Genistein Enhancement of Respiratory Allergen Trimellitic Anhydride-induced IgE Production by Adult B6C3F1 Mice Following In Utero and Postnatal Exposure

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INTRODUCTION

Genistein (GEN), a major isoflavone in most soy products, has been demonstrated to interact with estrogen receptors in vivo (Martin et al., 1978). Despite the hypothesized beneficial effects of GEN (e.g., decreased incidences of some hormone-related cancers), there are concerns about the potential long-term effects of this compound on human health, especially that of infants and young children. Infants fed soy milk formulas have plasma isoflavone levels that are orders of magnitude higher than those of infants fed human or cows’ milk (Setchell et al., 1997). The possible long-term effects of these relatively high levels of phytoestrogens during infancy are unknown. A retrospective multiple controlled cohort study has indicated that there was an increase in the use of asthma or allergy drugs in young adults who had been fed soy formula during infancy as compared to those who were fed cow milk formula from the age of less than 9 days old when both groups were healthy term infants whose mother elected not to breastfeed (Strom et al., 2001). Additionally, phytoestrogens have been detected in amniotic fluid (Doerge et al., 2001), suggesting that in utero exposure also occurs.

The prevalence of asthma has doubled in the past two decades (Robinson et al., 2004). One important mechanism for the development of asthma is that allergens repeatedly stimulate T helper (Th) 2-polarized T-cell immunity and change the immune response to a state of hypersensitivity (Cohn et al., 2004). Both natural killer (NK) and T cells have been demonstrated to contribute significantly to the disease’s persistence and progression in asthma and allergy (Cohn et al., 2004; Korsgren et al., 1999). In our previous studies, we have provided evidence that both the functions of T cells and NK cells were altered after oral exposure to GEN at physiologically relevant concentrations in experimental animals. Specifically, exposure to GEN in adult female B6C3F1 mice for 28 days by gavage increased the activities of cytotoxic T cells (CTLs) and NK cells (Guo et al., 2001). Additionally, increased splenic T cell number was observed in male and female Sprague-Dawley rats when the rats were exposed to GEN gestationally and lactationally by feeding the dams with GEN-containing diet, and via the diet from postnatal day (PND) 22 to PND 64 (Guo et al., 2002a).

Although there is considerable, and as yet unresolved, debate regarding the mechanisms by which chemicals may cause respiratory sensitization and occupational asthma, the measurement of total serum IgE levels is still recognized to be one of the best methodologies for identifying respiratory sensitizers (Lanier et al., 2003). We have validated the mouse IgE test...
model using B6C3F1 mice (Guo et al., 2002b). In addition, the expression of cytokines (e.g., IL-4 and IL-13) and CD86 surface marker by the draining lymph node cells has been analyzed to determine the underlying molecular mechanisms (Guo et al., 2002b). In the present study, a modified mouse IgE test using trimellitic anhydride (TMA) as the allergen was employed to determine if developmental exposure to GEN affected the production of IgE in adult B6C3F1 mice. Trimellitic anhydride is a strong respiratory sensitizer and a known cause of occupational asthma in humans (Zeiss et al., 1977). Occupational asthma has been proposed to be a good model for allergic asthma in the general population because the underlying mechanisms are similar (Mapp et al., 1999).

It was hypothesized that exposure to GEN during a sensitive developmental period, e.g. in utero exposure, would modulate the activation of T cells and NK cells and the production of Th1/Th2 cytokines, and thus lead to an increase in the IgE response to the respiratory allergen TMA in adult life. In this study, we have evaluated the effects of developmental GEN exposure on total serum IgE production in response to TMA stimulation in the F1 generation (B6C3F1) of female C57BL/6 × male C3H mice. The B6C3F1 mouse is the mouse strain of choice in conducting immunological studies, and it is the designated mouse strain of the National Toxicology Program (NTP). A large database exists on this strain, and the major components of the immune system in the mouse and human are the same; many agents that perturb the immune system in human perturb the immune system in the mouse in a similar manner. Moreover, the B6C3F1 mice are preferred over randomly bred mice because their use decreases the variation between individual animals’ response and reduces the number of animals needed for each experiment. The results demonstrated that GEN modulates the developing immune system in such a way that more IgE was produced upon exposure to TMA in adult mice.

**MATERIALS AND METHODS**

**Animals and treatments.** Both female C57BL/6 and male C3H mice (8–12 weeks old) were obtained from Charles River Breeding Laboratories (Raleigh, NC). Timed pregnant primiparous C57BL/6 mice (×male C3H mice) were generated through housing two female C57BL/6 mice and one male C3H mouse in one cage (plug date = gestational day 0). Pregnant mice were housed individually in standard plastic cages with hardwood chip bedding, and the animal room was maintained within a temperature range of 22–25°C and relative humidity of 50 ± 20 with 12-h light cycles (700–1900).

In the feeding studies, pregnant mice were randomized into two groups initially on GD 14 based on the body weight, and were provided with either the standard NIH-07 rodent diet or the GEN-containing feed or control feed. At this time, the offspring were housed up to four same-sex littermates per cage. To eliminate litter effect, one mouse from each litter for each sex was selected for the evaluation.

The diet in a powdered form (5K06, purchased from Purina Mills, St. Louis, MO) is based on the NIH-31 formula, except that casein replaces the protein contributed by soy and alfalfa, soy oil is replaced by corn oil, and the vitamin mix is adjusted for irradiation. The control diet was assayed for genistein and daidzein after hydrolysis of conjugates. The concentrations of both genistein and daidzein of this diet were determined by LC-ES/MS/MS to be approximately 0.5 ppm (Doerge et al., 2000). GEN (Toronto Research Chemicals, North York, Ontario, Canada) with purity greater than 99% was mixed into the standard 5K06 feed every 3 months by the Diet Preparation Staff, Bionetics at the National Center for Toxicological Research (NCTR, Jefferson, AR). Each batch of feed was analyzed by the Division of Chemistry, and it was stable for at least 6 months when stored refrigerated.

In the gavage studies, the mice consumed Harlan Teklad Laboratory Diets (NII-07; Madison, WI) and tap water from water bottles ad libitum. The diet contained crude protein (22.5%), crude fats (4.5%), and crude fiber (4.5%). The ingredients in the NIH-07 open formula rodent diet were as follows: dried skim milk 50 g/kg, fish meal 100 g/kg, soybean meal 120 g/kg, alfalfa meal 40 g/kg, corn meal 30 g/kg, ground #2 yellow shelled corn 235 g/kg, ground hard winter wheat 230 g/kg, wheat middlings 100 g/kg, dried brewers yeast 20 g/kg, dried molasses 15 g/kg, soybean oil 25 g/kg, salt mix 30 mg/kg, and vitamin mix 5 g/kg (Report of the American Institute of Nutrition Ad Hoc Committee on Standards for Nutritional Studies, 1977). Brown and Setchell (2001) found the dietary concentration of isoflavones in the NIH-07 diet to be approximately 33 ppm. GEN solutions were prepared fresh daily in 25 mM Na2CO3 at a concentration of 2 mg/ml (Guo et al., 2001). Mice were administered the GEN solution or the vehicle by gavage (0.1 ml/10 g body weight) via an 18 G gavage needle.

Mice were shaved on both flanks prior to dermal treatment with TMA, and any animals with broken skin were excluded. Sensitization to TMA (Aldrich-Sigma) was achieved by treating individual mice with 50 μl of 10% TMA on the shaved back in acetone:olive oil (4:1) 14 days prior to sacrifice. Seven days after sensitization, the mice were challenged with 25 μl of TMA (10%) on the dorsal side of both ears. On PND 42 and PND 84, mice were bled by cardiac puncture under carbon dioxide anesthesia and serum was collected. The spleens were obtained for immunological evaluations. All animal procedures were conducted under an animal protocol approved by the Virginia Commonwealth University (VCU) Institutional Animal Care and Use Committee (IACUC).

**Determination the numbers of splenocytes.** The quantification of immune cells was performed as previously described (Guo et al., 2001). Briefly, splenocytes were prepared by washing the spleens between two slides with frosted ends. After washing, the cells were resuspended in RPMI (Roswell Park Memorial Institute) complete medium and counted using a Coulter Counter ZII, with the red blood cells lysed with a ZAP-OGLOBIN II lytic reagent (Coulter Corporation, Miami, Florida).

**Flow cytometric analysis of splenocytes.** To determine the percentages of splenocyte subsets, the respective cell types were labeled with an appropriate monoclonal antibody (mAb), conjugated with a fluorescent molecule for visualization as previously described (Guo et al., 2001). All the antibodies were obtained from BD Pharmingen (San Diego, CA). These included a phycoerythrin (PE) conjugated mAb specific for the CD4 cell surface protein and a fluorescein isothiocyanate (FITC)-conjugated mAb specific for the CD8 marker. Additionally, FITC anti-mouse IgM and PE anti-mouse CD3 were also used to label splenocytes. Isotype-matched irrelevant antibodies were used as controls. Following the addition of the reagents, the cells were incubated at 4°C in the dark for at least 30 min. After incubation, the cells were washed 2×, and propidium iodide (PI) was added as a viability stain. After incubation with PI for 5 min, the cells were washed and enumeration was performed on a Becton Dickinson FACScan Flow Cytometer. Nonviable cells were eliminated through setting a live gate excluding red fluorescence emerging from PI; a forward scatter threshold was set high enough to eliminate red blood cells. For each sample, 5,000 PI negative events were counted.
Natural killer cell activity. Our previous study suggested that GEN affected IL-2–augmented NK cell activity (Guo et al., 2001); thus, similar assays were performed in this study. Briefly, single cell suspensions were adjusted to six concentrations: $2 \times 10^3$, $1 \times 10^3$, $5 \times 10^3$, $2.5 \times 10^3$, $1.25 \times 10^4$, and $0.625 \times 10^4$ cells/ml in a 96-well U-bottom plate with two replicate wells (0.1 ml/well) for each concentration. Recombinant IL-2 (Chiron, Emeryville, CA) at a volume of 50 μl was added to each well so that the final concentration of IL-2 was 5000 IU/ml. The plates were cultured overnight at 37°C in 5% CO2 and then assayed for NK cell activity using $^{51}$Cr-labeled YAC-1 cells as the target cells. The $^{51}$Cr–YAC-1 cells were added to each well of a 96-well plate in a volume of 50 μl to obtain effector: target (E:T) ratios of 200:1, 100:1, 50:1, 25:1, 12.5:1, and 6.25:1. The spontaneous release and the maximum release were determined by adding 0.1 ml of medium and Triton X-100 (0.1%) to each of 12 replicate wells containing the target cells, respectively. After a 4-h incubation period, the plates were centrifuged, and 0.1 ml of the supernatant was removed from each well and the radioactivity counted. The mean percentage of cytotoxicity at each effector concentration was determined.

Anti-CD3 antibody-mediated spleen T-cell proliferation. The proliferation of splenocytes in the presence of anti-CD3 antibody was performed as described elsewhere (Guo et al., 2001). Briefly, a single spleen cell suspension was prepared and resuspended in RPMI medium supplemented with fetal bovine serum (FBS, 10%; Hyclone Laboratories, Logan, UT), sodium bicarbonate (GIBCO), HEPES (GIBCO BRL, Grand Island, NY), L-glutamine, gentamicin, and 2-mercaptoethanol (0.00035%). The splenocytes ($2 \times 10^6$/well) were cultured in microtiter wells coated with anti-CD3 antibody (1 μg/ml; PharMingen), or in wells without antibody coating, at 37°C in 5% CO2. Prior to harvest on day 3, the cells were pulsed with $^3$H-thymidine for 18–24 h. The incorporation of $^3$H-thymidine into the proliferating cells was used as the endpoint of the assay, and the data were expressed as CPM/2 x $10^5$ cells.

Measurement of cytokine and serum Ig levels by ELISA. ELISAs for IgE, IFN-γ, IL-2, IL-4, IL-5, TGF-β, and TNF-α were performed according to the manufacturer’s instructions (Pharmingen, San Diego, CA). Briefly, 100 μl of diluted capture antibody was added to each well in a 96-well plate (MAXisorb) and allowed to adhere overnight at 4°C. Plates were washed, then blocked in 10% FBS-PBS for 1 h at room temperature. After washing, serial dilutions of the standard and samples were prepared in the plates and then allowed to adhere for 2 h at room temperature. After washing, 100 μl of working solution including detector antibody and avidin-HRP reagent was added to each well and then incubated for 1 h at room temperature. The detector antibodies were biotinylated anti-mouse IgE, IFN-γ IL-2, IL-4, IL-5, TGF-β, and TNF-α monoclonal antibodies. After washing, 100 μl of tetramethylbenzidine substrate solution was added to each well. After incubation in the dark for 30 min at room temperature, the absorbance was read at 650 nm. For IL-13, the ELISA was performed according to the manufacturer’s instructions (R&D Systems Inc, Minneapolis, MN). For the IgM ELISA, goat anti-mouse IgM was used for capture, and goat anti-mouse IgM-HRP was used for detection (Southern Biotechnology Associates, Birmingham, AL). For the IgG ELISA, rabbit anti-mouse IgG (ICN Biomedicals, Inc., Aurora, OH) was used for capture, and goat anti-mouse IgG-HRP (Biorad Laboratories, Hercules, CA) was used for detection. The substrate used for the IgM and IgG ELISAs was 2,2′-azinobis (3-ethylbenzothiazoline 6-sulfonate (ABTS; Sigma, St Louis, MO). One ABST tablet (10 mg) was dissolved in 50 ml phosphate citrate with urea hydrogen peroxide, pH 5.0.

Culture of draining lymph node cells. For cell culture, bilateral draining lymph nodes (auricular) were removed from mice in individual groups. These draining lymph nodes were mashed and suspended in RPMI medium supplemented with 10% heat-inactivated FBS, 25 mM HEPES, 0.2% sodium bicarbonate, and gentamicin. Lymph node cells ($5.5 \pm 10^6$ cells/2 ml per well) were cultured in a 24-well plate in the presence of concanavalin A (Con A, 2 μg/ml; Sigma) overnight at 37°C in a humidified atmosphere of 5% CO2. Supernatants were collected for the measurement of cytokine levels using ELISA.

Statistical analysis. Data are expressed as means ± standard error of means. Results were tested for variance homogeneity using Bartlett’s test. Homogeneous data were analyzed using a one-way analysis of variance, and all the data reported were homogeneous; when significant, Dunnett’s t-test was used to determine differences between the experimental and vehicle control groups. Experiments were performed multiple times, and p values of 0.05 or less were considered statistically significant.

RESULTS

Developmental GEN Exposure on IgE Production by Adult B6C3F1 Mice (PND84)

The feeding studies were initially employed to determine if developmental exposure to GEN altered the production of total serum IgE levels in response to TMA treatment in adult B6C3F1 mice. As shown in Figure 1, a significant increase in total serum IgE level was observed in male mice when mice were exposed to GEN in utero from GD 14 to the time of birth (Fig. 1A). Exposure to GEN from GD 14 to PND 84 did not affect the production of total serum IgE in response to TMA treatment when compared to the controls (Fig. 1A). In female mice, a relatively small but significant increase in serum IgE level was observed when the animals were exposed to GEN from GD 14 to PND 84 (Fig. 1B). No significant changes in total serum IgE levels in response to TMA treatment were observed in female mice when the animals were exposed to GEN in utero only (Fig. 1B).

To further confirm if in utero exposure to GEN affected IgE production by adult B6C3F1 mice, animals were exposed to GEN (20 mg/kg) from GD 14 to the time of birth by gavaging the dams as described. In response to TMA treatment, a significant increase in total serum IgE level was observed in female mice (Fig. 1D) but not in male mice (Fig. 1C) at PND 84. When these mice received TMA challenge on the ears but not TMA sensitization on the back, three times less serum IgE was produced, and in utero exposure to GEN did not affect the IgE level (data not shown).

In Utero Exposure to GEN Had No Effect on Total Serum IgM and IgG Production by Adult B6C3F1 Mice (PND 84)

To determine if in utero exposure to GEN had any effects on IgM and IgG production by adult male and female B6C3F1 mice, animals were exposed to GEN by feeding the dams at 500 ppm or by gavaging the dams at 20 mg/kg from GD 14 to parturition, and then treated with TMA on the back at PND 70 and on the ears at PND 77, respectively. The levels of total serum IgM and IgG were measured at PND 84. No significant alterations in total serum IgM and IgG levels were observed in the GEN treatment groups when compared to the corresponding controls (data not shown).

Effect of Developmental GEN Exposure on the Body Weight and Organ Weights in Adult B6C3F1 Mice (PND 84)

In utero exposure to GEN either by feeding or by gavage did not affect the terminal body weight (Table 1 and 2). However,
a decrease in the terminal body weight was observed in both male and female mice when the animals were exposed to GEN by feeding from GD 14 to PND 84 (Table 1). In addition, no significant alteration in spleen weights (both absolute and relative weights) was observed in any of the GEN treatment groups when compared to the control (Table 1 and Table 2).

**Effect of Developmental GEN Exposure on T-Cell Activation by Adult B6C3F1 Mice (PND 84)**

The proliferative response of splenocytes was evaluated in the presence or absence of anti-CD3 antibody, a T-cell stimulator. A significant increase in the basal splenocyte proliferation was observed in female pups at PND 84 in the following treatment groups when compared to controls: (1) exposure to GEN by feeding from GD 14 to PND 84 (Fig. 2B); and (2) exposure to GEN from GD 14 to the time of birth by gavaging the dams (Fig. 3B). No effect of the basal splenocyte proliferation was observed in male mice (Fig. 2A and Fig. 3A). In the presence of anti-CD3 antibody, significant increases in T-cell proliferation were observed in both male and female mice when the animals were exposed to GEN by feeding from GD 14 to PND 84 (Fig. 2C and 2D). Neither the anti-CD3 antibody-mediated T-cell proliferation was observed in male pups at PND 84 in the following treatment groups when compared to controls: (1) exposure to GEN by feeding from GD 14 to PND 84 (Fig. 2B); and (2) exposure to GEN from GD 14 to the time of birth by gavaging the dams (Fig. 3B). No effect of the basal splenocyte proliferation was observed in male mice (Fig. 2A and Fig. 3A). In the presence of anti-CD3 antibody, significant increases in T-cell proliferation were observed in both male and female mice when the animals were exposed to GEN by feeding from GD 14 to PND 84 (Fig. 2C and 2D). Neither the anti-CD3 antibody-mediated T-cell proliferation was observed in male pups at PND 84 in the following treatment groups when compared to controls: (1) exposure to GEN by feeding from GD 14 to PND 84 (Fig. 2B); and (2) exposure to GEN from GD 14 to the time of birth by gavaging the dams (Fig. 3B). No effect of the basal splenocyte proliferation was observed in male mice (Fig. 2A and Fig. 3A). In the presence of anti-CD3 antibody, significant increases in T-cell proliferation were observed in both male and female mice when the animals were exposed to GEN by feeding from GD 14 to PND 84 (Fig. 2C and 2D). Neither the anti-CD3 antibody-mediated T-cell proliferation was observed in male pups at PND 84 in the following treatment groups when compared to controls: (1) exposure to GEN by feeding from GD 14 to PND 84 (Fig. 2B); and (2) exposure to GEN from GD 14 to the time of birth by gavaging the dams (Fig. 3B). No effect of the basal splenocyte proliferation was observed in male mice (Fig. 2A and Fig. 3A). In the presence of anti-CD3 antibody, significant increases in T-cell proliferation were observed in both male and female mice when the animals were exposed to GEN by feeding from GD 14 to PND 84 (Fig. 2C and 2D). Neither the anti-CD3 antibody-mediated T-cell proliferation was observed in male pups at PND 84 in the following treatment groups when compared to controls: (1) exposure to GEN by feeding from GD 14 to PND 84 (Fig. 2B); and (2) exposure to GEN from GD 14 to the time of birth by gavaging the dams (Fig. 3B). No effect of the basal splenocyte proliferation was observed in male mice (Fig. 2A and Fig. 3A). In the presence of anti-CD3 antibody, significant increases in T-cell proliferation were observed in both male and female mice when the animals were exposed to GEN by feeding from GD 14 to PND 84 (Fig. 2C and 2D). Neither the anti-CD3 antibody-mediated T-cell proliferation was observed in male pups at PND 84 in the following treatment groups when compared to controls: (1) exposure to GEN by feeding from GD 14 to PND 84 (Fig. 2B); and (2) exposure to GEN from GD 14 to the time of birth by gavaging the dams (Fig. 3B). No effect of the basal splenocyte proliferation was observed in male mice (Fig. 2A and Fig. 3A). In the presence of anti-CD3 antibody, significant increases in T-cell proliferation were observed in both male and female mice when the animals were exposed to GEN by feeding from GD 14 to PND 84 (Fig. 2C and 2D). Neither the anti-CD3 antibody-mediated T-cell proliferation was observed in male pups at PND 84 in the following treatment groups when compared to controls: (1) exposure to GEN by feeding from GD 14 to PND 84 (Fig. 2B); and (2) exposure to GEN from GD 14 to the time of birth by gavaging the dams (Fig. 3B). No effect of the basal splenocyte proliferation was observed in male mice (Fig. 2A and Fig. 3A). In the presence of anti-CD3 antibody, significant increases in T-cell proliferation were observed in both male and female mice when the animals were exposed to GEN by feeding from GD 14 to PND 84 (Fig. 2C and 2D). Neither the anti-CD3 antibody-mediated T-cell
proliferation in male mice nor that in female mice was significantly altered after in utero GEN exposure either by feeding (Fig. 2C and 2D) or by gavage (Fig. 3C and 3D).

**Effect of Developmental GEN Exposure on NK Cell Activity in Adult B6C3F1 Mice (PND 84)**

The activity of IL-2-augmented splenic natural killer cells was examined using 3^1^Cr-labeled YAC-1 cells as the target. The NK activity was not significantly altered after in utero GEN exposure (Fig. 4A and 4B; Fig. 5A), with the exception that an increase in NK activity was observed in female mice when the animals were exposed to GEN in utero by gavaging the dams (Fig. 5B). Exposure to GEN from GD 14 to PND 84 by feeding produced a slight increase in NK activity, with significant changes observed in the E:T ratios of 50:1 and 100:1 for male mice and at 50:1 for female mice (Fig. 4A and 4B).

**Effect of Developmental GEN Exposure by Feeding on Cytokine Production by Adult B6C3F1 Mice (PND 84)**

The supernatants from overnight ConA-treated draining lymph node cell cultures were evaluated for the presence of differential cytokines. As shown in Figure 6A, a significant increase in the level of IL-2 was observed in male mice when the animals were exposed to GEN by feeding from GD 14 to PND 84. The same GEN treatment also increased the levels of IL-4 and IFN-\(\gamma\) in male mice (Fig. 6C and 6E). Exposure to GEN in utero alone did not significantly affect the production of these three cytokines (IL-2, IL-4 and IFN-\(\gamma\)) when compared...
to the controls (Fig. 6A, 6C, and 6E). In female mice, increased levels of IL-2 and IL-4 but not IFN-γ were observed when the animals were exposed to GEN from GD 14 to PND 84 (Fig. 6B, 6D, and 6F). No significant changes in the levels of IL-2, IL-4, and IFN-γ were observed when the female mice were exposed to GEN in utero alone (Fig. 6B, 6D, and 6F). No significant changes in the levels of IL-5, IL-13, TGF-β and TNF-α were observed in either male or female mice in any treatment groups when compared to the controls (data not shown).

Effect of In Utero Exposure to GEN by Gavage on the Percentages of Splenic CD4⁺CD25⁺ Regulatory T Cells in Adult B6C3F1 Mice (PND 84)

To further explore the possible underlying mechanism that GEN modulated IgE production, a flow cytometric analysis of T-regulatory cells (CD4⁺CD25⁺) in the spleen was performed (Fig. 7A). Exposure to GEN from GD 14 to the time of birth by gavaging dams at 20 mg/kg produced a significant decrease in the percentage of CD4⁺CD25⁺ T cells in female mice (Fig. 7C) but not in male mice (Fig. 7B). The percentages of total
T cells and B cells were not affected in either sex (data not shown).

**Effect of In Utero Exposure to GEN by Gavage on the Expression of CD86 by B Cells in Adult B6C3F1 Mice (PND 84)**

Surface expression of costimulatory molecules B7.2 (CD86) by B cells in the draining lymph nodes was evaluated as an indicator of whether or not B cells were further activated by GEN in response to TMA treatment. As shown in Figure 8, when compared to vehicle controls, there was a significant increase of B7.2 (CD86) expression on B cells in GEN-treated female mice, as reflected by an increase in the observed mean fluorescence intensity (MFI). No such effect was observed in male mice (data not shown).

**In Utero Exposure to GEN by Gavage Did Not Affect IgE Production and Other Immune Parameters in B6C3F1 Mice at PND 42**

To determine if *in utero* exposure to GEN had any effects on TMA-induced total serum IgE production in younger animals (PND 42), mice were treated with TMA on PND 28 for the back exposure (sensitization), and on PND 35 for the ear exposure (challenge). Both male and female mice were sacrificed on PND 42 for measurement of serum IgE levels and other immune parameters. No significant changes were observed for the following parameters (data not shown): (1) total serum IgE; (2) splenocyte proliferation (± anti-CD3 Ab); (3) IL-2–augmented NK cell activity; (4) percentage of splenic CD4\(^+\)CD25\(^+\) regulatory T cells; (5) MFI of CD86 expression by the draining lymph node B cells; (6) body weight and spleen weights (both absolute and relative).

**DISCUSSION**

One of the key findings in the studies reported here was that *in utero* exposure to GEN increased serum total IgE production by adult B6C3F1 mice (PND 84) after dermal exposure to the respiratory allergen TMA. The increases were observed in both male and female mice administered TMA by a physiologically relevant exposure route, e.g., oral exposure. Furthermore, these increases were demonstrated with two different modes of oral exposure, feeding and gavage. Isoflavones have been identified in amniotic fluid, suggesting they can pass the placental barrier (Doerge *et al*., 2001). For a 25-g mouse consuming 2 g of chow every day, the concentrations of GEN at 500 ppm are approximately equivalent to doses of 40 mg GEN/kg/day, respectively.
For a 4-month-old infant who consumes soy formula as directed by the manufacturers, approximately 6–9 mg/kg body weight of isoflavones can be achieved (Irvine et al., 1998). The doses between 20 and 40 mg/kg in mice are comparable to the exposure levels in humans. This amount of GEN in a mouse is much lower than a clinical human treatment dose (approximately 100 mg/day) in terms of milligrams per square meter of body surface, which usually gives more accurate interspecies extrapolation (Hodgson, 1997). Additionally, the serum levels of GEN in mice that have been fed 1000 ppm GEN-containing diet is equivalent to that in men who received 50 mg genistein per day (Yellayi et al., 2002; Djuric et al., 2001).

On the one hand, an increase in IgE production was observed in adult male but not female mice after in utero exposure in our feeding study; however, an increase in IgE production was observed in adult female but not male mice after exposure to GEN by feeding from GD 14 to PND 84. On the other hand, in utero exposure to GEN increased the levels of total serum IgE in adult female but not male mice in our gavage study. The apparent discrepancies between feeding and gavage studies might be explained by the diets employed. The NIH-07 rodent diet that the mice consumed in our gavage study contained 120 g/kg soybean meal and 40 g/kg alfalfa meal, which suggested that these mice were constantly exposed to a medium level of phytoestrogens. Thus, in female mice, continuous exposure to GEN from GD 14 to PND 84 was required to produce an increase in serum total IgE. In contrast, in utero exposure to GEN was sufficient to increase serum total IgE production in male mice, and continuous exposures to GEN postnatally diminished the in utero exposure-induced enhancement in serum total IgE production.

In our study, increases in the total serum IgE levels in female mice correlated with the increases in splenocyte proliferation, NK cell activity, and IL-4 production. Increased levels of total serum IgE were observed in adult female mice that had been exposed to GEN-containing feed continuously from GD 14 to PND 84. However, the changes in total serum IgE levels were not significant in adult female mice that had received GEN from the time of birth to PND 84 (data not shown), which suggests that the in utero GEN exposure was indispensable for increased IgE production. Differentiation of naïve T helper cells into mature Th1/Th2 cells is associated with chromatin remodeling and DNA methylation of the cytokine genes such as IL-4 (Agarwal and Rao, 1998). DNA methylation also regulates the expression of CD86 by B cells (Kohm et al., 2002). Neonatal exposure to estrogen diethylstilbestrol (DES) alters uterine gene expression through modulating the methylation pattern (Alworth et al., 2002; Li et al., 1997), and importantly, GEN exposure alters methylation patterns in the mouse genome in vivo (Day et al., 2002). There is evidence that estrogen might enhance the development of asthma by driving the immune response from a Th1 type immune response to a Th2 type (Salem et al., 2000). It is possible that the increased level of estrogen in fetuses following in utero GEN exposure, which had been reported by Harrison et al. (1999), modulated IL-4 expression, splenocyte proliferation, CD86 expression, and NK cell activity, and thus, increased the IgE response using DNA methylation or other epigenetic mechanisms in our female mice.

The CD4+CD25+ regulatory T cell has been shown to regulate immune responses by maintaining peripheral tolerance against antigens, including autoantigens and allergens (Karagiannidis et al., 2004; Thornton et al., 2004). There was a significant decrease in the percentage of CD4+CD25+ T suppressor cells but not in CD4+CD25+ T cells in GEN-treated female mice when compared to the controls. Estrogen treatment has been reported to promote immune tolerance by increasing the numbers of CD4+CD25+ T cells (Polanczyk et al., 2004). In our study with younger female mice (PND 42), in utero exposure to GEN did not affect the total serum IgE production in response to TMA, suggesting that exposure to GEN after sexual maturation was necessary for enhanced IgE production. Thus, GEN might decrease the percentages of CD4+CD25+ T cells by functioning as an anti-estrogen in adult female mice. In support of this hypothesis, there is evidence that GEN exposure in adult females decreases the levels of estrogen (Kumar et al., 2002; Wood et al., 2004). However, a decrease in the percentages of CD4+CD25+ T cells was not the only change induced by GEN to promote IgE production, because it has been shown that only a decrease in FoxP3 expression (the specific marker for CD4+CD25+ T-regulatory cells) was not sufficient to cause an increase in IgE levels (Thornton et al., 2004). Thus, a decrease in the percentages of CD4+CD25+ T cells induced by GEN during postnatal exposure, together with the changes induced by in utero exposure as described above, may have increased the IgE response in our female mice.

In contrast, the increases in IgE production in male mice after in utero exposure to GEN were not associated with these immunological changes as described above for female mice. Interestingly, stimulation of IgE production by hydrocortisone is also independent of T cells and differential cytokines including IL-4, IL-5, IL-6, IL-8, IL-13 and TNF-α (Kimata et al., 1995). In utero exposure to GEN has been shown to increase the levels of estrogen in monkey fetuses (Harrison et al., 1999). It was possible that in utero exposure to GEN increased the level of estrogen in our male mouse fetuses and caused a permanent change in the male reproductive system, and thus, led to decreased testosterone production in adulthood, as reported by Wisniewski et al. (2003). It has been reported that a disturbance of the androgen–estrogen balance by DES in neonatal male rats also causes changes in Leydig cell development and function that last through puberty into adulthood (Sharpe et al., 2003). Because testosterone has been demonstrated to decrease the production of IgE in response to an antigen (Yamamoto et al., 2001), an increase in TMA-induced IgE production was produced in our adult male mice after in utero exposure. However, the increase in IgE...
production by adult male mice was lost when the animals were continuously exposed to GEN postnatally, which suggested that the changes induced by GEN during in utero exposure could be compensated or attenuated by the changes induced by GEN during postnatal exposures. There is evidence that exposure to GEN after PND 21 decreases the production of cortisol in male rats (Ohno et al., 2003), and corticosteroid enhances the Th2 and IgE responses, although it has been used to treat symptoms associated with allergies (Kimata et al., 1995; Wiley et al., 2004). Thus, continuous treatment with GEN postnatally would also decrease the cortisol level in our male mice and attenuate the IgE enhancement mediated by in utero GEN exposure.

In conclusion, our results demonstrated that in utero exposure to GEN produced an increased IgE response to TMA in adult B6C3F1 mice. Furthermore, male and female mice might have employed differential endocrine and immune mechanisms in their responses. Although the total serum IgE levels in asthmatics are approximately four times higher than those in nonasthmatic individuals (Siroux et al., 2004), the magnitude of IgE enhancement after GEN exposure in our study is sufficient to increase asthma severity. Belessis et al. (2004) have reported that one of the risk factors for an intensive care unit admission is a 1.6-fold increase in serum total IgE levels. Future study to determine the human susceptibility to develop asthma, autoimmunity, and subsequent autoimmune diseases after GEN exposure is therefore warranted.

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