Humoral Immune Response in Mice Over-expressing or Deficient in Growth Hormone[1]

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Effects of growth hormone (GH) levels on the humoral immune response were investigated in metallothionein I (MT)-bovine (b) GH-transgenic (*tg*) and GH-deficient Ames dwarf (*Prop1 df*$^{-/-}$) mice. Four-month-old mice were given primary and secondary injections of either normal saline or tetanus toxoid (TT) to induce specific antibody (Ab) production. MT-bGH-*tg* mice with high peripheral levels of bGH produced less TT-specific Ab than normal nontransgenic (*Ntg*) littermates, *df*, or nondwarf (*Ndf*) control mice. Titers reached maximum levels at 3–4 weeks post-primary immunization (PPI) and declined gradually through 24 weeks PPI in all groups of mice. Peripheral CD4$^+$ and CD8$^+$ T cell populations were significantly lower in *tg* than in *Ntg*, *df*, or *Ndf* mice. No significant differences were found in B cell numbers between *tg*, *Ntg*, or *df* mice. T helper 2 (Th2) cell populations were significantly greater in *df* mice compared to *Ntg* control mice. No significant differences were found in CD4$^+$:CD8$^+$ T cell ratios, interleukin (IL)-4 concentrations or interferon (IFN)-γ levels between *tg*, *Ntg*, *df*, and *Ndf* mice. No patterns of significant sexual dimorphism were found for any of the immune parameters studied. Elevated levels of corticosterone were investigated as a possible immunosuppressant mechanism responsible for low Ab responses in the *tg* mice. Ab production was not enhanced by decreasing corticosterone in *tg* mice. Thus, high endogenous GH levels inhibit specific Ab production and peripheral T cell populations but not peripheral B cell numbers, Th2 cell populations, or IL-4 or IFN-gamma production. Elevated corticosterone levels do not appear to be responsible for suppressed humoral immune responses. Low levels of endogenous GH do not inhibit specific Ab production but may contribute to increased peripheral Th2 cell numbers.

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The existence of a neuro-endocrine–immune axis was first postulated in 1930 when experiments involving removal of the rat anterior pituitary resulted in thymic atrophy (1). Developments in neuroendocrinology and immunology within the past twenty years have led to the emergence of an interdisciplinary field of neuroendocrine immunology and a basic awareness that neuroendocrine system mediators affect the immune system (2–4). However, direct evidence for significant physiological effects of individual neuroendocrine mediators on immune system development and function remains very limited.

Growth hormone (GH) is a 22-kDa peptide that is primarily produced and secreted by the anterior pituitary (5). It is one of several neuroendocrine proteins that interacts with immune system components such as B and T lymphocytes (6–8). GH is required for normal linear growth and metabolism (5, 9). Children and adults diagnosed with GH deficiency or impaired release of GH from the pituitary have been administered GH to promote growth and improve body composition, respectively (5, 9–12). GH has also been administered to trauma patients and individuals infected with human immunodeficiency virus (HIV) to improve metabolic functions such as protein synthesis, gain lean body mass and boost immune functions (13–17). However, the long-term effects of such GH usage are unknown. Excessive GH production has been found in adults with acromegaly, a condition that is marked by enlargement of the hands, feet, and facial features. It is accompanied by weakness of proximal muscles and associated with a high incidence of cardiomyopathy, hypertension, diabetes, and coronary disease (18). High levels of GH have also been found in children who suffer from Hutchinson-Gilford progeria syndrome (HGPS), a rare premature aging disease (19).

Growth hormone promotes thymic growth and T cell development, improves T cell function, and may contribute to increased T cell receptor (TCR) diversity (20, 21). While

interaction of GH with cell-mediated immune parameters has been more extensively documented, the effects of GH on B cell development and humoral immunity are more obscure. The introduction of extra GH-producing genes into laboratory animals using molecular techniques (22–28) and the availability of GH-deficient mice have provided novel models for investigating the role of GH in immune system development and function (2, 20, 29–32).

Understanding the effects of GH levels on the immune system requires further investigation at both the cellular and the molecular levels. This study aimed at gaining a better understanding of the interactions between the immune and neuroendocrine systems as they affect the humoral immune response. Specific antibody production was measured in transgenic mice having high levels of endogenous GH throughout their lives, due to over-expression of the bovine GH gene (22, 23, 27, 33), and in Ames dwarf mice, which are genetically deficient in GH, prolactin (PRL), and thyrotropin (TSH) (31, 34, 35). Several other parameters of the immune response were investigated to identify possible mechanisms responsible for differences in antibody responses in these animals.

Materials and Methods

Animals. Transgenic mice over-expressing bovine GH (bGH) under the control of the mouse metallothionein I (MT) promoter were derived from transgenic males kindly provided by Dr. T. Wagner (22, 23). Hemizygous transgenic males (from an MT-bGH20 male × a [C57BL/6J × C3H/HeJ] F_1 female) were mated with C3H/SnJ females (The Jackson Laboratory, Bar Harbor, ME) to produce approximately equal numbers of MT-bGH20-transgenic (*tg*) and normal nontransgenic (*Ntg*) progeny.

Mice homozygous for the mutated gene responsible for Ames dwarfism (*Prop1* $df^{-/-}$) (31, 34) were also used in this study. They were produced in a closed colony by mating non-littermate heterozygous animals (36). Approximately 25% of offspring were homozygous dwarf mice, while the remainder were either normal heterozygous carriers or normal homozygous noncarriers. Both heterozygous and homozygous normal nondwarf (*Ndf*) mice were used as littermate controls for homozygous dwarf (*df*) mice.

All animals were housed and bred in a temperature-controlled (22°C) room with a daily photoperiod of 12 hr:12 hr light:dark. Rodent food (Formulab Laboratory Diet, PMI Nutrition International, Inc., St. Louis, MO) and tap water were supplied *ad libitum*. All animal procedures were approved by the Southern Illinois University at Carbondale Animal Care Committee. From each of the MT-bGH20 and the Ames dwarf lines, 10 mice per group (i.e., 10 each *tg*, *Ntg*, *df*, and *Ndf*) were investigated per assay when possible. Equal numbers of male and female mice were tested when possible.

Identification of Transgenic and Dwarf Animals. MT-bGH20-*tg* mice were identified using a polymerase chain reaction (PCR). Tail samples (~200 mg) were taken at 10 days of age and incubated in lysis buffer (400 μl) containing proteinase K (200 μg), 0.45% NP40, 0.45% Tween 20, 100 m*M* Tris (pH 8.0), 500 m*M* KCl, and 15 m*M* $MgCl_2$ at 55°C, overnight with vigorous agitation (37). Proteinase K inactivation was carried out at 95°C for 15 min. The DNA extracted from these tail samples was stored at 4°C until utilized as a template for amplification of the MT-bGH transgene in PCR.

A primer specific for the 5′ end of the MT promoter region (primer sequence, 5′-TCCTCACTTACTCCG-TAGCTC-3′) and an equimolar concentration of a reverse primer specific for the 3′ end of the bGH region (primer sequence, 5′-AAGCTCTGAACACATCCAT-3′) of the transgene were synthesized by Operon Technologies (Alameda, CA) and utilized in each 25-μl PCR mixture. Also present in the reaction mixture were DNA template (3 μl), deoxynucleotides (200 μ*M*) (Amersham Life Science, Inc., Cleveland, OH), $MgCl_2$ buffer (1.5 m*M*), and 0.5 μl of *Taq* DNA polymerase (Perkin Elmer-Cetus, Norwalk, CT) (37). The PCR was carried out in a Perkin Elmer-Cetus thermocycler with the following conditions: 96°C for 3 min, 37 cycles of 96°C for 1 min, 62°C for 1.5 min, and 72°C for 2 min, with a final extension step of 72°C for 5 min.

Each PCR product was mixed with 2 μl of loading buffer (1000 parts 15% Ficoll, 12.5 parts bromophenol blue, 12.5 parts xylene cyanol), and 10 μl was loaded into a 2% agarose (0.0001% ethidium bromide) gel. A 123-bp DNA ladder (GIBCO BRL, Life Technologies, Grand Island, NY) was used. Electrophoresis was carried out at 100 V for 50 min, and amplified DNA was visualized under ultraviolet light. MT-bGH20-*tg* mice were identified by the presence of a 183-bp amplicon.

Dwarf (*df*) mice were identified and differentiated from normal nondwarf (*Ndf*) littermates at 21 days post-partum. At this age, *df* mice are obviously smaller and have juvenile characteristics when compared to their normal *Ndf* siblings (2, 31).

Immunizing Antigen. Tetanus toxoid (TT), aluminum phosphate adsorbed (Wyeth Laboratories, Inc., Marietta, PA), was given sc as the immunizing antigen to mice in primary and secondary doses (at weeks 0 and 2, respectively). A volume of 50 μl of TT, equal to one-tenth (38) of the human immunizing dose, was given in 200 μl of normal saline (0.15 *M* NaCl) for a total dose volume of 0.25 ml (39). Protein concentration was determined by the Lowry assay (39, 40) to be 33 μg/ml or 1.6 μg per dose.

Sham injections (0.15 *M* NaCl) were given to at least four each *tg*, *Ntg*, *df*, and *Ndf* mice. For sham injections at week 0, normal saline was administered in an equal volume of complete Freund's adjuvant (CFA; GIBCO BRL, Life Tech.) in a total injection volume of 0.25 ml (39). At week 2 post-primary immunization (PPI), incomplete Freund's adjuvant (IFA; GIBCO BRL, Life Tech.) was used in place of CFA in sham injections. All injections were given subcutaneously at several different sites on the dorsal side of the mouse to avoid development of necrotizing lesions.

Plasma. After anesthesia with ether, mice were bled by puncturing the retro-orbital plexus using heparinized capillary tubes as described previously (39). Blood was drawn prior to primary and secondary immunizations (weeks 0 and 2, respectively), at 3 and 4 weeks post-primary immunization (PPI), and every 4 weeks thereafter through 24 weeks. Heparinized blood was stored at 4°C overnight. Plasma was separated from red blood cells by centrifugation (850*g*) and stored at −80°C until tested in an enzyme-linked immunosorbent assay (ELISA).

Nonimmunized normal mice were bled to obtain negative control plasma. Plasma was processed as indicated above and the nonimmune mouse plasma samples (NMS) were pooled and aliquoted before storage at −80°C. Positive control serum (immune human serum [IHS]) was processed from whole blood obtained from a human volunteer having recently received a tetanus immunization. Guidelines approved by the Human Subjects Committee, Southern Illinois University at Carbondale, were followed.

ELISA. An ELISA was used to measure levels of TT-specific antibody in mouse plasma (41). TT, unadsorbed (Wyeth Laboratories, Inc., Marietta, PA), was diluted 1:10 in 0.1 *M* sodium carbonate, pH 9.8, 0.02% sodium azide, and bound (100 μl/well) to 96-well flat-bottom microtiter plates (ICN, Costa Mesa, CA) overnight at 25°C. Excess antigen was removed, and 0.2% gelatin (porcine) in PBS, pH 7.4, was added (200 μl) to each well and incubated at 37°C for 1.5 hr to block subsequent nonspecific binding. Wells were washed three times with 150 μl of 10 m*M* Tris, 0.15 *M* NaCl, 0.3% Brij 35, pH 7.4.

Positive (IHS) and negative (NMS) controls were serially diluted from 1:100 and mouse plasma was serially diluted from 1:10 in PBS, pH 7.4, 0.2% gelatin (porcine). Diluted control and test samples were added (100 μl) to wells in triplicate. Incubation proceeded at 4°C overnight, followed by another wash. Alkaline phosphatase-conjugated secondary antibody (sheep anti-human IgG or goat anti-mouse IgG, A, and M; The Binding Site, Inc., San Diego, CA; or Zymed Laboratories, Inc., So. San Francisco, CA; respectively) was diluted according to the manufacturer's recommendations and monitored for optimum performance, and then added (100 μl) to appropriate wells. Incubation at 25°C for 2 hr followed. After washing, disodium *p*-nitrophenyl phosphate (Sigma, St. Louis, MO) was reconstituted to 1 mg/ml in 1 *M* Trizma base, 0.3 m*M* $MgCl_2$, pH 9.8, and added to each well (100 μl). The substrate was allowed to react for 20 min at 25°C in the dark with gentle rotation (85 rpm).

The absorbance of each well was read at 405 nm using a Titertek Multiskan MC microtiter plate reader (Flow Laboratories, Inc., McLean, VA) and corrected for background absorbance. Triplicate values were averaged for each control or mouse plasma sample dilution tested. The TT-specific antibody titer for a given sample was the reciprocal of the highest dilution of the sample that produced a corrected absorbance of ≥0.300 at 405 nm.

Enumeration of Lymphocyte Populations. B and T cells were enumerated from peripheral blood of mice at 3 weeks PPI using flow-cytometry techniques and a fluorescence-activated cell sorter (FACS, FACSVantage apparatus, Becton Dickinson, San Jose, CA) located at SIU-School of Medicine (Springfield, IL). After red blood cell lysis (PharM Lyse, PharMingen, San Diego, CA), lymphocytes were stained with fluorochrome-labeled monoclonal antibodies (Mab; PharMingen). A Mab specific for the surface marker CD19 (i.e., phycoerythrin [PE]-labeled anti-CD19) was used to stain B cells and an anti-CD4 Mab (i.e., fluorescein isothiocyanate [FITC]-labeled anti-CD4) was used to stain $CD4^+$ T cells. A Mab specific for CD3 (i.e., cychrome [PE + cyanine dye Cy 5]-labeled anti-CD3ϵ) was used to count overall T cells, and one specific for CD8 (i.e., FITC-labeled anti-CD8) was used to enumerate $CD3^+$ $CD8^+$ T cells.

T helper 2 (Th2) cells were enumerated using FITC-labeled anti-CD4 Mab and a Mab to surface marker CD45RB on $CD4^+$ T cells (42, 43). $CD45RB^+$ cells that yielded low or dim fluorescence were indicative of Th2 cells, whereas $CD45RB^+$ cells that had high or bright fluorescence represented non-Th2, $CD4^+$ populations. These cells include naive $CD4^+$ T cells, undifferentiated $CD4^+$ T cells, T helper 1 (Th1) cells, and likely some memory Th2 cells that reverted back to the naive CD45RB high phenotype. All stained cells were resuspended in PBS with 0.5% paraformaldehyde prior to FACS.

Measurement of Th1- and Th2-Associated Cytokines. Peripheral lymphocytes were isolated from heparinized blood collected from mice at week 2 PPI, just prior to secondary immunization with TT. The cells were resuspended at approximately 2×10^6 cells/ml in RPMI-1640 tissue culture medium (Sigma)-complete (i.e., supplemented with 5% heat-inactivated fetal calf serum, 2 m*M* L-glutamine, 2 m*M* sodium pyruvate, 50 μg/ml gentamicin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 pg/ml amphotericin) (44, 45). Lymphocytes (50 μl) were then stimulated *in vitro* with TT (25 μl) in 125 μl RPMI-1640-complete (for a total culture volume of 200 μl) at 37°C with humidity and 5% CO_2 in air for 96 hr. After centrifugation at 400*g* for 15 min, supernatants were carefully removed and analyzed by ELISA (OptEIA Mouse IL-4 Set and Mouse IFN-γ Set, PharMingen) for Th2- and Th1-associated cytokine concentrations (interleukin [IL]-4 and interferon-γ [IFN-γ], respectively).

Determination of Corticosterone Levels. Corticosterone concentrations were measured in pre-primary immunization mouse plasma using a RIA (Corticosterone ^{125}I RIA Kit, ICN Biomedicals, Inc., Costa Mesa, CA) to determine baseline corticosterone levels for each group of mice under investigation (24, 28). Plasma corticosterone levels were also quantitated for *tg* and *Ntg* mice that underwent adrenalectomies. In these mice, corticosterone was measured at four to 18 days prior to the procedure, at 7–9 days post-adrenalectomy but prior to primary immunization

with TT, at 2 weeks PPI but prior to secondary immunization, and at 3 and 4 weeks PPI. This was carried out to determine baseline corticosterone concentrations in these animals prior to any procedures and to monitor corticosterone levels post-adrenalectomy. All plasma samples were tested in duplicate.

Removal of Adrenal Glands. Adrenalectomies were performed to investigate the role of high corticosterone levels as an immunosuppressant with respect to specific antibody production in the MT-bGH20-*tg* mouse line. Adrenal glands were surgically removed from 12 *tg* and 10 *Ntg* 4-month-old mice as follows. Mice were anesthetized with ether prior to surgery and provided anesthetic via a nose cone throughout surgery as necessary. Bilateral incisions were made to remove both adrenal glands. Each incision was closed with sterile sutures and wound clips.

During recovery and for the duration of their postoperative lives, the adrenalectomized animals were given tap water supplemented with 0.15 *M* NaCl as their drinking solution. Within 7–9 days, immunization with TT of nine each adrenalectomized *tg* and *Ntg* mice and plasma collection followed as described above.

Statistical Analysis. Statistical comparisons were made using a one-way analysis of variance (ANOVA) with JMP Version 4 software (JMP Sales, Cary, NC) when means of more than two groups of mice were compared. Statistical comparisons were made using the Student's *t*-test (unpaired) with Microsoft Excel Version 5.0 software (Microsoft Corp., Redmond, WA) when means of only two groups of mice were compared. A P value ≤ 0.05 was considered significant.

RESULTS

ELISA. TT-specific antibody titers were higher in four-month-old mice relative to 1-, 2- and 6-month-old mice of both the MT-bGH-transgenic and Ames dwarf lines in a preliminary study (data not shown). Therefore, the present study investigated mice given primary immunization at 4 months of age. In all antibody assays, positive control (IHS) titers were 800 or 1600 and negative control (NMS) titers were <100. Inter-assay control titers were reproducible within one titer unit. TT-specific antibody was very low to undetectable (titer $\leq$ 20) in plasma from all mice receiving sham injections.

For all mice receiving TT, specific antibody titers increased significantly from weeks 0 and 2 to weeks 3 or 4 PPI ($P < 0.05$). Titers peaked at 3 or 4 weeks and declined gradually to 24 weeks PPI (Fig. 1). For all groups of mice

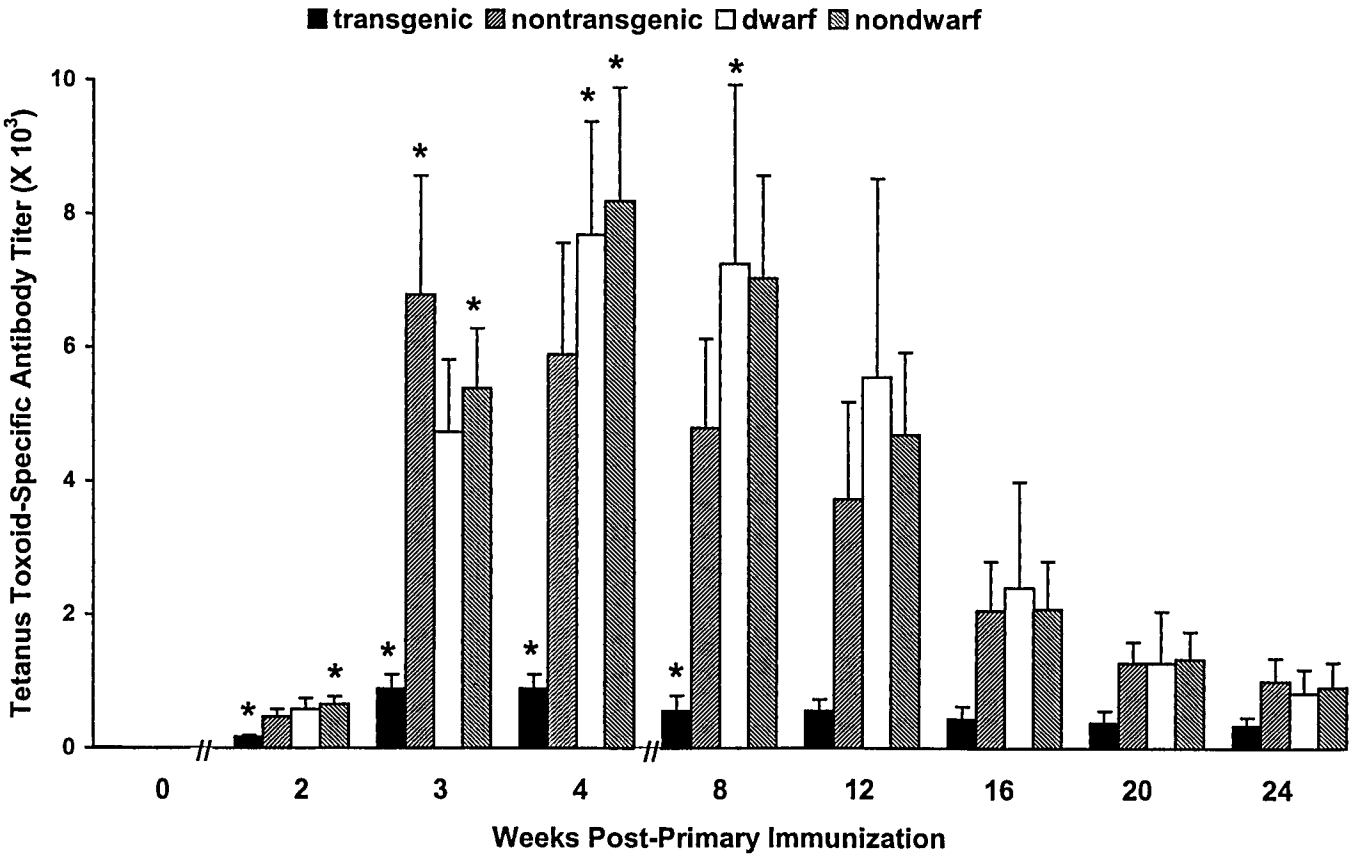

Figure 1. Tetanus toxoid-specific antibody titers in plasma from MT-bGH20-transgenic, normal nontransgenic, Ames dwarf, and normal nondwarf mice at pre-immunization (week 0) and 2, 3, 4, 8, 12, 16, 20, and 24 weeks post-primary immunization. Values represent means ± SEM for 10 mice in each group. *Represents significantly different antibody titers for transgenic versus nontransgenic, dwarf or nondwarf mice at either 2, 3, 4, or 8 weeks post-primary immunization ($P < 0.05$).

at all time points tested, no significant differences were found in specific antibody levels between male and female mice within a particular group with one exception. Titers were higher in *tg* males (1280 ± 784) than in *tg* females (512 ± 175) at 4 weeks PPI ($P < 0.04$).

MT-bGH-*tg* mice produced significantly less TT-specific antibody than *Ntg* littermates, *df* mice or *Ndf* control mice at 2, 3, 4, and 8 weeks PPI ($P < 0.05$; Fig. 1). At weeks 12, 16, 20, and 24 PPI, no significant differences were found in specific antibody levels between any of the groups of mice.

Lymphocyte Populations. To investigate possible mechanisms responsible for reduced specific antibody production in the *tg* mice, peripheral B and T lymphocytes were enumerated at 3 weeks PPI. No significant differences were found in B cell ($CD19^+$) numbers between *tg*, *Ntg* littermate control, and *df* mice (Fig. 2). However, *Ndf* control mice had a higher percentage of B cells in their periphery than did *Ntg* controls ($P < 0.05$). This difference most likely represents inter-strain variability as the normal animals are from different strains. Peripheral T cell ($CD3^+$) populations were lower in *tg* mice relative to those of *Ntg*, *df*, and *Ndf* mice ($P < 0.05$). Similarly, $CD4^+$ T cell numbers were lower in *tg* mice relative to those of *df* mice, and $CD8^+$ T cells were less numerous in *tg* mice compared to those of *Ntg*, *df*, and *Ndf* mice ($P < 0.05$). Interestingly, *df* mice appeared to have higher percentages of $CD3^+$, $CD4^+$, and $CD8^+$ lymphocytes than did their *Ndf* littermates. However, these differences were not statistically significant.

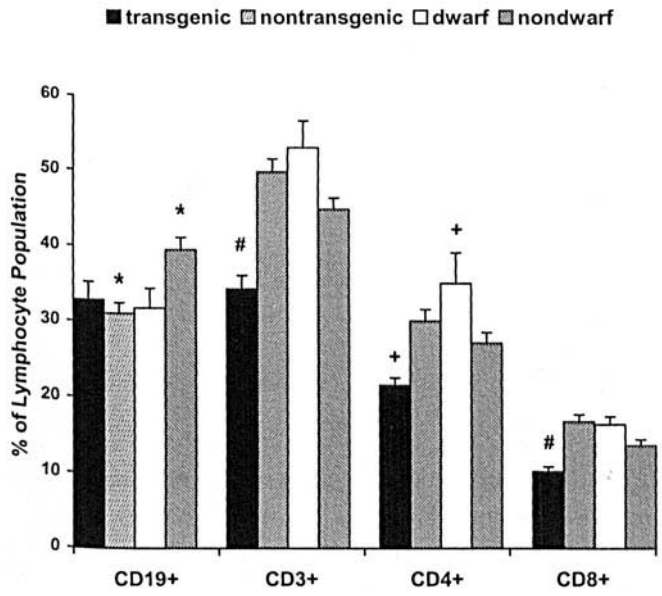

Figure 2. Lymphocyte phenotypes in peripheral blood of MT-bGH20-transgenic ($n = 12$), normal nontransgenic ($n = 11$), Ames dwarf ($n = 12$), and normal nondwarf ($n = 11$) mice at 3 weeks post-primary immunization with tetanus toxoid. Values represent means ± SEM. *Represents significantly different values for nondwarf versus nontransgenic mice ($P < 0.05$). #Represents significantly different values for transgenic versus nontransgenic, dwarf, and nondwarf mice ($P < 0.05$). +Represents significantly different values for transgenic versus dwarf mice ($P < 0.05$).

$CD3^+$ populations correlated well with the sum of $CD4^+$ plus $CD8^+$ T cells (Fig. 2). More than 91% of the $CD3^+$ lymphocyte population was accounted for when $CD4^+$ and $CD8^+$ populations were enumerated for all groups of mice. The remaining 8–9% of the $CD3^+$ lymphocytes counted may have been NK cells, as they also express the CD3ϵ surface antigen (46). There were no genotype-related differences in $CD4^+$ to $CD8^+$ T cell ratios (approximately 2:1; Table I).

Surprisingly, *df* mice had a higher percentage of peripheral Th2 cells than did a control group (*Ntg*) of mice ($P < 0.05$; Table I). No significant differences were found for lymphocyte populations between male and female mice except for the following. *Ntg* females had more $CD8^+$ T cells (18 ± 2%) in their periphery than did *Ntg* males (15 ± 3%; $P < 0.02$). Ratios of $CD4^+$ to $CD8^+$ T cells were higher in *tg* females (2.45 ± 0.52) than in *tg* males (1.90 ± 0.31) and lower in *Ntg* females (1.58 ± 0.15) than in *Ntg* males (2.19 ± 0.67; $P < 0.03$).

Th1- and Th2-Associated Cytokines. Correlation coefficients for all cytokine ELISA standard curves were 0.9997–0.9998. No significant differences were found in IL-4 concentrations or IFN-γ levels in lymphocyte culture supernatants between *tg*, *Ntg*, *df*, or *Ndf* mice (Table I). The only significant differences between male and female mice for IL-4 and IFN-γ concentrations were that lymphocytes from *df* males produced higher levels of IL-4 (26 ± 6 pg/ml) than those from *df* females (16 ± 2 pg/ml; $P < 0.02$), and lymphocytes from *Ndf* females produced higher levels of IFN-γ (193 ± 15 pg/ml) than those from *Ndf* males (115 ± 61 pg/ml; $P < 0.05$).

Corticosterone Levels. Results for duplicate plasma samples varied < 9% and correlation coefficients were 0.9999 or 1.0000 for all assay standard curves. MT-bGH20-*tg* mice had higher levels of corticosterone in plasma than did their *Ntg* littermates and the *Ndf* control

Table I. Means ± SEM for Peripheral Ratios of $CD4^+$ to $CD8^+$ T cells, Th2 Cell Populations, IL-4 Levels, and IFN-γ Concentrations Produced by MT-bGH20-Transgenic (*tg*), Normal Nontransgenic (*Ntg*), Ames Dwarf (*df*), and Normal Nondwarf (*Ndf*) Mice (n = Number of Animals Tested)

Genotype	$CD4^+$:$CD8^+$ T cells	Th2 cells (% of $CD4^+$ T cells)	IL-4 (pg/ml)	IFN-γ (pg/ml)
tg	2.17 ± 0.14 (12)	11 ± 1 (8)	15 ± 2 (6)	140 ± 11 (5)
Ntg	1.86 ± 0.16 (11)	8 ± 1* (8)	13 ± 1 (10)	108 ± 21 (6)
df	2.22 ± 0.25 (12)	12 ± 1* (11)	21 ± 2 (7)	117 ± 19 (3)
Ndf	2.12 ± 0.23 (11)	9 ± 1 (10)	19 ± 3 (6)	148 ± 23 (7)

* Represents significantly different values for dwarf versus nontransgenic mice ($P < 0.05$).

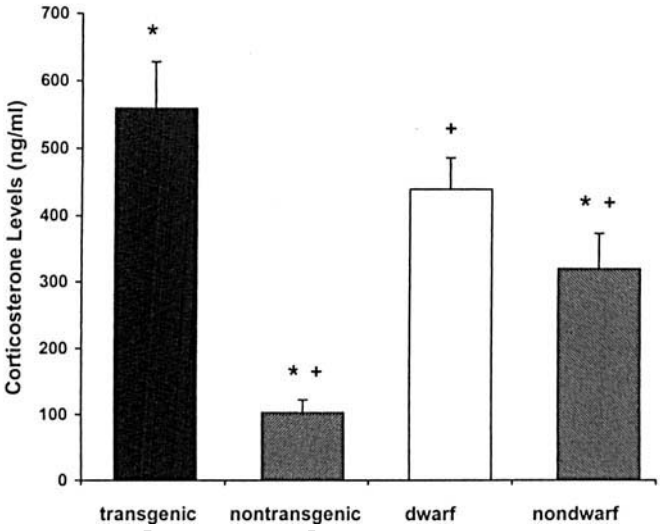

Figure 3. Corticosterone levels in plasma from MT-bGH20-transgenic, normal nontransgenic, Ames dwarf, and normal non-dwarf mice prior to primary immunization with tetanus toxoid. Values represent means ± SEM for 10 mice in each group. *Represents significantly different values for transgenic versus nontransgenic and nondwarf mice ($P < 0.05$). +Represents significantly different values for nontransgenic versus dwarf and nondwarf mice ($P < 0.05$).

mice ($P < 0.05$; Fig. 3). Interestingly, *df* mice, as well as *Ndf* mice, also had higher levels of corticosterone than did the *Ntg* control mice ($P < 0.05$). Numerical differences in corticosterone levels found in *df* versus *tg* or versus *Ndf* mice were not significant. There were no significant differences in corticosterone levels between males and females in any genotype group.

In the adrenalectomized groups of mice, pre-adrenalectomy corticosterone levels in plasma from *tg* mice were higher than those in their *Ntg* littermates ($P < 0.04$) (Fig. 4). After adrenalectomies, only mice that underwent a reduction of greater than 50% ($P < 0.01$) in plasma corticosterone levels and in which the reduction was maintained throughout the immunization schedule ($n = 5$ each *tg* and *Ntg*) were included in the analysis (Figs. 4 and 5). Although significantly decreased, post-adrenalectomy corticosterone levels in *tg* mice were significantly greater than those in their *Ntg* littermates at 0, 2, 3, and 4 weeks PPI ($P < 0.04$). No significant differences were found in corticosterone levels between 0, 2, 3, or 4 weeks PPI in adrenalectomized *tg* or *Ntg* mice.

Specific Antibody in Adrenalectomized Mice. Adrenalectomized *tg* mice produced lower levels of TT-specific antibody than their adrenalectomized *Ntg* littermates at 3 and 4 weeks PPI (Fig. 5). However, the difference was only significant at week 4 ($P < 0.05$). Antibody titers increased significantly for the adrenalectomized *tg* and *Ntg* mice from week 0 to weeks 2, 3, or 4 PPI ($P < 0.05$).

Discussion

The present study investigated several parameters of the humoral immune response in MT-bGH20-transgenic (*tg*) mice that over-express GH (27) and in Ames dwarf (*df*) mice that are GH, PRL, and TSH deficient (31, 34, 35). TT was used as an immunizing antigen in these mice because of

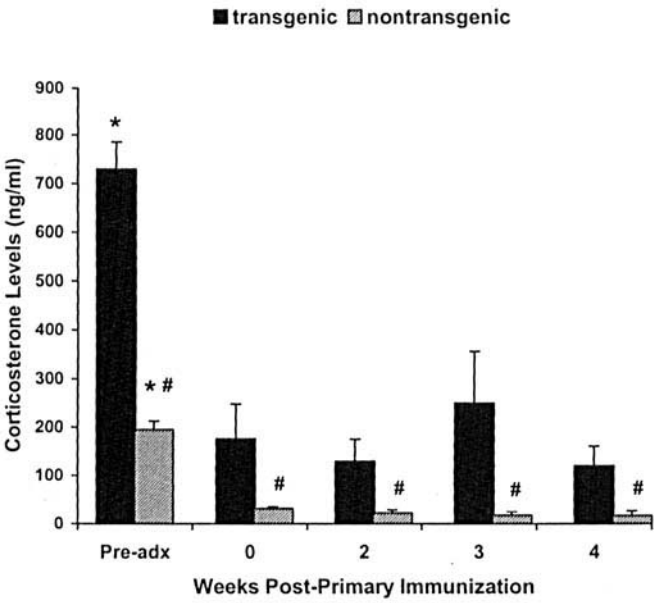

Figure 4. Corticosterone levels in plasma from MT-bGH20-transgenic mice and normal nontransgenic littermates prior to adrenalectomy (pre-adx) and post-adrenalectomy at 0, 2, 3, and 4 weeks post-primary immunization with tetanus toxoid. Values represent means ± SEM for 5 mice in each group. *Represents significantly different values for transgenic and nontransgenic mice pre-adrenalectomy versus respective transgenic and nontransgenic mice post-adrenalectomy at 0, 2, 3, and 4 weeks post-primary immunization ($P < 0.01$). #Represents significantly different values for transgenic versus nontransgenic mice ($P < 0.04$).

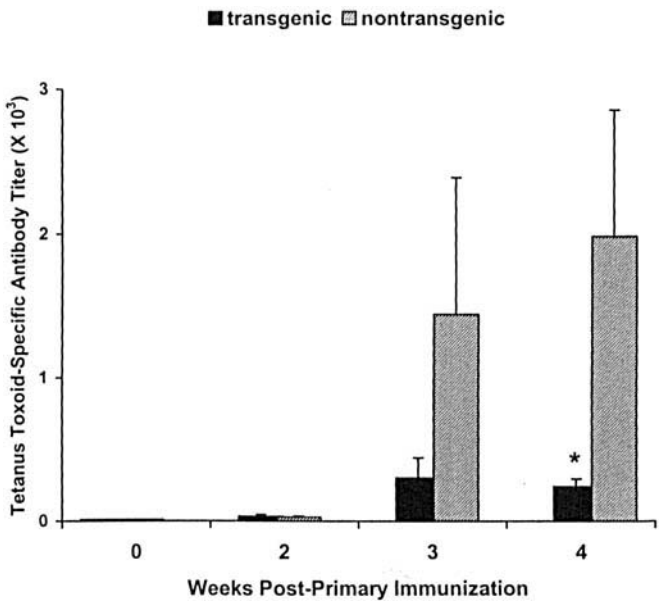

Figure 5. Tetanus toxoid-specific antibody titers in plasma from adrenalectomized transgenic mice and nontransgenic littermates at pre-immunization (week 0) and 2, 3, and 4 weeks post-primary immunization. Values represent means ± SEM for 5 mice in each group. *Represents significantly different antibody titers for transgenic versus nontransgenic mice at 4 weeks post-primary immunization ($P < 0.05$).

its clinical relevance in vaccine research (47–49). Relative to nontransgenic (*Ntg*) siblings that express GH at normal levels (33, 50), MT-bGH20-*tg* mice grow to adult body weights that are significantly greater (51) and have significantly reduced life spans (25). Ames *df* mice are GH-deficient due to a mutation in a pituitary-specific, paired-like homeodomain transcription factor gene, the *Prophet of Pit-1* (*Prop1*) (34). Body weights for adult Ames *df* mice are only 40–50% of those of their nondwarf (*Ndf*) littermates that express normal levels of GH (35, 52). Ames *df* mice live significantly longer than their normal siblings (53).

After receiving primary and secondary immunizations with TT, GH-*tg* mice produced significantly less specific antibody than their *Ntg* littermates, *df* mice or *Ndf* control mice throughout 8 weeks PPI. Ames *df* mice produced specific antibody at levels that were not significantly different from those of their *Ndf* littermates and *Ntg* control mice. This suggests that a high level of GH does not enhance and may inhibit specific antibody production, and that congenital GH deficiency does not affect antibody production. These findings are in contrast to previous studies which indicated that GH administration leads to increased antibody titers to TT in aged rhesus monkeys (54) and enhances Ig production in human cells (55, 56), and that treatment with a GH secretagogue (a synthetic inducer of GH secretion by the pituitary) enhances specific antibody production (57). Our findings are also in contrast with earlier suggestions that GH-deficient mice are immunodeficient (2, 32, 58). However, our data are consistent with other findings. For example, implantation of GH-secreting GH_3 cells does not boost production of antibody to sheep erythrocytes in nude rats (59), rat MT-GH-transgenic rats do not produce higher levels of specific antibody to sheep erythrocytes than normal littermates (59), recombinant human (rh) GH therapy does not increase serum Ig levels in GH-deficient children (11), and rhGH therapy in young adults with childhood-onset GH deficiency resulted in a decrease in circulating Ig (4).

Another important finding from this study is that at 3 weeks PPI (1 week after secondary immunization), peripheral $CD3^+$ and $CD8^+$ T cell populations were lower in *tg* mice than in *Ntg*, *df*, and *Ndf* mice, and that $CD4^+$ T cell numbers were lower in *tg* than in *df* mice. There were no significant differences in T cell numbers between *df*, *Ndf*, and *Ntg* mice. This suggests that high levels of GH do not stimulate and may inhibit T cell proliferation. It also suggests that T cell proliferation is not inhibited by GH-deficiency. These findings do not support earlier studies which indicated that GH administration stimulates splenic T cell proliferation in aged rats (59), GH augments T cell proliferation in rat MT-GH-transgenic mice (59), recombinant GH administration increases splenic T cell percentages in aged female rhesus monkeys (54), and GH deficiency is associated with reduced peripheral T cell numbers in mice (3, 21). However, our findings do correlate with other findings. For example, implantation of GH_3 cells does not augment progenitor T cell numbers in nude rats (59), T cell percentages decrease during GH therapy in GH-deficient children (10), and splenic $CD4^+$ and $CD8^+$ T cell populations are lower in MT-bGH20-transgenic mice than in their normal littermates (51).

Peripheral $CD19^+$ B cell numbers were very similar in *tg*, *Ntg*, and *df* mice. The only significant difference found in B cell populations was between *Ndf* and *Ntg* control mice. These findings demonstrate that high levels of GH do not augment or inhibit B cell numbers, and that B cell populations in GH-deficient *df* mice are not significantly reduced relative to those of normal mice.

Also of interest in this study is that $CD4^+$ to $CD8^+$ T cell ratios were virtually 2:1 for all genotypes. In addition, no significant differences were found in peripheral Th2 cell numbers between *tg*, *Ntg*, or *Ndf* mice, or between *tg*, *df*, or *Ndf* mice. However, peripheral Th2 cell populations were significantly higher in *df* mice compared to normal *Ntg* controls. In lymphocyte culture supernatants, no genotype-related differences were found in concentrations of Th2- or Th1-associated cytokines, IL-4 or IFN-γ, respectively. These findings suggest that low antibody titers in the *tg* mice are not due to low $CD4^+$ to $CD8^+$ T cell ratios, low Th2 cell numbers, low IL-4 concentrations, or high IFN-γ levels. Thus, low antibody responses in the MT-bGH20-*tg* mice do not appear to be the result of a switch to higher Th1 responses relative to Th2 responses in these animals. Also, elevated Th2 cell populations and normal IL-4 production may contribute to normal levels of specific antibody produced by the GH-deficient *df* mice.

It is possible that the examined parameters of immune function were influenced by alterations in the secretion of hormones other than GH. As was mentioned above, Ames *df* mice have primary deficiencies of PRL and TSH (36). Transgenic mice over-expressing bGH tend to release increased amounts of PRL (26). However, it seems exceedingly unlikely that PRL deficiency and hypothyroidism counteracted or masked the effects of GH on immune function in Ames *df* mice, or that mild hyperprolactinemia of bGH-*tg* mice was responsible for inhibition of antibody production and peripheral T cell populations in these animals. Isolated PRL deficiency in homozygous PRL knock-out mice has essentially no effect on their immune function (60, 61).

Corticosterone, the principal adrenal glucocorticoid in the mouse, is capable of suppressing certain immune functions. Previously, corticosterone levels were found to be elevated in different lines of GH-*tg* mice (24, 28). Therefore, corticosterone levels were investigated as a possible immunosuppressant mechanism that may be responsible for the low specific antibody titers observed in the *tg* mice. Interestingly, corticosterone levels are not only elevated in the *tg* mice relative to their *Ntg* littermates and *Ndf* controls, but are also significantly higher in the *df* and *Ndf* mice

relative to the *Ntg* mice. Given the relatively normal levels of specific antibody produced by the *df*, *Ndf*, and *Ntg* mice, our findings suggest that elevated corticosterone levels in *df* and *Ndf* mice do not suppress specific antibody production.

After decreasing plasma corticosterone levels in *tg* and *Ntg* mice at least 50% via adrenalectomy, specific antibody levels produced by adrenalectomized *tg* mice were still significantly lower than those produced by adrenalectomized *Ntg* littermates at 4 weeks PPI. Persistence of relatively high (100–200 ng/ml) plasma levels of corticosterone after surgical removal of the adrenals suggests the intriguing possibility of ectopic glucocorticoid production in *tg* mice. It is possible that ectopic corticosterone expression in the *tg* mice after adrenalectomy played a role in inhibiting specific antibody production. However, persistence of compromised production of specific antibody in adrenalectomized *tg* mice in which plasma corticosterone levels were greatly reduced indicates that high plasma corticosterone levels in intact MT-bGH20-*tg* mice are not solely responsible for low specific antibody production.

In addition, immune function is known to decline with age (62). From the present study and earlier findings (25), there are some indications of early aging in GH-*tg* mice. Given the lower specific antibody production and reduced life span (25) in the MT-bGH20-*tg* mice relative to their normal littermates, it can be suggested that at a given age MT-bGH20-*tg* mice are biologically older than normal mice. Also, given the relatively normal levels of specific antibody produced by the Ames *df* mice in this study, recent findings of improved persistence of immune function in old Snell dwarf mice relative to normal littermates (63), and significantly greater life spans in Ames *df* mice compared to normal littermates (53), it can be suggested that at a given age GH-deficient dwarf mice are biologically younger than normal mice.

In conclusion, the complete role of GH in the development and function of the immune system still remains unclear. Here, GH-*tg* and Ames *df* mouse lines were used to investigate the effects of high levels of GH and GH deficiency on the humoral immune response. High endogenous GH levels in the MT-bGH20-transgenic mouse inhibit specific antibody production. These effects may be mediated by low peripheral T cell populations, but not by low B cell numbers, Th2 cell numbers or IL-4 concentrations. Elevated plasma corticosterone levels correlate with high endogenous GH concentrations in MT-bGH20-*tg* mice but are not solely responsible for suppressed specific antibody production. Deficiency of endogenous GH in the Ames *df* mouse (along with PRL and TSH deficiency) does not suppress specific antibody production, IL-4 concentrations or peripheral $CD4^+$ T cell numbers, but may enhance humoral immunity via elevated Th2 cell populations. Finally, the humoral immune parameters investigated in these mouse models do not exhibit significant sexual dimorphism. Further investigation is necessary to thoroughly understand the physiological effects of GH in both cell-mediated and antibody-mediated immunity.

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