Immune Function in Transgenic Mice Overexpressing Growth Hormone (GH) Releasing Hormone, GH or GH Antagonist

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Abstract. **Effects of life-long exposure to high levels of homologous or heterologous growth hormone (GH) and effects of GH resistance on selected parameters of immune function were studied in adult male transgenic mice overexpressing GH releasing hormone (GHRH), bovine (b) GH or an antagonistic bGH analog. In metallothionein I (MT)-bGH transgenic mice with high peripheral levels of bovine GH, there were significant increases in the absolute weight of the thymus and the spleen and in the mitogenic responses of splenocytes to concanavalin A (ConA), lipopolysaccharide (LPS) and phytohemagglutinin (PHA), as compared to age-matched normal animals. There were no significant differences between MT-bGH transgenic and normal mice in splenocyte viability or in delayed-type hypersensitivity measured by the allergic contact dermatitis response to oxazolone. Similar results, including significant stimulation of splenocyte responses to ConA, LPS, and PHA, were obtained in MT-hGHRH transgenic mice in which overexpression of GHRH leads to striking pituitary enlargement and massive elevation of peripheral levels of homologous (mouse) GH. In MT-bGH-antagonist transgenic mice in which overexpression of an antagonistic bGH analog interferes with the actions of endogenous GH, spleen weight was reduced but proliferative responses of splenocytes to ConA, LPS, and PHA were not affected. It is concluded that overexpression of heterologous or homologous GH in transgenic mice can lead to significant stimulation of some parameters of immune function, whereas antagonism of GH action by expression of an antagonistic GH analog does not affect splenocyte responses to mitogens.** [P.S.E.B.M. 1999, Vol 221]

Both growth hormone (GH) and insulin-like growth factor-I (IGF-I), the main mediator of GH actions, can act on the immune system and influence various aspects of its function. Growth hormone receptors were detected in the bone marrow, thymus, spleen, lymph nodes, and on peripheral blood lymphocytes (1, 2). Receptors for IGF-I are also present on peripheral blood lymphocytes including natural killer cells (3). Growth hormone was reported to influence lymphopoiesis, granulopoiesis, and erythropoiesis (4). In mice and rats, hypophysectomy produces atrophy of the thymus and alterations, almost exclusively suppressive, in many parameters of the immune system function ranging from antibody production to mitogen-induced lymphocyte proliferation (5). In the thymus, GH exerts a potent stimulatory effect on thymulin-producing cells and can induce proliferation of epithelial cells (6). Atrophy of the thymus resulting from GH deficiency or aging can be reversed by GH treatment (7). Indications of reduced thymus function are present in Ames and Snell dwarf mice that are GH, PRL, and TSH deficient (8, 9), and include reduction of thymic weight as well as decreases in some parameters of the immune response (10). Kappel *et al.*

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(11) and Postel-Vinay *et al.* (12) demonstrated that GH can stimulate proliferation of activated mouse T lymphocytes. In another study, Warwick *et al.* (13) showed that GH-treated macrophages release more H_20_2 when compared to unstimulated macrophages. Snell dwarf mice treated with bGH exhibit enhanced T-cell activation in the popliteal lymph node (14).

In the human, therapy with recombinant homologous GH is used to treat congenital GH deficiency and various growth disorders (15), and the hormone shows considerable promise for the treatment of various catabolic states, including burn injuries (16) and AIDS (17). Human GH therapy is also being evaluated for the treatment of various symptoms of aging (18) and as an antiobesity agent (19). In this context, understanding the effects of GH on immune function acquires additional significance.

In the present study, several measures of the immune response were evaluated in transgenic mice with life-long GH excess due to overexpression of the bovine (b) GH gene (20) or the human GHRH gene (21). Overexpression of GHRH leads to overstimulation of somatotrophs, enlargement of the pituitary, and massive elevation of peripheral levels of homologous GH (21), thus resembling many clinical features of giantism and acromegaly. Additional studies were conducted in transgenic animals with partial GH resistance induced by overexpression of an antagonistic bGH analog (bGH-Ant) (22, 23).

Materials and Methods

Animals. Transgenic mice expressing bGH, hGHRH, or G119K bGH antagonist (20–22) under control of the mouse metallothionein I (MT) promoter were derived from transgenic males kindly provided by Drs. T.E. Wagner and J.S. Yun, K. Mayo and J. Hyde, and J.J. Kopchick, respectively. The animals were produced by crossing hemizygous transgenic males with C57BL/6xC3H F_1 hybrid females purchased from the Jackson Laboratory (Bar Harbor, ME). These crosses provided approximately equal numbers of hemizygous transgenic (*Tg*) and normal (*N*) progeny. Adult males were used in the present study at the age of 4–5 months (MT-bGH and MT-hGHRH) or 4–10 months (MT-bGH-Ant). The animals were kept in a temperature-controlled (22°C) room on a 12:12 light:dark cycle. Tap water and rodent laboratory diet (Lab Diet Formulab #5008, PMI Feeds, St. Louis, MO; 23% protein, 6.5% fat) were provided *ad libitum*.

From the MT-bGH line, 8 *N* and 8 *Tg* mice were used for lymphocyte proliferation assays, and 12 *N* and 10 *Tg* for the oxazolone test. From the MT-hGHRH line, 8 *N* and 8 *Tg* mice were used for lymphocyte proliferation assays, and 11 *N* and 9 *Tg* animals for the oxazolone test. From the MT-bGH-Ant line, 5 *N* and 6 *Tg* were used for lymphocyte proliferation assays. All animal procedures were approved by the Animal Care and Use Committee of Southern Illinois University at Carbondale.

Mouse Genotyping. MT-bGH and MT-hGHRH *Tg* mice were identified using a polymerase chain reaction (PCR). Approximately 200 mg of either tail or ear sample were incubated in a lysis buffer (100 m*M* Tris, 500 m*M* KCl, 15 m*M* $MgCl_2$, 0.45% Nonidet-P40, 0.45% Tween 20, 50 μg Proteinase K) for 8 hr at 55°C in a shaking water bath, and subsequently at 100°C for 10 min to denature Proteinase K. All samples were stored at 4°C until used in the PCR reaction.

For the PCR reaction, 2 μl of the tail or ear lysate along with 10 m*M* Tris HCl, 1.5 m*M* $MgCl_2$, 50 m*M* KCl, 5 m*M* of each dNTP (Promega, Madison, WI), 0.5 μ*M* of 5′ primer, 0.5 μ*M* of 3′ primer, and 2.5 U of Taq DNA polymerase (Boehringer Mannheim, Indianapolis, IN) were used. For the MT-bGH mice, one primer was designed to anneal to sequences within the MT gene promoter (5′-TCCTCACTTA CTCCGTAGCTCC-3′), and a second primer was designed to anneal to a portion of the bGH sequence of the transgene (5′-AAGCTCTGAACACAT CCATCG-3′) (Brown-Borg, personal communication). For the MT-hGHRH mice, the primers used were as described by Mayo *et al.* (21). One primer anneals to a sequence within the MT gene promoter (5′-CCAGTCGTC-CCAAAGGGGCGG-3′), and a second primer (5′-ACAGGCGGGCGTTCGACGAGG-3′) anneals to the hGHRH sequence of the transgene. All oligonucleotide primers were synthesized by Cruachem (Dulles, VA). The PCR conditions for the MT-bGH line samples were as follows: 95°C for 3 min, 35 cycles of 95°C for 1 min, 60°C for 1.5 min, and 72°C for 2 min, and a final extension period at 72°C for 5 min. The PCR conditions for the MT-hGHRH line samples were as follows: 95°C for 3 min, 35 cycles of 95°C for 1 min, 62°C for 1.5 min, and 72°C for 2 min, and a final extension period at 72°C for 5 min.

A 10-μl sample from each PCR reaction was analyzed by electrophoresis through a 1% agarose gel. The DNA was stained with ethidium bromide and visualized under UV light. The MT-bGH transgenics were identified by the presence of a 183-base-pair band, whereas the MT-hGHRH transgenics were identified by the presence of a 642-base-pair band.

Oxazolone-Induced Delayed-Type Hypersensitivity Test. The procedure described by Chapman *et al.* (24) was used for this test. All animals received three doses of oxazolone (Sigma, St. Louis, MO). The first two were the sensitizing doses, and the third one was the challenging dose. Each animal was anesthetized with ether, the hind flank was shaved, and 25 μl of a solution consisting of 100 mg/ml of oxazolone in acetone:corn oil (4:1) were applied evenly on the shaven hind flank. The solution was applied using a micropipettor and was then gently spread on the skin surface using a rounded glass probe. The same procedure was repeated 24 hr later. Five days after application of the second dose, the third (challenging) dose consisting of 10 μl of a 10-mg/ml solution of oxazolone in acetone:corn oil (5:1) was applied on the dorsum of the right ear pinna. The

left pinna received 10 μl of vehicle (acetone:corn oil 5:1) and served as the negative control. Twenty-four hours after application of the last dose, each animal was anesthetized, and the thickness of the central section of each ear lobe was measured using a micrometer.

Splenocyte Culture. Splenocytes were isolated and prepared for culture using previously described protocols (25, 26). Briefly, RPMI 1640 was used as a base, and the following supplements were added for complete medium: 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 2 m*M* L-glutamine, and sodium pyruvate (Sigma), 50 μg/ml gentamycin, 100 U/ml penicillin (Gibco BRL, Gaithersburg, MD), 100 μg/ml streptomycin (Gibco), and 0.25 pg/ml ampotericin (Gibco). Erythrocytes were lyzed using Tris-buffered ammonium chloride method (25). A cell suspension sample was observed *via* light microscopy for presence of remaining erythrocytes, and the procedure was repeated if positive. The cells were washed using HBSS and enumerated using a hemacytometer. The final concentration was adjusted to 1×10^6 cells/ml using complete medium. The percentage of viable white blood cells was determined *via* the trypan blue exclusion test (25). Splenocyte proliferation was assayed by a tritiated thymidine uptake assay (25,26). Briefly, 100 μl of cells and 100 μl of mitogen (final concentration Con A – 2.5 μg/ml, Pharmacia, Uppsala, Sweden; LPS – 10 μg/ml, Sigma; PHA – 5 μg/ml, Pharmacia) were added to microtiter plates. Control wells received cells and complete medium only. At the end of a 40-hr incubation (37°C, 7% CO_2), 5 μl of medium containing 1 μCi [^{3}H]-thymidine was added to each well. The plates were incubated for 8 hr, transferred on FP-24 Whatman 934AH paper (Brandel, Giathersburg, MD) using a M24R Cell Harvester (Brandel), and counted.

Statistical Analysis. Statistical analysis was performed using the Student's *t* test with Microsoft Excel software. A *P*-value of ≤ 0.05 was considered significant.

Results

MT-bGH Transgenic Mice. The mean body weight of the MT-bGH *Tg* mice was significantly higher than that of age-matched *N* mice from the same line (Table I). The liver weight, a very good indicator of the level of transgene expression, was also significantly higher in the *Tg* when compared to the *N* mice (Table I). The thymus and spleen weights of *Tg* mice were heavier ($P \leq 0.05$, $P \leq 0.005$, respectively) when compared to normals.

Splenocyte viability was not different between *Tg* and *N* mice (96.4 ± 0.4 vs. 95.9 ± 0.3%); however, the splenocytes of MT-bGH *Tg* mice proliferated more extensively ($P \leq 0.001$) when compared to splenocytes from *N* mice and to control wells ($P \leq 0.001$) for every mitogen tested (Fig. 1). There was no difference in the ratio of the challenged over control ear thickness 24 hr after oxazolone challenge (Table I).

MT-hGHRH Transgenic Mice. The mean body weight of the MT-hGHRH *Tg* mice was higher ($P \leq 0.001$) when compared to the *N* mice from the same line (Table I). The mean pituitary weight of the transgenics was markedly and significantly higher when compared to the normals (Table I). The thymus was heavier ($P \leq 0.05$) compared to the *N* mice, and there was a significant difference in spleen weight, similar to the results obtained in the bGH line.

Splenocyte viability was comparable between *Tg* and *N* mice (95.9 ± 0.2 vs. 96.1 ± 0.3%). The proliferative capacity of splenocytes from *Tg* mice was elevated ($P \leq 0.005$) in response to each mitogen tested when compared to normal mice from the same line and to control wells ($P \leq 0.001$; Fig. 2).

MT-bGH-Ant Transgenic Mice. The mean body weight of the MT-bGH-Ant *Tg* mice was significantly lower than body weights of normal mice (Table I). There was no difference in mean thymus weights between *Tg* and *N* mice (Table I). In contrast, spleen weight in MT-bGH-Ant *Tg*

Table I. Body Weight, Organ Weights, and Responses to Oxazolone in Transgenic Mice Overexpressing Bovine Growth Hormone (bGH), Human GH Releasing Hormone (hGHRH) or bGH Antagonistic Analog (Ant), and in Normal Animals From the Same Lines

	Number of animals used for weights and proliferation assays	Body weight (g)	Liver weight (g)	Pituitary weight (mg)	Thymus weight (mg)	Spleen weight (mg)	Oxazolone ear thickness ratio (no. animals tested)
MT-bGH	8	47.6 ± 2.18	2.35 ± 0.09	ND	45.3 ± 3.40	152.5 ± 9.70	1.80 ± 0.03 (10)
Normal	8	32.2 ± 1.07	1.35 ± 0.03	ND	31.0 ± 1.40	92.0 ± 2.50	1.79 ± 0.04 (12)
P value	—	0.001	0.0001	—	0.05	0.005	NS
MT-hGHRH	8	46.2 ± 0.08	ND	6.17 ± 0.93	43.7 ± 3.03	259.8 ± 30.3	1.72 ± 0.03 (9)
Normal	8	32.6 ± 0.08	ND	1.84 ± 0.18	31.8 ± 2.04	107.3 ± 15.4	1.79 ± 0.04 (11)
P value	—	0.001	—	0.01	0.05	0.01	NS
MT-bGH-Ant	6	31.3 ± 1.78	ND	ND	33.6 ± 2.73	61.7 ± 5.50	ND
Normal	5	47.3 ± 2.05	ND	ND	35.4 ± 1.32	91.1 ± 5.00	ND
P value	—	0.001	—	—	NS	0.01	—

Note. Means ± SE.

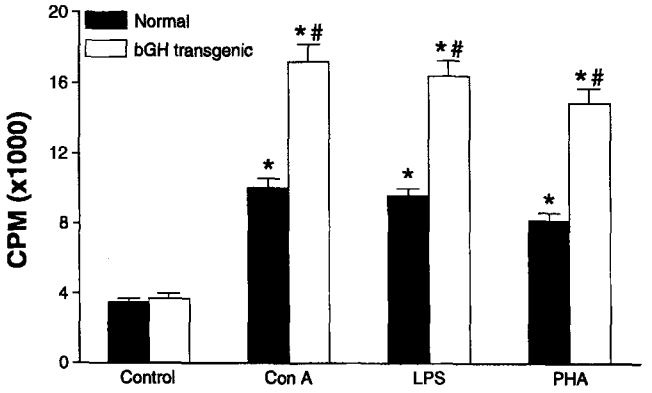

Figure 1. *In vitro* mitogenic responses of splenocytes from normal ($n = 8$) and MT-bGH transgenic mice ($n = 8$) to Con A, LPS, and PHA. Values represent means ± SEM. *Represents significantly different ($P < 0.001$) responses versus corresponding control wells. #Represents significantly different ($P < 0.001$) responses between splenocytes from normal and transgenic mice stimulated with the same mitogen.

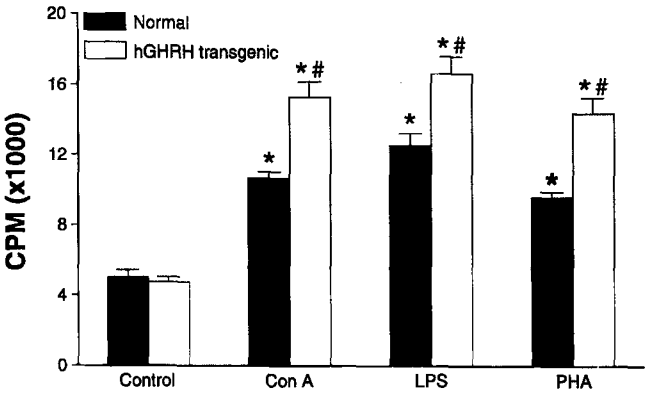

Figure 2. *In vitro* mitogenic responses of splenocytes from normal ($n = 8$) and MT-hGHRH transgenic mice ($n = 8$) to Con A, LPS, and PHA. Values represent means ± SEM. *Represents significantly different ($P < 0.001$) responses versus corresponding control wells. #Represents significantly different ($P < 0.005$) responses between splenocytes from normal and transgenic mice stimulated with the same mitogen.

mice was lower ($P \leq 0.01$) compared to *N* mice from the same line (Table I). No differences in splenocyte viability were observed (95.9 ± 0.2 vs. 95.7 ± 0.2%). Splenocytes of MT-bGH-Ant *Tg* and age-matched *N* mice proliferated to the same extent when stimulated with Con A, LPS, or PHA (Fig. 3).

Discussion

The main novel finding from the present study was the increase in the response of splenocytes to mitogens in transgenic mice overexpressing GH. This effect was observed in both MT-bGH mice with ectopic expression of heterologous GH and in MT-hGHRH animals in which eutopic (adenohypophyseal) expression of endogenous (mouse) GH was elevated as a consequence of overexpression of GHRH. Moreover, a significant increase in the proliferative response of splenocytes in GH-*Tg* and GHRH-*Tg* as compared to *N* mice was consistently detected with each of three

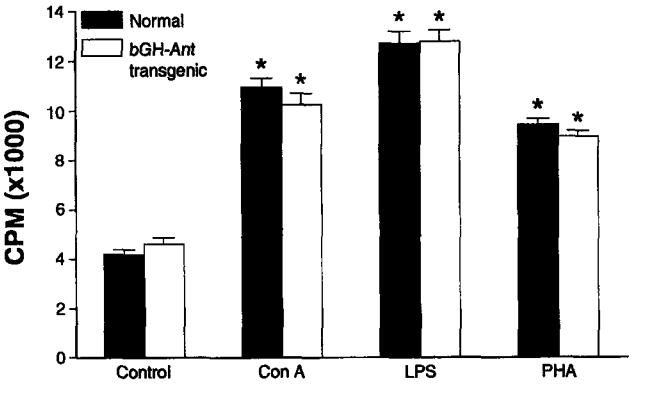

Figure 3. *In vitro* mitogenic responses of splenocytes from normal ($n = 5$) and MT-bGH-Ant transgenic mice ($n = 6$) to Con A, LPS, and PHA. Values represent means ± SEM. *Represents significantly different ($P < 0.001$) responses versus corresponding control wells.

different mitogens used in the present study. These results could not have been predicted from previous studies. Administration of GH for short periods of time (4 days to 2 weeks) was reported to exert various stimulatory effects on the immune system (11–13, 27, 28). However, life-long exposure to elevated GH levels in transgenic mice is associated with a reduced percentage of splenic T cells (29) and reduced antibody responses to tetanus toxoid (30).

In these experiments, high levels of plasma GH (MT-bGH and MT-hGHRH) appear to stimulate the proliferative capacity of both B (LPS) and T cells (Con A and PHA). Although absolute numbers of T and B cells were not determined in this study, previous reports (Ref. 29 and M. Hall, personal communication) indicate that the overall percentage of lymphocytes in the spleen is decreased in GH-*Tg* mice. The percentage of CD3 + T cells including both CD4 + and CD8 + subsets are equally reduced in mice overexpressing GH compared to normal mice (Ref. 29 and M. Hall, personal communication). High levels of circulating GH are accompanied by elevated corticosterone (31), which may contribute to the overall decrease in the number of peripheral T cells found in these mice. However, it has been shown that the number of T cells expressing Vβ8, a commonly used variable region of the TCR β chain, is increased in GH transgenic mice (29). The number of B cells is not altered in mice expressing the GH transgene when compared to normal counterparts (M. Hall, personal communication). Although the number of NK cells in spleens of *Tg* mice has not been determined, we do know that natural killer cell activity (chromium release assay using Yac-1 cells) is greater in *Tg* (PEPCK-bGH) when compared to *N* mice (unpublished data). These data indicate that GH differentially affects immune cell numbers and function in the spleen and may affect overall immune defense against pathogens (30).

Differences between the effects of injecting GH in *N* animals and overexpression of GH in *Tg* animals can be due to the life-long versus short exposure, higher peripheral GH levels, and nearly constant rather than pulsatile GH release in the transgenics (32). Moreover, GH-*Tg* mice have el-

evated plasma corticosterone levels (31), and the immunosuppressive effects of this glucocorticoid could conceivably mask or reverse the immunostimulatory action of GH. The functional status of the immune system in GH transgenic mice remains to be fully characterized, and it is unclear whether alterations in immune function in these animals are in any way related to their reduced life expectancy (33, 34). Histopathological studies suggest that GH-*Tg* mice die most often of renal failure consequent to glomerulonephritis, of tumors, or cardiovascular complications (33), and it has been suggested that some of these changes (in particular, glomerulonephritis) may represent an autoimmune disease (I. Berczi and J. Martinko, personal communication). It has been implied that the altered Vβ8 expression in these mice may represent an early onset of autoimmune disease via an aging-associated dysregulation of apoptotic pathways in the thymus (29). This altered expression may be related to the higher levels of circulating plasma corticosterone known to occur in the GH-*Tg* mice (31).

In both MT-bGH and MT-hGHRH *Tg* mice, there was a significant increase in the absolute weight of the thymus, but the relative thymic weight was not altered. Apparently, chronic major elevation of peripheral GH levels does not have a specific trophic effect on thymus weight. However, a modest increase in the relative spleen weight was seen in the transgenics when compared to the normals. This increase in weight is probably due to a higher number of epithelial cells that in turn increase the total surface area of the spleen allowing the spleen to accommodate more cells entering from the bloodstream. There was no difference in splenocyte viability between the *N* and *Tg* mice. As mentioned above, an increased response of splenocytes from transgenics to mitogens was observed in lymphocyte proliferation assays. Unexpectedly, no differences were detected between *Tg* and *N* animals in the allergic contact dermatitis test (oxazolone challenge) in either line of *Tg* mice. Similarity of the effects of overexpression of hGHRH and bGH on the examined parameters of immune function suggest that these effects were due to elevation of peripheral GH levels, rather than to direct action of GHRH on the immune cells of MT-hGHRH *Tg* mice (35).

The effects of partial GH resistance associated with overexpression of GH antagonists on the immune system have not been studied. Ames and Snell dwarf mice, which are GH-, PRL-, and TSH-deficient (8, 9), have been used to study the effects of GH on the immune system. It is unclear whether dwarf mice are immunocompromised or not (10, 26, 36, 37). The endocrine phenotype of dwarf mice is due to a mutation of Pit-1, or to a failure of Pit-1 producing pituitary cells to develop (9). However, extrapituitary GH expression can be independent of Pit-1 (38). If this applies to leukocytes, perhaps the amount of GH produced by lymphocytes involved during an immune response is sufficient for the normal progression of the response. This could also mean that GH plays an autocrine or paracrine role in the activation and/or progression of an immune response. The numerical differences in thymus and spleen weight in the MT-bGH-Ant *Tg* versus *N* mice did not correspond to the effects in the MT-hGHRH and MT-bGH transgenic animals. Thus, the ratio of thymus weight to body weight was higher in the bGH-Ant transgenics, and there was no difference in the ratio of spleen weight to body weight between transgenics and normals. Substantial differences in body weight of normal (control) animals from the different lines are most likely due to age differences and age-related obesity. However, differences in the genetic background of the MT-bGH-Ant, as compared to the other two lines of mice, may have also contributed to these differences. These results, along with the results from the bGH and hGHRH transgenics, indicate that thymus development and maintenance is not dependent upon GH produced outside of the thymic environment. When the results from the three lines are combined, there is an indication that GH seems to have a trophic effect since bGH antagonists can inhibit growth of the spleen. There was no difference in the proliferative responses of splenocytes from normal and bGH-Ant transgenic animals when stimulated with mitogens. It is not known what the level of expression of this transgene is in the splenocytes. If expression of the transgene is low, then GH that is produced by the splenocytes could probably overcome the effect of bGH-Ant. It is also possible that the effects of GH resistance on immune function in MT-bGH-Ant *Tg* mice were not detected because of the limited number of animals from this line available for the present study, as well as the wide range of their ages.

In conclusion, the data collected in this study indicated that the MT-hGHRH and the MT-bGH transgenic mice were not immunocompromised when compared to normal mice, and that some measures of the immune system function were stimulated in these animals. Mice expressing a GH antagonist gene did not show any differences in immune function when compared to normal mice.

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