The phytoestrogen genistein suppresses cell-mediated immunity in mice

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Abstract

The soy phytoestrogen, genistein, induces thymic atrophy when administered to ovariectomized mice by injection or in the diet. Injected genistein also causes decreased humoral immunity, but the effects of genistein on cell-mediated immunity have not been addressed. Here we examined effects of injected and dietary genistein on cell-mediated immune responses. Female C57BL/6 mice (25- to 27-days-old) were ovariectomized, then placed on phytoestrogen-free feed 5 days later. Seven days after ovariectomy, they were given daily subcutaneous injections of either dimethylsulfoxide (DMSO) or genistein (8, 20, 80 mg/kg) for 28 days; some mice were given 80 mg/kg genistein plus the anti-estrogen ICI 182,780 (5 mg/kg/week). Cell-mediated immune response was tested by analyzing the delayed-hypersensitivity (DTH) response to a hapten, 4-hydroxy-3-nitrophenyl acetyl succinimide (NP-O-SU), at the end of treatment. Reversibility of the effects of genistein was tested by measuring the DTH response in mice that were given genistein (20 or 80 mg/kg) for 28 days, then allowed to recover for 28 days. To determine if dietary genistein could affect cell-mediated immunity, mice ovariectomized as above were fed genistein at 0, 1000 or 1500 parts per million (ppm) for 28 days. There was a 46–67% decrease in the DTH response in the footpads of mice injected with 8–80 mg/kg genistein compared with controls (P<0.05 vs control for all treatment groups); these effects were reversible. On histopathological examination of the feet, there was decreased cell infiltration in genistein-treated animals compared with controls, and the numbers of CD4+ and CD8+ T cells in popliteal lymph nodes were reduced. The effects of genistein are mediated through both estrogen receptor (ER) and non–ER pathways, as the anti–estrogen ICI 182,780 only partially blocked the effects of genistein on the DTH response. Dietary genistein (1000 or 1500 ppm) decreased cell-mediated immunity while producing serum genistein concentrations in the physiological range for humans under certain nutritional conditions. Further work is needed to determine if dietary genistein and phytoestrogen exposure can produce effects on cell–mediated immunity in humans or other animals under various nutritional conditions. Journal of Endocrinology (2003) 176, 267–274

Introduction

Genistein is an isoflavone present in high quantities in soy. Like other phytoestrogens, genistein binds to the classical estrogen receptor (ER), ERα, and produces increases in parameters such as uterine weight (reviewed in Whitten & Patisaul 2001). In addition, genistein binds to the recently discovered second estrogen receptor, ERβ, with higher affinity than for ERα, which has led to the suggestion that genistein signaling through ERβ may be important for its biological actions (Kuiper et al. 1997, 1998).

Soy and products derived from soy, such as soy protein and isoflavone supplements, are being consumed in increasing quantities by humans. Similarly, high quantities are used as a feed ingredient for laboratory, companion and food animals (Brown & Setchell 2001, Court & Freeman 2002). Soy consumption has been suggested to have a variety of human health benefits based on laboratory and epidemiological evidence (reviewed in Patisaul & Whitten 1999). Many of these potential health benefits may be due to the soy isoflavones genistein and daidzein (reviewed in Adlercreutz 1998, Patisaul & Whitten 1999). However, there has been concern that the estrogenic effects of these compounds might have deleterious effects in some situations. For example, consumption of high levels of soy by captive cheetahs was linked to infertility
and a veno-occlusive disease in these animals (Setchell et al. 1987), and Sharpe et al. (2002) have recently shown that consumption of soy formula by neonatal marmosets led to decreases in serum testosterone. The high levels and ubiquity of soy consumption in certain human and animal populations emphasize the need to more fully understand the actions of the isoflavones.

We have previously shown that injected and dietary genistein administered to mice can produce decreases in thymic weight of up to 80%; dietary genistein also produced thymic atrophy (Yellayi et al. 2002). In addition, animals injected with genistein had decreased overall numbers of thymocytes. These thymic changes were accompanied by decreases in relative percentages of certain T cells in the spleen and a systemic lymphocytopenia.

The adaptive immune system involves both humoral and cell-mediated immunity. Humoral immunity results from the coordinated actions of CD4+ T cells and B cells, and injected genistein produced a dose-dependent suppression of humoral immunity (Yellayi et al. 2002). In contrast, cell-mediated immunity involves CD4+ and CD8+ T cells, which are both reduced in the thymus by genistein injection (Yellayi et al. 2002). These cell types, as well as macrophages, express estrogen receptor (Cohen et al. 1983, Gulshan et al. 1990, Suenaga et al. 1998), and cell-mediated immunity is decreased by estrogen in mice (Holmdahl & Jansson 1988, Salem et al. 2000) and women (Hoek et al. 1995), raising the possibility that genistein could have similar effects. The objective of the present study was to determine whether genistein had effects on cell-mediated immunity. Our results show that injected and dietary genistein suppress cell-mediated immunity, and dietary genistein does so even when fed in amounts that produce serum genistein levels commonly obtained in both humans and animals.

Materials and Methods

Animals

Female C57BL/6 mice were obtained from Harlan (Indianapolis, IN, USA) or produced in our colony using parental stock purchased from Harlan. Teklad rodent Chow and tap water were available ad libitum. All animals were housed under controlled lighting (12 h light, 12 h darkness) and temperature (21–22 °C) conditions and maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Illinois.

Animal treatments

Mice (25– to 27-days-old) were ovariectomized one week before initiation of genistein treatment to minimize circulating 17β-estradiol (E2) levels. They were fed a casein-based phytoestrogen-free diet (AIN-93 G), available ad libitum, starting 2 days before injections began. Mice (32–34-days-old when injected) were given one subcutaneous injection/day for 28 days of 0.02 ml dimethylsulfoxide (DMSO) (control) or genistein (Indofine Chemicals, Somerville, NJ, USA) at 8, 20 or 80 mg/kg/day. Some animals were also given genistein (80 mg/kg/day) in combination with the anti-estrogen ICI 182,780 (AstraZeneca, Macclesfield, Cheshire, UK; 5 mg/week/animal). We have previously shown that this dose of ICI 182,780 totally blocks the uterotropic effects of the genistein dosage administered here (Yellayi et al. 2002).

To determine the effects of dietary genistein on cell-mediated immunity, we fed 32–34-day-old, ovariectomized mice for 28 days with the phytoestrogen-free AIN-93 G diet (control), available ad libitum, or this diet supplemented with 1000 or 1500 parts per million (ppm) genistein.

Cell-mediated immune responses

Cell-mediated immunity was tested by analyzing the delayed-type hypersensitivity (DTH) response to a hapten, 4-hydroxy-3-nitrophenyl acetate succinimide (NP-O-SU), at the end of the genistein treatment period. Animals were primed by injecting a sensitizing dose of 50 µl NP-O-SU (7 g/100 ml of DMSO) in the flank on day 21 of genistein treatment. Six days after sensitization, mice were challenged by injecting 25 µl NP-O-SU (2 g/100 ml of DMSO) into the right footpad, while a control solution of DMSO and PBS vehicles was injected into the left footpad. One day later, the thickness of left and right footpads was measured in triplicate using a peacock dial gauge. The injection of the second dose of hapten induced a pronounced inflammatory response in the right foot, while the left foot did not show this response and served as the control. The results are expressed as the difference in footpad thickness between the right and left feet by subtracting left footpad thickness from that of the right. If a treatment decreases cell-mediated immunity, the difference between the right and left feet in these animals will be reduced compared with controls, while an increase in the difference between right and left feet would indicate increased cell-mediated immunity. Following the footpad measurements, animals were killed and both footpads were processed for histopathology.

Popliteal lymph nodes, the draining lymph nodes of the footpad, were collected from the hapten-injected limb of the animals that were injected with 80 mg/kg genistein or the vehicle control. The popliteal nodes were also collected from animals given dietary genistein at 0, 1000 or 1500 ppm. A single cell suspension was made for flow cytometric analysis of CD4+ and CD8+ T cells. Direct immunofluorescence was used to analyze lymphocyte subpopulations in these nodes using FITC-conjugated anti-mouse CD4 (L3T4 Pharmingen, San Diego, CA, USA) monoclonal antibody and phycoerythrin-conjugated anti-mouse CD8 (L3T8 Pharmingen, San Diego, CA, USA) monoclonal antibody.
anti–mouse CD8 (LY–2, Pharmingen) monoclonal antibody, as we have described (Yellayi et al. 2002).

Reversibility of the effects of genistein on cell-mediated immune responses

The reversibility of the effects of genistein on cell-mediated immune responses was determined by measuring the DTH response in mice that had been given genistein treatment followed by a recovery period without genistein exposure. Mice were injected with DMSO or genistein (20 or 80 mg/kg body weight) for 28 days. All groups were then allowed to recover for an additional 28 days, then cell-mediated immunity was measured. All mice were sensitized by s.c. injection of 0·05 ml NP-O-SU, then footpad thickness in both feet was determined 24 h later, as above.

Statistical analysis

Results were expressed as means ± S.E.M., and the *P values for the various groups are given in the figure legends. Data were analyzed by one-way ANOVA followed by the Student–Newman–Keuls Multiple Comparisons test, and differences were considered significant at *P<0·05. In experiments involving only a control and a treated group, results were compared by Student’s *t*-test.

Results

Genistein injections (8–80 mg/kg) in mice produced 46–67% decreases in footpad thickness compared with controls (Fig. 1). In mice treated with genistein (80 mg/kg/day)+ICI 182,780, the anti-estrogen partially reversed the genistein effects such that differences in footpad thickness were greater than in mice treated with genistein alone. However, differences in mean footpad thickness were not restored to normal in mice given genistein+ICI 182,780, indicating that ICI 182,780 only partially blocked genistein’s effects on the DTH response. In contrast, uterine weights in the genistein (80 mg/kg/day)+ICI 182,780 group were totally inhibited and not different from those of control mice that did not receive genistein (not shown).

The observed effects of genistein on footpad thickness were corroborated by histopathological analysis of feet from the various groups (Fig. 2). Extensive cell infiltration was seen in right feet from control animals, typical of a robust DTH response (Fig. 2A). Cell infiltration was markedly reduced in right feet from mice treated with genistein (80 mg/kg), while the anti-estrogen ICI 182,780 partially reversed this genistein effect (Fig. 2B and C respectively).

Dietary genistein treatment for 28 days resulted in a diminished DTH response compared with control animals that had received only the phytoestrogen-free diet without genistein supplementation (Fig. 3). Although the overall diminution in the DTH response was not as pronounced as those seen following injections of high levels of genistein, the overall DTH response was decreased almost 50% in mice given 1000 or 1500 ppm of genistein.

The numbers of both CD4+ (Fig. 4a) and CD8+ (Fig. 4b) T cells in the popliteal lymph nodes of mice fed genistein at either 1000 or 1500 ppm for 28 days were reduced approximately 50% compared with those in control mice. Numbers of CD4+ and CD8+ T cells in the popliteal lymph nodes of mice injected with 80 mg/kg genistein were decreased approximately 75% for both cell types compared with the vehicle control (data not shown). Thus, in addition to the attenuated DTH response following genistein treatment, lymphocyte populations in the popliteal lymph nodes of these animals were decreased.

The genistein effects on cell-mediated immunity were rapidly reversed following cessation of this treatment (Fig. 5). Twenty-eight days after termination of genistein treatment, cell-mediated immunity had recovered even in mice given a high dose (80 mg/kg/day) of genistein.

Discussion

Our results show that injected genistein reduces the DTH response, indicating decreased cell-mediated immunity in
these animals. This decrease is similar to the decrease in humoral immunity that we recently reported following genistein injections (Yellayi et al. 2002), showing that genistein has suppressive effects on both the cell-mediated and humoral components of the adaptive immune system. It is well known that E2 suppresses cell-mediated immunity in mice and women (Holmdahl & Jansson 1988, Hoek et al. 1995, Salem et al. 2000). The present results are the first data indicating that phytoestrogens such as genistein can produce similar decreases, although the 85% decreases in the DTH response seen with E2 (Salem et al. 2000) were more pronounced than those observed here.

Our previous work demonstrated that dietary genistein decreased thymic weight but effects on immune function were not examined. Our present findings indicate that in addition to decreasing thymic weight, dietary genistein is capable of decreasing cell-mediated immunity. These results are consistent with the recent report that either a high-soy diet or intravenous genistein could delay the rejection of rat cardiac allografts, which also indicates that genistein can have immunosuppressive properties in vivo (O’Connor et al. 2002).

Genistein binds to both ERα and ERβ, and estrogenic effects of genistein have been reported on reproductive and other organs (Kuiper et al. 1998, Whitten & Patisaul 2001). The thymus expresses both forms of ER, and ERα has been shown to be involved in thymic development (Kuiper et al. 1997, Yellayi et al. 2000, Erlandsson et al. 2001). Our previous results showing that genistein reduces the number of developing CD4+ and CD8+ thymocytes suggests that this could be one mechanism for genistein effects on cell-mediated immunity (Yellayi et al. 2002). In addition, peripheral CD4+ and CD8+ T cells and macrophages all express ER (Cohen et al. 1983, Gulshan et al. 1990, Suenaga et al. 1998), and estrogen has been shown to modulate the activities of these cell types (Ansar Ahmed et al. 1985, Dean et al. 1986, Cutolo et al. 1995).

Treatment of mice with the anti-estrogen ICI 182,780 did not completely restore the genistein-induced decreases seen in the DTH response. We have previously examined the effects of genistein on thymic atrophy, adipose deposition and uterine weight in mice (A Naaz & P S Cooke, unpublished data; Yellayi et al. 2002). Dietary genistein produces decreases in thymic weight at 1000 ppm or above (Yellayi et al. 2002), but does not produce thymic effects at 500 ppm or lower (A Naaz & P S Cooke, unpublished data). Genistein produces effects on adipose tissue at 500 ppm or above, while uterotrophic effects of
Genistein are seen at 300 ppm or above. Similar results have been seen with injected genistein, where a 2 mg/kg/day dose produces uterotrophic effects even though no thymic effects are seen with this dose (Yellayi et al. 2002). Thus, the uterotropic effect of genistein is the most sensitive indicator of estrogenic genistein actions. Based on the ability of ICI 182,780 to block the typical uterine weight increase induced by genistein, we concluded that the ICI 182,780 completely inhibits ER-mediated effects, and that effects on cell-mediated immunity seen here even in the presence of ICI 182,780 were not mediated through ER.

Genistein inhibits protein tyrosine kinases, topoisomerase II and certain other cellular processes through non-ER-mediated mechanisms (reviewed in Adlercreutz 1998, Patisaul & Whitten 1999); these non-ER-mediated pathways appear to contribute to the genistein effects on cell-mediated immunity. The incomplete blockage of the inhibitory effects of genistein on cell-mediated immunity by ICI 182,780 is similar to that seen with genistein effects on thymic atrophy (Yellayi et al. 2002), indicating that the effects of genistein on both processes involve both ER-mediated and non-ER-mediated components. Consistent with this finding, Guo et al. (2002) have recently shown that the thymic changes resulting from feeding genistein to rats during gestation and lactation were not entirely the result of estrogenic effects. Therefore, the ability of genistein to induce thymic and immune effects appears to involve non-ER-mediated components in both rats and mice.

Previous results have indicated that E2 treatment of adult mice decreases leukocyte numbers in the draining lymph nodes from the injected limb in the DTH response (Salem et al. 2000). The measurements of CD4+ and CD8+ T cell numbers in the popliteal lymph nodes of control and genistein-treated mice indicate that both of these cell types are significantly reduced following genistein treatment. The decreased T cell numbers may be a critical aspect of the mechanism by which genistein produces decreased inflammation and the attenuated DTH response, although other changes (e.g. decreased T cell function) could be involved. The decreased lymph node

**Figure 4** Effect of genistein on CD4+ and CD8+ T cells numbers in the popliteal lymph nodes. Numbers of (a) CD4+ and (b) CD8+ T cells in the popliteal lymph nodes of the right limb of mice fed for 28 days with the phytoestrogen-free diet (control), or this diet supplemented with genistein. When genistein was given at either 1000 and 1500 ppm, there were significant (*) decreases in both CD4+ and CD8+ T cell numbers compared with the control, but the genistein groups were not significantly different from each other (P<0.05). Data are means ± S.E.M. and are expressed as a percentage of control; n=4–5 for all points. The lymph node data shown in this figure was obtained from the same mice used for the DTH results shown in Fig. 3.

**Figure 5** Recovery of the DTH response following cessation of genistein treatment. Ovariectomized mice (32- to 34-days-old at initiation of treatment) were given 28 daily subcutaneous injections of DMSO or genistein (20 or 80 mg/kg). They were then allowed to recover for 28 days. Mice were sensitized on day 21 of recovery with NP-O-SU, then challenged 6 days later with NP-O-SU, and footpad thickness was measured one day later, which was day 28 of recovery. Data are means ± S.E.M. and are expressed as a percentage of control; n=6–8 for all points. The control and 20 mg/kg genistein groups were not significantly different, but the mean footpad thickness in the 80 mg/kg genistein group was significantly greater than the control (P<0.05).
Genistein and cell-mediated immunity

T cell populations reported here are consistent with the decreased thymocyte numbers and T cell numbers in the thymus and spleen respectively following genistein treatment (Yellayi et al. 2002).

Genistein injected at doses of 20 or 80 mg/kg/day for 21 days causes decreases in thymic weight of 40% and 70% respectively (Yellayi et al. 2002), and impairs cell-mediated and humoral immunity. Despite the marked genistein effects on thymic size and immune function, cell-mediated immunity recovers to normal when these treatments are stopped and the mice recover for 28 days. Thymic weight also recovers to normal (data not shown). Thus, thymic and immune changes induced by adult exposure to genistein are reversible. Perinatal estrogen treatments are stopped and the mice recover for 28 days. These levels exceed serum genistein levels in Japanese men (mean=0.38 µM) whose diets have historically included soy products (Adlercreutz 1998). However, consumption of one meal containing soymilk or soy/isoflavone supplements by humans produces peak serum genistein concentrations of up to 5 µM (Xu et al. 1995, Djuric et al. 1999), and human infants fed soy-based formula have serum genistein concentrations ranging from 1.5–4.4 µM (Setchell et al. 1997). Serum genistein concentrations in mice fed 1000–1500 ppm genistein exceed those in human populations eating high-soy diets, but there are a number of situations where humans have serum genistein concentrations comparable to those reported here for mice fed genistein.

The ability of serum genistein levels obtained in humans to induce changes in cell-mediated immunity in mice suggests the possibility of potential risk to humans under certain nutritional conditions. However, it must be emphasized that a critical question that remains to be answered is whether these results are relevant for humans or animals consuming high levels of isoflavones. The complexity of this question is clearly illustrated by recent work of Guo and colleagues which showed that rat dams exposed to genistein beginning early in gestation had decreased thymic weight on day 22 postpartum after exposure to 800 ppm genistein (Guo et al. 2002b) but there was no effect on thymic weight on postpartum day 51 in a subsequent study even when dams were exposed to up to 1250 ppm genistein (Guo et al. 2002a). In addition, there were variations in the responses of the dams vs the pups, and male vs female pups (Guo et al. 2002a). The results indicate that the effects of genistein on immune parameters can vary depending on the species, length of exposure, and the age and sex of the animal. Thus, extrapolation of results from one species to others, and especially potential extrapolation of rodent studies to humans, must be approached with the greatest caution.

Present literature on a possible link between high levels of soy consumption and immune impairments is contradictory. There are previous reports that T cell function, gamma globulin and immunoglobulins were decreased in infants fed soy formula compared with cow milk formula-fed controls (Zoppi et al. 1979, 1982). Soy-fed infants had reduced titers of antibodies against polio, tetanus, diphtheria and pertussis and increased morbidity compared with infants fed cow milk formula (Zoppi et al. 1982, 1983). In contrast, two recent companion papers did not confirm the extensive immune abnormalities described in the initial studies and reported that a number of parameters related to immune cell populations, vaccine response and morbidity were within the normal range in soy-fed infants (Cordle et al. 2002, Ostrom et al. 2002). There were some differences in soy preparations, experimental methodologies and endpoints in these two groups of studies, but an explanation for the diametrically opposite conclusions obtained by the two groups is not obvious.

Therefore, a firm conclusion regarding immune effects of soy formula on human infants is difficult to reach. Even if subsequent work demonstrates conclusively that soy formula does not impair average immune function in large groups of infants, the possibility of immune effects in certain subsets of infants must still be considered. Serum isoflavone concentrations in soy–fed infants show about a threefold range, indicating that some infants may digest and/or absorb isoflavones more efficiently than others, or may metabolize these compounds more slowly (Setchell et al. 1997). In addition, only about 30–40% of humans are able to metabolize daidzein to the more potent estrogen equol (Adlercreutz 1998), and we have recently shown that equol injected for 7 days at 20 mg/kg produces a 25% decrease in thymic weight (P S Cooke, unpublished data). We have shown that female mice are more responsive than males to the thymic effects of genistein (Yellayi et al. 2002), and peak serum genistein concentrations are higher in females compared with males when both are given the same amount of genistein (Doerge in females compared with males when both are given the same amount of genistein (2002), and peak serum genistein concentrations are higher in females compared with males when both are given the same amount of genistein (Doerge et al. 2002), suggesting that female infants might be more susceptible to immune effects of genistein than male infants. Therefore, subsets of infants with greater isoflavone responsiveness, higher isoflavone concentrations and/or those who make equol may be more susceptible to thymic/immune effects of isoflavones and potentially show immune effects even if a suppression of average immune function in large populations of soy–fed infants is not seen.

Most commercial rodent diets contain high amounts of soy and isoflavones (Boettger–Tong et al. 1998, Thigpen et al. 1999), and recent work by Brown and Setchell (2001) reported up to 830 µg/kg total isoflavones in widely-used commercial chows. In addition, rodents fed soy–based diets have circulating isoflavone levels of up to 8·5 µM (Brown & Setchell 2001), which equals or exceeds those reported to cause thymic and immune suppression in mice in our studies. Although the majority of the plasma isoflavone was equol rather than genistein in these animals, our preliminary results show that injected equol is similar to genistein in its ability to induce thymic atrophy. Thus, feeding mice normal commercial diets containing high amounts of soy could potentially have immune effects, and this needs further investigation.

Some commercial dog and cat diets contain high levels of soy isoflavones, and food animals such as swine are fed high levels of soy, beginning at weaning (Cook 1998, Court & Freeman 2002). Total isoflavone intake of cats consuming these diets can be up to 4·5 mg/kg/day (Court & Freeman 2002), in the range of isoflavone consumption by human infants fed soy–based formula (Setchell et al. 1997). Cats have a significantly reduced capacity to metabolize and excrete compounds such as isoflavones by glucuronidation (Court & Greenblatt 2000) and infertility and a veno-occlusive disease have been reported in another felid, the cheetah, fed a high-soy diet (Setchell et al. 1987). Serum concentrations of isoflavones such as genistein have not been reported in cats fed commercial cat foods, but the combination of their high isoflavone consumption and decreased capacity to metabolize and excrete these compounds indicates they may reach serum genistein levels above those shown here to produce decreased cell-mediated immunity in mice, although whether this would produce immune effects in cats or other species consuming high levels of soy remains to be investigated.

In summary, injected and dietary genistein can impair cell–mediated immunity in mice. The relevance of these findings to humans and laboratory, companion and food animals is uncertain, but the serum genistein concentrations that produce these effects in mice can be obtained in humans and animals under various nutritional conditions.

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