receptor compared with ESTR and diethylstilbestrol (DES) (Fig. 3). The CB50 (concentration required to displace 50% of the 3H-labeled estradiol) of these compounds were: ESTR = 1.34 nmol/L; DES = 1.46 nmol/L; Dein = 1.6 μmol/L; DG = 14.7 μmol/L; Gein = 0.154 μmol/L and GG = 7.27 μmol/L. Compared to the two glucuronides, DES had a much higher binding affinity with 91.8% of the binding affinity of ESTR (Table 3). GG had a 100% greater binding affinity than DG. These results paralleled those of the respective aglycone compounds in that genistein had greater estrogen receptor binding affinity than did daidzein.

**NK.** For the sake of standardization, individual variation of NK activity in the absence of IL-2 and isoflavone was set to 100% (at 25:1 effector:target ratio: 13–41% cytotoxicity). All data were transformed to percentage baseline. IL-2 dose was chosen to give moderate rather than maximal activation. The activation by IL-2 was similar for all individuals and ranged from 107–117% of baseline NK activity (Fig. 4). The mean activation with IL-2 alone was 113% which is shown in Figure 4 as “baseline + IL-2.”

### Table 1

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Daidzein glucuronide</th>
<th>Genistein glucuronide</th>
</tr>
</thead>
<tbody>
<tr>
<td>nm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30% acetonitrile</td>
<td>250</td>
<td>262</td>
</tr>
<tr>
<td>Ethanol</td>
<td>262</td>
<td>262</td>
</tr>
<tr>
<td>Methanol</td>
<td>262</td>
<td>262</td>
</tr>
<tr>
<td>Methanol/NaOMe</td>
<td>282</td>
<td>272</td>
</tr>
<tr>
<td>Methanol/AlCl3</td>
<td>264</td>
<td>272</td>
</tr>
<tr>
<td>Methanol/AlCl3:HCl</td>
<td>264</td>
<td>274</td>
</tr>
<tr>
<td>Methanol/NaOAc</td>
<td>262</td>
<td>264</td>
</tr>
<tr>
<td>Methanol/NaOAc:H3BO3</td>
<td>262</td>
<td>264</td>
</tr>
</tbody>
</table>

No hydrolysis of DG and GG was found during the 4.5 h incubation with K562 cancer cells, NK cells alone, or in a reaction containing both K562 and NK cells (data not shown).

Genistein activated NK cell killing maximally at 0.1–0.5 μmol/L. With increasing genistein concentration above 5 μmol/L, NK activity decreased linearly. Genistein in the presence of IL-2 activated NK activity in a more narrow range (< 0.5 μmol/L). The activity of IL-2-activated NK cells were

### Table 2

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product</th>
<th>Km2,3 (μmol/L)</th>
<th>Vmax2,4 (μmol/(mg protein·min))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daidzein</td>
<td>Daidzein glucuronide</td>
<td>9.0 ± 3.6a</td>
<td>0.7 ± 0.0a</td>
</tr>
<tr>
<td>Genistein</td>
<td>Genistein glucuronide</td>
<td>7.7 ± 1.4b</td>
<td>1.6 ± 0.0b</td>
</tr>
</tbody>
</table>

1 Parameters were obtained by hyperbolic fit to the data according to the method of Wilkinson (1961). Values were means ± SD, n = 2.
2 Values in a column bearing different letters were significant different.
3 The least significant difference (LSD) for Km was 0.737 and P < 0.03.
4 The LSD for Vmax was 0.024 and P < 0.001.
suppressed by genistein linearly in a dose-dependent manner from 5–50 μmol/L.

In the absence of IL-2, DG and GG activated NK cell activities as much as IL-2 within the range of 0.1–10 μmol/L. The enhancement with glucuronide alone was equivalent to enhancement of NK activity by IL-2 alone. In the presence of IL-2, both glucuronides additively increased NK activity at concentrations of 0.1–10 μmol/L. Across all concentrations, genistein gave significantly less activation than DG, and GG gave significantly more activation than DG (*P*, 0.01). There were significant interactions between isoflavones and IL-2 (*P*, 0.01). IL-2 in the presence of DG and GG activated NK killing more than did genistein with IL-2 (*P*, 0.01). Although individual responses to the isoflavones were significantly different (*P* < 0.01), probably due largely to interindividual baseline differences, the difference was due to the magnitude of change and not a difference in the pattern of response curves.

### DISCUSSION

Isoflavone glucosides constitute over 97% of the isoflavones in soybeans (Wang and Murphy 1994). After consumption, glucoside isoflavones reach the lower intestine and can be cleaved by bacterial glucosidases. The released free aglycones can be absorbed by the intestinal mucosa where they are glucuronidated to a large extent (Sfakianos et al. 1997) and transported to the liver via the portal vein, where they can be further reacted with UDP-glucuronoyltransferase and sulfotransferase to form glucuronide and sulfate conjugates, such that the 7-O-glucuronide of genistein is by far the main biliary excretion product of an intestinal dose of genistein (Sfakianos...
Glucuronidation has low affinity but high capacity whereas sulfation has a high affinity but low capacity (Parkinson 1995). Following administration of relatively large doses of phenol compounds, glucuronidation would be the major pathway of biotransformation. For example, when rats were orally dosed with 50 mg/kg (0.3 mmol/kg) of 14C-2-hydroxybiphenyl, almost 90% of the dose was recovered in urine within 24 h and about 75% of the dose was excreted as the glucuronide or sulfate esters of the hydroxybiphenyl and other metabolites. As doses were increased from 5 to 500 mg/kg, the proportion of glucuronides in urine was increased by 50% (Reitz et al. 1983).

Numerous functional groups can undergo conjugations with glucuronic acid and form O-, N- and S-glucuronides, respectively. Certain nucleophilic carbon atoms form C-glucuronides. Isoflavones contain hydroxyl groups in their structure: daidzein at 7'- and 4'-positions, genistin at 5', 7', and 4'-positions. The hydroxyl in the 7'-position seemed to be the most active group and reacted with UDPGA to form isoflavone glucuronides. Our UV absorption results confirmed that 7-O-glucuronidation was the main isoflavone conjugation by rat liver UGT shown, especially by the absence of the bathochromic shift by DG and GG when sodium methoxide was added.

Analysis of enzyme kinetics demonstrated that the rat microsomal UDP-glucuronosyltransferase had a greater affinity for genistein than for daidzein. The relevance of the rat microsomal kinetics to humans remains to be determined.

It is generally assumed that nonsteroidal estrogen exerts their stimulatory effect on the estrogen receptor by binding to the same site occupied by steroidal estrogens such as ESTR. The distance between the two hydroxyl groups at the opposite poles of the isoflavone aglycone is similar to the distance between C-3 and C-17 hydroxyl groups of ESTR. Numerous studies showed that isoflavone phytoestrogens had estrogen receptor binding ability and weak estrogenic activity. Several major isoflavonoids (genistein, daidzein, equol, O-desmethylangolensin) and their precursors (biochanin A, formononetin) detected in human and animal urine bind to estrogen receptor. The estrogenicity of daidzein and genistein was roughly 103 to 104 times greater than that of DES in mice (Bickoff et al. 1962).

In a sheep uterine estrogen receptor binding study, Shutt and Cox (1972) reported that the binding affinity of genistein and daidzein was 0.9 and 0.1%, respectively, of that of ESTR. Our in vitro mouse uterine binding study showed the same pattern as theirs. Our results agreed with previous experimental results that genistein was a much more potent estrogen than daidzein (Farmakalidis and Murphy 1985). Unlike the free form of isoflavones, for which the relative binding affinity of genistein was 10 times higher than daidzein, GG was only 100% greater than DG in relative binding affinity. If the glucuronides could pass through the cell membrane as efficiently as their aglycone compounds, at a high concentration such as 10^3 to 10^6 times that of estradiol, these isoflavone glucuronides could compete with the endogenous estrogens by competitive binding to estrogen receptors.

The question is whether these glucuronides could pass through cell membranes. Because the glucuronides are relatively hydrophilic, they are unlikely to pass through cell membranes, or might require a transporter protein. Assuming that the isoflavone glucuronides could be absorbed into cells (although there is no evidence at present for such a phenomenon), within the cell these compounds might exert some estrogenic effects. Even though no hydrolysis was found within a 2.5 h-incubation, it is possible these glucuronides could be rehydrolyzed during a longer incubation and the aglycone isoflavones would be transported into cells and exert their effects. How the glucuronides might exert estrogen receptor-mediated effects remains to be determined.

NK cell activity plays an essential role in immune surveillance against tumor development and progression as well as against infectious agents. Tyrosine kinase activity is crucial for the activation of NK cells and genistein is a specific inhibitor of tyrosine kinase. Based on tyrosine kinase inhibition, it is reasonable to hypothesize that soybean isoflavones can inhibit NK activity, and NK cell activity may vary with plasma isoflavone concentrations. Genistein inhibited purified epidermal growth factor (EGF) receptor and pp60c-scr tyrosine kinase (PTK) with an IC50 of 22–26 µmol/L and inhibited intact human A431 PTK with an IC50 of 148 µmol/L (Akiyama et al. 1991). It was also found that genistein could suppress both direct and Fc receptor (FcR)-mediated human NK cellular cytotoxicity in a dose-dependent manner (Einspahr et al. 1991). In our experiment, we found that at a genistein concentration of 5.0 µmol/L, NK cell activity decreased with increasing dose. However, at concentration of 0.1–0.5 µmol/L, genistein increased NK cell activity above baseline. This was also true for the DG and GG, but over a wider concentration range than for genistein. Only at 50 µmol/L did the isoflavone glucuronides not increase NK activity above baseline. As we observed, the plasma concentrations of isoflavones could reach 5 µmol/L after soy feeding (Xu et al. 1994 and 1995). So, under physiologically achievable concentrations in humans, isoflavones which would be present as glucuronides would be less toxic than genistein. This aspect of our hypothesis was confirmed.

Olson et al. (1992) proposed several modes of action to explain the possible biological mechanisms exerted by retinyl glucuronides. They were (1) immediate hydrolysis to the parent compounds, which then act on cells, (2) transport into cells followed by intracellular hydrolysis to the parent compounds at appropriate sites and (3) direct activation of appropriate receptors on membranes or in the nucleus. In our study, the lowest genistein concentration used was 0.1 µmol/L. It is possible that lower concentrations of genistein than 0.1 µmol/L might have further activated NK cells. Because DG and GG were not hydrolyzed to their aglycones under the conditions studied, the effects of the isoflavone glucuronides were most likely not mediated by the conversion to aglycones. The enhancement of NK activity by isoflavone glucuronides over a wider dose range than the enhancement by genistein suggests that the glucuronides act by a separate mechanism from the aglycone genistein. We propose that this mechanism may be mediated by cell surface receptors because no transport mechanism is known for glucuronide conjugates.

IL-2 induces a rapid increase in the tyrosine phosphorylation of multiple proteins in NK cells. This function increases the cytotoxic activity of NK cells. In our study, after adding IL-2, NK cytotoxic activity was consistently increased. However, when IL-2 and isoflavone were both present, the NK cell activity was additively increased. This result suggested that physiological achievable concentrations of isoflavone glucuronides and genistein would not inhibit PTK, which agreed with Peterson and Barnes’ (1993) finding that genistein and biochanin A did not inhibit EGF receptor tyrosine autophosphorylation result. The enhancement of NK activity by isoflavones seems to occur at a site different from that acted upon by IL-2. Kniss et al. (1996) reported that genistein could suppress EGF-induced prostaglandin biosynthesis by abolishing the response to calcium ionophores, a mechanism independent of EGF receptor tyrosine kinase inhibition. Tumor cells can produce high levels of prostaglandins and downregulate NK cell activity by (1) inhibiting IL-2- and IL-2-
specific receptor production (Baxevanis et al. 1993), and (2) reducing protein tyrosine kinase C activity (Ohnishi et al. 1991), another transduction signal functioning in NK lytic activity (Hager et al. 1990). So, decreasing the level of prosta
glandins could explain several possible mechanisms of in-
creased NK activity by isoflavones. This prostaglandin-lower-
ing possibility of aglycone and glucuronide isoflavonides might be especially helpful for elderly people whose NK activity is decreased and prostaglandin production increased with age (Meydani et al. 1988).

El-Hag et al. (1986) reported that NK activity against K562
cells was susceptible to oxidative injury by H₂O₂. Genistein
and daidzein have antioxidant activities, as reported by Naim
(1976) and Wei et al. (1995). If the glucuronides of daidzein
and genistein also had antioxidant activity, they might also
enhance NK activity via reducing H₂O₂ formation or by
decreasing prostaglandins, shown to be a common activity of
many antioxidants (Lands and Rome 1976).

The NK cell assay was not performed with isolated NK

cells. It is possible that the effects of genistein and gluco-

uronic acid on NK activity were indirect and due to action upon
other mononuclear cells that modulated cell-cell or cytokine
mediated interactions to influence the ability of our human
mononuclear cell preparation to kill K562 cells.

In this experiment, we demonstrated that genistein, DG
and GG are not toxic to NK cells at physiological concen-
trations. The glucuronide forms are more active over a wider
concentration range than the parent isoflavone genistein in
activating NK cells. Overall, our study demonstrated that
isoflavone glucuronides might not only compete with endog-

enous estrogen to inhibit estrogen-dependent proliferation of
cancer cells, but also can activate NK cells at a site different
from IL-2 action to potentially increase the immune defenses
of the body against cancer.

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