Immune Enhancing Effect of a Growth Hormone Secretagogue

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Growth hormone (GH) is a 191-aa protein released by the pituitary gland. In addition to its effect in the promotion of growth, it was also found to have a positive influence on the immune system (for reviews, see Refs. 1–3). Earlier observations documented that thymic atrophy was often associated with ablation of the pituitary gland (4), and mice with the dwarf mutation had reduced immune functions (5, 6). Recently, in experimental animal models, administration of GH was found to enhance development of the thymus (1–3, 6) and promote the engraftment of murine or human T cells in SCID mice (7, 8). Some of the GH effects are indirectly mediated by insulin-like growth factor-I, synthesized in the liver, and induced by GH (3). Furthermore, GH and insulin-like growth factor-1 could synergize with other cytokines such as GM-CSF in hemogenesis (9), possibly leading to the various effects attributed to GH.

A class of synthetic nonpeptidyl compounds, the GH secretagogues (GHS), was recently discovered to synergize with the natural GH-releasing factor (10–12). GHS induces calcium flux in rat pituitary cells, and causes the release of GH (10). The release of GH is similar to physiological release of GH in that it is under pulsating regimen and is subjected to feedback mechanism (11). In vitro experiments showed that GH promotes GH release by acting on a specific G protein-coupled receptor located in the hypothalamus and pituitary gland (13). The natural or endogenous ligand for this receptor was recently identified and it was named ghrelin (14). Ghrelin, a 28-aa peptide, was found in rat and human stomach, and binding of ghrelin to pituitary cells induces secretion of GH.

Therapeutically and conceptually, an orally bioavailable, small m.w. compound such as GHS would be advantageous over the GH peptide if the biological effects associated with GH could be duplicated with GHS. Indeed, encouraging results from preclinical and clinical studies have indicated that GHS are efficacious in facilitating muscle development and mobilization in dogs whose hind legs were surgically immobilized (15). In a controlled clinical trial of elderly individuals, it improved the well being of the treated group (16). Markers of bone turnover were elevated in the 12-wk trial of these elderly people, suggesting that GHS will strengthen bones in the elderly. In this study, we focused on the immune enhancing effect of GHS, an area that has not been explored. We treated young and old mice with GHS, and examined various immunological parameters. We assayed for changes in peripheral blood cells, proliferative responses of T and B cells,Ab, cytolytic responses, and tumor resistance in the treated mice. We also tested the effect of GHS in promoting bone marrow engraftment, and found that one mechanism of immune enhancing effect of GHS was in the increase of cycling lymphoid cells in the treated mice.
Compound dosage

The GHS compound was previously published (10) and prepared at Merck. It is an analog of MK677, which was in clinical trials (11, 16). It was dissolved in water and given to the mice daily by gavage. The oral bioavailability is 16% in rat, and the \( t_{1/2} \) in rat is 2.9 h. (K. Leung, Merck Research Laboratories, Rahway, NJ, unpublished observations) (Fig. 1).

Six mice per group were treated with the compound at 5–10 mg/kg or water as the vehicle controls. The old BALB/c mice were treated daily for 3 weeks, and the old B6 mice were treated for 5 wk. In the study with tumor resistance, the old mice were treated with compound at 1 mg/kg i.p. every other day or every day from Monday to Friday. For i.p. dosing, the compound was solubilized in DMSO in 10 mg/ml, and diluted in Dulbecco’s PBS (Life Technologies, Grand Island, NY). Vehicle was then PBS dissolved in some of the studies.

Hematology

Hematology profiles, including the standard total white blood cells (WBC), RBC, WBC differentials, and hemoglobin, were determined by Bayer H1E Hematology analyzer (Bayer Diagnostics, Tarrytown, NY) at the beginning and end of the study. At the termination of the study, thymuses and spleens were removed and weighed, and cell suspensions were made for various in vitro assays.

Proliferation assays

Spleen cells at \( 2 \times 10^6 \)/well were cultured in RPMI 1640 (Life Technologies), supplemented with 10% FBS (Sigma, St. Louis, MO), 1% each of penicillin-streptomycin, glutamine, nonessential amino acid, and sodium pyruvate (Life Technologies), and \( 5 \times 10^{-5} \) 2-ME (Sigma). For T cell stimulation, anti-CD3 (2C11 at 10, 20, and 50 ng/ml, purified from hybridoma; a gift from J. Bluestone, University of California, San Francisco) and anti-CD28 (29.6 at 10, 20, and 50 ng/ml, purified from hybridoma; a gift from H. Shevach, National Institutes of Health) were added to B6 splenocytes at a ratio of 3:1 of splenocytes to EL4. Five days later, CTL activity was assessed by using the \( ^{51} \text{Cr} \)-labeled EL4, similar to that described above for allogeneic CTL. Lytic units were calculated by linear regression analysis of the lytic data to determine the number of cells required to achieve 20% lysis in \( 10^5 \) cells (18). Student’s \( t \) test or paired \( t \) test was used for statistical analyses.

Bone marrow transplant in SCID mice

CB17/SCID (3–4 mo old) mice depleted of NK cells with rabbit anti-AsGm-1 Ab (25 \( \mu \)g/mouse; WAKO, Richmond, VA; Ref. 7) on day −1 were dosed with vehicle or GHS 1 mg/kg i.p. −1 h before i.v. infusion of \( 3 \times 10^7 \) bone marrow cells (BMC) from normal BALB/c mice. BMC were flushed from femurs with RPMI 1640 medium, supplemented with 10% FBS, 1% penicillin-streptomycin, and 10 U/ml of sodium heparin, and washed twice before infusion. Mice were then dosed with PBS vehicle or GHS at 1 mg/kg i.p. (Monday-Friday) for 16 days. Thymus and spleen were analyzed for subset phenotype. Proliferation assays were performed with the splenocytes as described above.

GH determination

At the termination of the study, mice were injected i.p. with 1 mg/kg of the compound and bled 10 min later. Sera were stored at −70°C until time of the assay. Levels of GH were determined by RIA at Merck Research Laboratories, according to previously described procedures (10).

Anti-5-bromo-2’-deoxyuridine (anti-BrdU) staining for FACS analyses

The procedures were modified from a combination of published protocols (19, 20). Briefly, after surface staining by conventional techniques, cells were washed with FACS buffer, resuspended in residual medium, and fixed by dropwise addition of 1 ml cold 95% ethanol, incubated for 30 min on ice, pelleted, and then washed with 1 ml cold PBS and fixed with 1 ml containing cold 1% paraformaldehyde and 0.01% Tween 20 for 1 h on ice. Cells were pelleted and washed with 2 ml of PBS one time. Cells were then incubated with 0.5 ml of freshly made 200 Kunitz units DNase I (Sigma) in 0.15 M NaCl, 4.2 mM MgCl\(_2\), pH 5, at 37°C for 30 min. After washing in 2 ml of FACS buffer with 0.01% Tween, cells were blocked with IgG1 and incubated with 15 \( \mu \)l FITC-conjugated anti-BrdU mAb (PharMingen) or FITC-IgG1 (PharMingen) and analyzed on a FACSScan flow cytometer (Becton Dickinson) using two- or three-color analysis.

Results

GHS increases PBLs in young mice

We have previously observed increased peripheral WBC when pigs were dosed with the compound during a study in the effect of GHS on body weight. Therefore, we started the treatment of 5- to 6-wk-old B6 mice by orally dosing them daily at 5 mg/kg for 3 wk. The IC\(_{50}\) of the compound is ~0.1 nM on rat pituitary cells, and the bioavailability of the compound is ~16% in the rats. The mice were weighed and randomized in the various experimental groups such that there was an even distribution of mice with different body weights. There was significant weight gain (15% of vehicle control, \( p < 0.04 \)) in the first week of treatment, and this initial weight gain contributed to the total weight gain at the end of 3 wk. We have consistently observed 30% higher in lymphoid cells in the
peripheral blood, compared with the control mice (Fig. 2). From 4–7 wk of age, the peripheral WBC and lymphoid cells generally decreased as the mice matured (21), and treatment with GHS maintained the level of lymphopoiesis in these mice. In one of three experiments, there was a 30% increase in the proliferative response with α-CD3; control animals had 32,628 ± 3,365 cpm and treated animals had 44,797 ± 3,049 cpm (p < 0.05, n = 6). Concomitantly, there was a slight decrease in response to goat anti-mouse IgM; control animals had 26,766 ± 1,783 cpm and treated animals had 20,434 ± 1,879 cpm (p < 0.05, n = 6). We have also observed some increase in NK activity or Ab-dependent cytolytic activity in one experiment, but these observations were not consistently observed. Therefore, in the young mice the compound increased the production of lymphoid cells as well as some functions of cellular immunity.

GHS increases thymic cellularity in old mice

In the first three experiments, 20- to 24-mo-old BALB/c or B6 mice were orally dosed with the compounds similar to the study with the young mice. Contrary to the effect of the compound in the young mice (5 mg/kg), the WBC of the treated mice were not significantly altered, and there was no significant weight gain. In all three experiments, the compound did not affect the T or B cell proliferation. The generation of allogeneic CTL was also not affected in the B6 mice. The lytic activity of NK cells or Ab-dependent cytotoxic cells was not significantly different in the treated groups (data not shown).

However, in all three studies there was a statistically significant increase of the thymic cellularity in the treated groups, as shown in Fig. 3A (p < 0.05 in all experimental groups). In addition to the increase in cellularity, all of the thymic subsets in two experiments were increased in the treated group. In particular, the percentage of double negative (DN, CD4^−/CD8^−) cells of the treated groups were significantly increased in both studies (Fig. 3B, p < 0.05 in both experiments).

In a subsequent study, a group of 16-mo-old B6 mice were treated with GHS at 1 mg/kg i.p. from Monday to Friday for 3 weeks, the thymus weight of these vehicle-treated old mice was 25.6 ± 5.3 mg (n = 5), and that of compound-treated mice was 40.2 ± 7.8 (p < 0.023, n = 4). Histological sections of the thymus showed normal thymic lobular architecture in both control (Fig. 4, A and B) and treated B6 mice (Fig. 4, E and F). However, thymic size and cortical-cellular density were obviously lower in the 16-mo-old B6 mice (Fig. 4, C-F) in comparison to that of a normal 6-mo-old B6 mouse (Fig. 4, A and B). Peri-thymic adipose tissue replaced thymic tissue in the 16-mo-old mice (Fig. 4, C and E). Compared with the normal young (6 mo) thymus (Fig 4A), the 16-mo-old thymus lost considerable cellularity (Fig 4B), evident in the loss of cortex region. Upon treatment for 3 wk, the cortex began to regain cellularity. (Fig. 4, E and F). These data imply that either more stem cells entered the thymus or GHS enhanced thymocyte differentiation, resulting in an increase of cellularity.

GHS enhances Ab production and tumor resistance in old mice

To assess the effect of GHS on immune responses, mice were immunized with sheep erythrocytes (SRBC) 4–7 days after initiation of treatment, lasting for 3 wk, and sensitized again 7 days before the termination of the study. Sera were titered in Dulbecco’s...
PBS, and SRBC was added to assess the ability of Ab to agglutinate the SRBC. The treated 18-mo-old BALB/c mice had higher Ab titers (1/1633 ± 352) than SRBC, compared with the controls (1/756 ± 305, p = 0.05, n = 4–6). We also studied the primary Ab production to allogeneic cells in two groups of animals, and the treated 21-mo-old B6 mice responded better to the allogeneic cells and demonstrated higher Ab levels as detected by FACS analyses. Relative mean fluorescence of control animals was 14.5 ± 6.3 and of treated animals was 56 ± 18 at 1/20 dilution of the sera (p < 0.058, n = 4–5). However, there were not significant differences in the number of B cell population (data not shown).

After we observed encouraging results in the treated aging mice, we next examined the effect of GHS in a disease model. Because the elderly human population generally had a higher incidence of tumor (22), we studied the effect of GHS in resistance to a transplantable tumor, EL4 (H-2b, derived from B6 mice). EL4 cells proliferate rapidly in culture, with a doubling time of 16–18 h. Before using EL4 in this tumor model, we cultured the cells in various concentrations of GHS, from 1 pM to 100 nM, and no effect on cell growth was found. Therefore, the in vivo effect of GHS on EL4 would be mediated through the host, inoculated with the tumor cells. Transplanted EL4 is an aggressive tumor in the syngeneic B6 mice, causing mortality in adult mice in 3–5 wk. In these studies, 1–10 × 10^5 EL4 cells were given s.c. in the flank, after the mice were treated with GHS for 2–3 wk. In each experiment, five young adult mice (3–6 mo old) were similarly injected with EL4 to serve as controls for comparison. At the end of the study, the GHS-treated mice were in general more active compared

FIGURE 4. Cellularity was increased in GHS-treated thymus. Histologic (H&E stain) thymic sections of three B6 mice. A and B, 6-mo-old B6 mouse thymus, weight = 66.5 mg. C and D, 16-month-old vehicle control B6 mouse, thymus weight = 31.4 mg. E and F, GHS-treated 16-mo-old B6 mouse, thymus weight = 47.4 mg. Photomicrographs of A, C, and E were taken at low (×3.12); B, D, and F were taken at high (×50) magnification. The dense thymic cortical region of the young mouse (A and B) was markedly diminished in old vehicle control mouse (C and D) and partially replenished in old GHS-treated mouse (E and F). The ratio of thymic cortical-medullary area varied accordingly, being greatest in the younger mouse (A), and least in the old vehicle control mouse (C). The thymic cortical-medullary zone in the old vehicle control mouse (D) was less defined in comparison to that of the young mouse (B) and the old GHS-treated old mouse (F). The medullar of the old vehicle control mouse (D) exhibited a conglomeration of lymphoid, epithelioid, and reticular cell types, in contrast to the uniform lymphoid and epithelioid cells in medullar of the younger mouse (B) and the GHS-treated old mouse (F).
with the lethargic and cachectic vehicle control mice. In these 5–6 wk studies, we also determined the levels of GH in the placebo and GHS-treated mice, and we found a 2-fold increase in serum GH.

Table I shows the summary of four studies, and the results were tabulated in Fig. 5. In the vehicle group, progression of the tumor was faster, and some mice became moribund and died before termination of the study. The vehicle controls of the 22-mo-old mice developed tumor in 10 days, and no metastasis was observed in the treated mice. In the 24-mo-old mice, we again observed extensive metastases in 6/10 mice in the untreated group, and three mice were moribund at the end of the study. In comparison, 4/9 of the treated group had smaller metastatic lesions, and they all appeared healthy at the termination of the study. On the average, primary tumor mass of the treated mice was also smaller, although it was not statistically significant. Even though the number of mice studied was limited, these data showed that treatment with GHS significantly decreased metastases by 42% (p < 0.02), and it also reduced mortality in these aging mice (Table I).

Because we detected some delay in the establishment of EL4 in the old mice, we inoculated 16-mo-old mice with a lower tumor burden, 1 × 10^5 EL4 cells (Table I). These mice were treated with compound or vehicle for 3 wk before inoculation of the tumor cells s.c. The treatment was continued for another 13 days until the end of the study on day 14. We measured the progression of the tumor, and found a significant delay (p < 0.04) in the onset of the tumor (Fig. 6). At the time of euthanasia, the average tumor size was half that of the vehicle control (control: 690 ± 90 mg, treated: 320 ± 50 mg, n = 5). The GH level of the pooled serum of treated mice was 7.5 ng/ml and of the control mice was <0.95 ng/ml at the end of the study. Similar observations were made in a second experiment.

In some experiments, at the time of euthanasia, we tested for splenic lytic activity to EL4, and there appeared to be no significant difference between vehicle control and treated mice. Spleen cells were also cocultured with irradiated allogeneic BALB/c (H-2^d) spleen cells to assess the generation of lytic activity in microtiter plates. After 5 days, lytic activity was determined with 51Cr-labeled H-2^d target, RAW. In this assay, there was no significant difference between treated and vehicle control mice in their CTL response to allogeneic cells (data not shown).

When generation of anti-EL4 CTL was tested, we found higher lytic activity to EL4 cells in the treated mice. The lytic units of the treated mice (43.6% of young mice, n = 12) was higher than vehicle-treated mice (23.4% of young mice, n = 9), but it was comparable to that of the naive young mice (46.5% of young mice, n = 3) with no prior EL4 exposure. However, we did not detect significant anti-EL4 Ab in the mice inoculated with EL4. Under these circumstances, humoral responses to EL4 were not generated. It is interesting to note that GHS did not enhance tumor development in this lymphoma system, contrary to some speculation on the possible tumorigenic effect of GHS (20).

Finally, we have dosed a group of 2-year-old BALB/c mice s.c. at 1 mg/kg continuously for 320 days, every other day. Even though our number (n = 5) was small, we found that there was a significant increase (p < 0.009) in the life span of these treated mice. The median survival time for the vehicle group was 97.8 days, whereas that of the treated group was 134 days, beyond the 2 years, when we initiated the treatment with GHS. Therefore, the compound was not toxic and the treated mice looked healthy.

**GHS promotes engraftment of BMC in SCID mice**

In an earlier study, Murphy et al. (7) found that GH promoted the engraftment of murine and human T cells in SCID mice. Therefore, in the next series of studies, we transplanted SCID with BMC from normal mice, with and without GHS treatment of the recipients, to test whether GHS would promote engraftment of BMC. CB17/SCID mice were used as recipients of BALB/cJ BMC. Mice were treated with GHS at 1 mg/kg i.p. from the day of transplant.

![FIGURE 5.](http://www.jimmunol.org) GHS decreased the incidence of metastases (p < 0.02) and mortality in treated mice.
for 16 days, and various immunological assays were performed on day 21. Thymuses were tested for cellularity, and thymic subsets were analyzed by FACS (Fig. 7). Again, the most striking and significant effect was in the thymic development, and cellularity was significantly different in the treated group ($p < 0.01$, Fig. 7A). All of the subsets of thymocytes in the treated group were significantly increased ($p < 0.01$), with the exception of CD8$^+$/CD4$^-$ population (Fig. 7B). At the end of the study (21 days), we observed a numerical but statistically insignificant increase in proliferation of splenic T or B cells induced by anti-CD3 and anti-IgM Abs, respectively.

GHS increases cycling cells in the spleens of 9- to 10-month-old mice

To understand the effect of GHS on lymphoid cells, we adapted the protocol of Tough and Sprent (20) with the hope to track cells exiting from the thymus. After some initial attempt, we discovered that actually there was already an increase in cell number in spleen and BMC of 9- to 10-mo-old mice when they were treated with GHS for 3 days. There was a 130–140% increase in spleen cell number during this short treatment. By injecting BrdU into the mice we were able to label newly dividing cells in the spleen. We found that there was a $\sim$2-fold increase of BrdU$^+$ cells in the spleen (Fig. 8, $p < 0.0019$), whereas the increase in BrdU$^+$ cells in bone marrow was not statistically significant. The splenic BrdU$^+$ cells were both CD3$^+$ and B200$^+$. Therefore, it appears that one mechanism of the effect of GHS is promoting lymphoid cells to enter the cell cycle.

**Discussion**

Our studies show that GHS could generally duplicate the effect observed by treating mice with GH (1–3). We have assessed the immunological status of the mice to measure beneficial effect of GHS in the immune system. We observed statistically significant changes in immunological parameters in the young and old mice treated with GHS for 3–5 wk. The young mice showed an increase in the peripheral lymphocytes. The old mice showed an increase in thymic cellularity, when treated with GHS at 10 mg/kg orally or 2 mg/kg i.p. FACS analyses of thymic cell subsets showed an increase in the immature DN cells. There was some increase in Ab titer in the treated groups. These effects were apparent in the aging mice, as well as in the BMC-transplanted SCID mice, where the aging process and genetic circumstance put the thymus in a deficient mode. GHS was able to revert the deficiency and improve thymic development. Therefore, these results confirmed and extended all earlier findings with GH in the immune system.

A physiological consequence of the enhanced thymic development was the improved tumor resistance observed in the treated old mice inoculated with EL4. Although earlier data with GH consistently showed an effect on lymphoid cells, the physiological relevance of such enhancement was not addressed (3). It is well documented that tumor incidence increased with age (22), and our
results with the EL4 tumor system clearly show that metastases were rare in younger mice in this tumor model (Table 1). Upon treatment with GHS, old mice had a significantly lower (42% lower, p < 0.05) incidence of tumor metastases and no mortality (Table 1). We further showed that by limiting the tumor inoculum, the progression of the tumor was significantly retarded by GHS administration (Fig. 5). This improved resistance to EL4 was partly attributed to better cellular immunity in the generation of CTL to EL4. Taken together, GHS appeared to confer better immune resistance to a transplanted tumor in aging or geriatric mice. We plan to confirm these results in a spontaneous tumor system.

The mechanism of the effect of GH or GHS in thymic development is not very well understood. GHS receptor could not be detected in the thymus (13), and GHS does not affect proliferation of T or B cells in vitro (our unpublished observation). However, lymphoid cells are known to have GH receptors, which may contribute to the immune enhancing effect (1–3). It is intriguing that the effect is most profound on thymocytes. Perhaps GH induces some cytokine production, such as IL-7, that has been proposed to be a mediator of thymic development (23, 24). Using BrdU to label dividing cells in the spleen, we observed a higher number of dividing cells in treated mice, suggesting that GHS is inducing these lymphoid cells to enter the cell cycle. Consistent with these findings, there was a higher number of lymphoid cells in the peripheral blood of the treated young mice (Fig. 2). Similarly, thymuses of GHS-treated old and SCID mice had higher thymic cellularity than the untreated controls (Figs. 3 and 7). Both sets of data are compatible with increased cycling cells, as defined by BrdU in this study. The higher number of dividing cells was reminiscent of the transgenic mice with bovine GH, which also have enlarged spleen and more dividing cells in the periiphery (25). A more detailed study, focusing on the effect of cytokines, will decipher the downstream effect of GHS treatment on the lymphoid system.

In summary, we have shown a general immune enhancing effect of GHS in the mouse in various immunological compromised situations. Although preclinical studies in rodents does not always predict efficacy in humans, recent studies in humans have also suggested that treatment with GH releasing hormone showed better immunological responses in elderly people (26). Significant increase was observed in IL-2* T cells and CD20* B cells and T and B cell proliferation and basal IL-2 production in the treated individuals. Furthermore, a study using GH in AIDS for adjudant effect demonstrated some improvement over placebo, in producing Abs to HIV vaccine (27). Certainly more extensive clinical trials on GHS, focusing on immunological parameters, will eventually validate the efficacy of GH in patients undergoing thymic atrophy. Recent reviews on aging, AIDS, and other drug or irradiation depletion of thymus and T cell repertoire (28) imply that it would be beneficial to induce thymic activity to regenerate peripheral T cell repertoire. Our findings would suggest that GHS could fulfill that clinical need for AIDS patients and individuals undergoing chemotherapy and irradiation. GHS will, of course, have the advantage of being orally bioavailable, whereas GH is administered s.c. (29). Furthermore, GHS assumes a more physiological route of releasing GH, with the pulsating characteristics, as well as the feedback mechanism (11, 12), that appear more beneficial than bolus GH administration.

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References