Mechanisms of Ageing and Development, 57 (1991) 87—100
Elsevier Scientific Publishers Ireland Ltd.

EFFECTS OF LONG-TERM, LOW-DOSE GROWTH HORMONE THERAPY ON IMMUNE FUNCTION AND LIFE EXPECTANCY OF MICE

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(Received June 1st, 1990)
(Revision received July 25th, 1990)

SUMMARY

We have studied effects of long-term, low-dose growth hormone therapy on the immune function and life expectancy of Balb/c mice. Sixty male Balb/c mice were aged up to the time when they started showing signs of senescence and casual death (deaths started when they became 17 months old). The aged mice were divided into two groups of 26 mice each. One group received growth hormone (30 μg/mouse) subcutaneously twice a week for 13 weeks. The control group received an equal volume of saline for the same period. During this treatment period, 16 control mice died (61%) whereas only 2 of the hormone-treated mice died (7%). Four mice from each group were killed and immunological functions of splenocytes were evaluated. Hormone-treated mice had higher stimulation indices for pokeweed mitogen but not for Concanavalin-A. Total IgG production was decreased but IL-1, IL-2 and TNF production was increased. After a lag period of 4 weeks, growth hormone therapy was continued for another 6 weeks. One of the growth hormone treated mice died while the control group no longer existed. Splenocyte functions of the growth hormone treated mice were compared to those of young mice. The results showed no significant difference between cytokine production (IL-1, IL-2, TNF and IgG) in the young and the hormone treated groups. Stimulation induced by concanavalin-A and pokeweed mitogen however, was higher in the young group than the old group. The mortality curve obtained suggests that long-term low-dose growth hormone treatment prolongs life expectancy.

Key words: Growth hormone; Life span; Aging; Immune response; Longevity; Immunologic theory of aging

0047-6374/91/$03.50
Printed and Published in Ireland

INTRODUCTION

It is accepted that immune response declines with advancing age [1—3]. This decline may be responsible for the increase incidence of age-associated diseases, *i.e.*, late-occurring neoplasia, autoimmune and immune complex diseases [4]. Age-related disfunction of the immune system has been documented in almost all phases of immune function at the organ, systemic, and cellular level in the mouse, rat and human [5—10]. The first and perhaps fundamental, changes noted in an organ system is the involution of thymus that occurs shortly after sexual maturity [11]. Since one of the major lymphoid populations concerned with immunological recognition and regulation is the T-cell, the implications for maintenance of immune function are evident. Shortly after an individual reaches sexual maturity, the thymic epithelial cells are replaced by adipose tissue [11], leading to decreased thymic hormone secretion [12]. The best described thymic peptides involved in the maturation and regulation of T-cells are thymopoietin, thymulin and thymosin (fraction 5) [13—15]. Decreased thymosins are inversely correlated to an increase in age-associated diseases [11]. Whether the involution of the thymus and the decline of thymic hormones with advancing age are controlled as the function of the "biological time clock" [16] or by age-associated endocrinological imbalances [17] has not been fully clarified.

Life span in mice is correlated to the major histocompatibility complex, the master gene of the immune system [18]. In addition, regimens that have shown to increase longevity, such as calorie restriction [19] and lowering body temperature [20], also delay the age-related decline of immune functions [21]. Indeed, some investigators have shown that modulation of the immune function(s) may increase mean life expectancy [22—24]. These findings reinforce the "immunologic theory of aging" which was advanced by Walford in 1969 [25].

A pituitary-thymic axis to regulate immunologic functions has been postulated [26]. Thymic hormones modulate the pituitary gland [27] and pituitary hormones affect the function of lymphoid cells [28,29]. The observation that supports this network is that maximal thymic size occurs at puberty, the time of maximal secretion of growth hormone (GH) [30,31]. A decrease in thymic size, accompanied by a decline in plasma GH, is observed in aging subjects [32].

There are many reports suggesting that GH and possibly prolactin are required for the normal development and maintenance of some aspects of the immune system [33,34]. The important findings that reinforce this notion are: lymphoid cells have receptors for both GH [35,36] and prolactin [37] and both GH and prolactin can be used to augment a variety of immune responses [38,39]. Indeed, research in the 1960s showed that injection of young animals with pituitary extracts with certain hormonal activities could augment thymic size [40,41]. Furthermore, thymic implants or thymic hormones are known to partially restore the decline in several immune events that occur during aging [42,43].

We became interested in studying the effect of GH therapy on aging animal immune function and life expectancy following reports that GH therapy in aged rodents [44] and dogs [65] restores thymus structure and potentiates some of the immune functions. We found that long-term, low-dose GH therapy appears to prolong average life expectancy of experimental mice but the effect on immune functions was less conclusive.

MATERIALS AND METHODS

Mice

Male Balb/c mice were bred in our own facilities and maintained until they reached 15 months. This mouse strain has an average life span of approximately 21 months [45]. The aged mice were divided into two groups of 35 each, five mice per cage. One group of young adults (8—10 week-old) was also included for this experiment. All groups were kept for 8 weeks before starting hormone or saline therapy. At the time therapy was begun, 26 mice from each group of aged mice were still alive. Throughout the experiment all animals were maintained under conventional (nonbarrier) conditions of 20—24°C, 50—60% humidity and a 12—hour lighting cycle. The mice were monitored for common pathogens, including sendi virus.

Growth hormone therapy

One group of aged mice received two 30 μg subcutaneous injections of human GH (Sigma Chemicals, Co., St. Louis) per week. The second group and young group received an equal volume of saline solution. The treatment lasted 13 weeks after which four mice from each group were killed (by cervical dislocation) for assessment of immune function. The remaining mice were kept without treatment for 4 weeks (GH supplies were depleted). At the end of this lag period, all of the saline treated mice (control) were dead. Four mice from hormone treated or young groups were sacrificed. Splenocytes were isolated and immune function was evaluated. The second period of GH therapy lasted 6 weeks, then all mice were killed and splenocyte function was evaluated.

Assessment of splenocyte function

In this experiment we assessed cytokine production by: splenic macrophages (IL-1 and TNF), T-cells (IL-2) and B-cells (IgG). We also looked at stimulation response of splenocytes to concanavalin-A (Con-A) and pokeweed mitogen (PWM).

(a) Proliferation assay. Spleens were removed aseptically and placed in cold RPMI 1640 cell culture media. A single cell suspension was made by teasing the spleen apart and then passing it through a 26 gauge needle. The cells were washed three times with cold medium and resuspended (5×10^6 cells/ml) in complete medium (RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf

serum, 2 mM/ml L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin). All the cultures were incubated at 37 °C in a 5% CO_2 humidified incubator unless otherwise stated.

The lymphocyte proliferation assay was performed as described by Khansari *et al.*, [46]. Briefly, spleen cells were resuspended in complete medium to make a 5×10^6 cells/ml cell suspension. One hundred microliters of the cell suspension were placed into each well of a 96-well flat bottom microtiter plate (quadruplicate). Each well received 100 μl of medium containing either 1.0 μg/ml PWM or 2.0 μg/ml Con-A. Control cultures received 100 μl of the media only. Plates were incubated for 48 h and then pulsed with 0.60 μCi/well [^{3}H]thymidine (^{3}H-TdR). After incubation for an additional 18 h, cells were harvested onto a fiber glass filter disk using an automatic cell harvester, and the radioactivity of the filter disks was measured by a beta-counter. The stimulation index (SI) was determined by the formula:

$$SI = \frac{\text{Mean CPM (stimulated cells)}}{\text{Mean CPM (contol cells)}}$$

(b) Cytokine production. For interleukin-1 (IL-1) production, spleen cells were resuspended in complete RPMI 1640 medium to make a 10×10^6 cells/ml cell suspension containing 1 μg/ml Con A. One half of one milliliter of the cell suspension was placed in each well of a 24-well microtiter plate, then incubated for 2 h. At the end of incubation period the nonadherent cells were removed, the adherent cells were gently rinsed with warm RPMI medium. Then 500 μl of fresh complete medium, containing 10 μg/ml lipopolysaccharide (LPS) plus 10^{-5} M indomethacin, was added to each well. Following an incubation period of 48 h, the supernatants were recovered and stored at −20 °C until needed.

For interleukin-2 (IL-2) production, spleen cells were resuspended in complete RPMI medium to make a 5×10^6 cells/ml suspension. One milliliter of the cell suspension was placed in each well of a 24-well microtiter plate and incubated for 48 h. At the end of incubation, the supernatants were recovered and stored at −20 °C until needed.

Tumor necrosis factor (TNF) production was accomplished by resuspending spleen cells in complete medium to make a 10×10^6 cells/ml suspension. One half of 1 ml of the cell suspension was placed in each well of a 24-well microtiter plate, then incubated for 2 h. At the end of the incubation period, the nonadherent cells were removed and the adherent cells were gently rinsed. Then 500 μl of complete medium containing 50% rat growth factor (supernatant from Con-A stimulated rat spleen cells containing 0.1 M α-methyl mannoside) were added to each well. After a 24-h incubation, the supernatants were replaced with 500 μl of complete medium containing 10^{-5} M indomethacin and 1 μg/ml LPS. Cells were incubated for an additional 24 h. Supernatants were then recovered and stored at −20 °C until needed.

Immunoglobulin G (IgG) production was done by culturing spleen cells (1×10^6 cell/ml) in a 24-well microtiter plate in total volume of 0.5 ml per each well in the presence of 2.5 μg/ml PWM in complete medium. Cultures were incubated for 7 days. Eighty microliters of nutritional cocktail [47] were added to cultures daily. Culture supernatants were recovered at the end of the incubation period and stored at −20°C until needed.

(c) Cytokine activity assays. The IL-1 activities of the spleen cell culture supernatants were determined using the method described by ConIon [48]. Briefly, LBRM-33-1A5 cells were treated with mitomycin-C to abolish DNA synthesis [49]. The cells were then cultured in a 96-well microtiter plate in the presence of 0.5 μg/ml phytohemagglutinin (PHA) and various dilutions of supernatants recovered from LPS stimulated spleen cells. Control wells received either PHA only or media only. After 24 h incubation, 50 μl of a 8×10^4 cells/ml CTLL-2 (an IL-2 dependent cell line) cell suspension was added to each well. Cultures were incubated at 37°C for an additional 20 h then pulsed with 0.60 μCi ^{3}H-TdR for 4 h. At the end of incubation, cells were harvested onto fiber glass filter disks and radioactivity was measured by a beta-counter.

The IL-2 activities of supernatants recovered from Con-A stimulated spleen cells was determined by the method described by Gillis *et al.* [50]. Briefly, 1×10^4 CTLL-2 cells (in 100 μl complete RPMI 1640) were cultured in each well of a 96-well microtiter plate in the presence of various dilutions of Con-A stimulated culture supernatant. Cultures were incubated for 20 hours then pulsed with 0.60 μCi of ^{3}H-TdR for 4 h and harvested onto fiber glass filter disks. The radioactivity of the disks was measured by a beta-counter.

The TNF activities of supernatants were determined using the method described by Espevik and Nissen-Meyer [51] using WEHI 164 cell line (kindly provided by Dr. K.W. Kelley).

The IgG content of culture supernatants, recovered from PWM stimulation of spleen cells for 7 days, was measured by an ELISA as described elsewhere [52].

Statistical analysis

A Fisher's test was used to determine mortality differences between the GH-treated old group and the control (old saline-treated) group. Duncan's multiple range test was used to determine differences in immune functions among all groups. Differences were considered statistically significant when the *P*-value was 0.05 or less.

RESULTS

Effects of GH or saline treatment on Balb/c mice life expectancy

Two groups of 17-month-old mice (26 mice each group) were injected twice a week with either 30 μg/mouse GH or saline, respectively, for 13 weeks. At the end of

the treatment period four mice from each group were sacrificed and splenocyte functions were assayed. During this treatment period, 16 mice from the saline-treated (control) group died (61%) whereas only 2 of the GH-treated group died (7%). As shown in Fig. 1, the remainder of saline treated group died by the end of lag period whereas only one mouse from GH treated died.

Hormone therapy was continued for another 6-week period. During this time one mouse died. The remainder were sacrificed and splenocyte functions were assayed and compared with those of young (6-month-old) mice.

Using Fisher's test, we found that mortality in the control group was significantly ($P < 0.001$) higher than in the hormone treated group. These results suggest that long-term GH therapy prolongs the average life expectancy of the hormone treated mice significantly.

Effects of GH or saline treatment on splenocyte function

Four mice from each group (old saline-treated, old GH-treated and young were sacrificed at the end of the first treatment period. Splenocytes were isolated and their functions were assessed by determining various cytokines (IL-1, IL-2, TNF, and IgG) production and lymphocyte proliferation in response to Con-A and PWM. Results are presented in Table I. We found no difference in IL-1 and TNF production between the young and old group but the GH-treated group had higher IL-1 and TNF production. Interleukin-2 production was decreased significantly in the old group. The hormone-treated group had a higher level of IL-2 production compared to the old saline-treated group but still less than that of the young group. In contrast, total IgG in the old group was significantly higher than in both the young and hormone-treated groups. It appeared that hormone therapy, however, has reduced levels of IgG comparable to that of the young group.

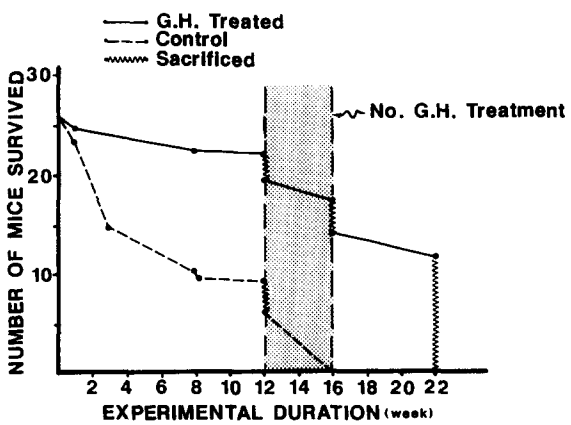

Fig. 1. Mortality curve of aged 19-month-old mice treated with either 30 μg/mouse of human GH (●——●) or saline (●------●) twice per week for the period shown.

Stimulation of splenocytes in response to Con-A and PWM is shown in Table I. There is a significant difference in stimulation indices between young and old saline-treated groups for both mitogens. Growth hormone therapy did not improve the stimulation index for Con-A, but it enhanced the stimulation index for PWM.

Due to lack of GH, the second treatment started one month after the first treatment was completed. This treatment lasted six weeks. At the end of treatment, all mice were sacrificed and splenocyte function was assessed by determining various cytokine production and lymphocyte proliferation. A group of young mice (6-month-old) was also included in these experiments. Results are summarized in Table II. We found no significant differences in cytokine (IL-1, IL-2, TNF and IgG) production between 6-month-old (young) and 25-month-old groups. In contrast, proliferation of splenocytes in response to Con-A and PWM was significantly lower in GH-treated old mice than in young mice. These data suggest that GH therapy has rejuvenated some of the immunocyte functions.

DISCUSSION

The primary objective of gerontologic research is to explore the mechanisms of aging and the methods to prevent or at least delay the onset of age-associated diseases. If successful, the period of productive life will be enhanced and average life expectancy will be prolonged. Many investigators were able to prolong life expectancy by genetic manipulation [53], splenectomy [54], elimination of suppressor cells [55], caloric restriction in rats [56] and lowering body temperature [57]. All these manipulations also potentiate the immune system of the animal.

Advancing age parallels decline in immune competence (reviewed in Ref. 58). The clinical evidence of this immunosenescence is the increased susceptibility to certain types of infections, autoimmune and immune complex diseases and cancer. Furthermore, there is evidence that vaccination is less successful in inducing immunity in older subjects than in young. These observations have led investigators to employ immunologic intervention to delay the immunosenescence process. Methods employed include cell grafting [59], chemotherapy [60], thymic peptide therapy [11] and oral antioxidant intake [23,61]. Even though all of these immunomodulators possess some immunorestorative activities, their effect on age-associated diseases, hence effect on mean life span, has been marginal.

Berczi [26] reviewed the effects of pituitary hormones on the immune system. It seems that GH is an intermediary in the pituitary-thymic axis. An observation that supports such an axis is that maximal thymic size (at puberty) parallels the time of maximal GH secretion. Conversely, a decrease in thymic size is accompanied by a decline in plasma GH in the postpubescent period.

We became interested in GH as an immunopotentiator for boosting immune function of aging mice. We chose a dose (30 μg/mouse) that does not induce an antibody response (preliminary observation) to be administered for long-term therapy.

TABLE I

EFFECT OF GH TREATMENT ON MOUSE SPLENOCYTE FUNCTIONS (FIRST TREATMENT)

Mice group	*Cytokine activity*								*Proliferation assay*			
	IL-1[a] (cpm)	*P<*	*IL-2[a] (cpm)*	*P<*	*TNF[b]*	*P<*	*IgG[c] (ng/ml)*	*P<*	*Con-A[d] (SI)*	*P<*	*PWM[d] (SI)*	*P<*
Young	8105 ± 443	N.S.*	3115 ± 1645	0.05	47 ± 4	N.S.	10 ± 4	0.05	367 ± 85	0.05	58 ± 20	0.05
Old	7332 ± 534	0.05	107 ± 24	0.05	51 ± 3	0.05	30 ± 9	0.05	75 ± 95	N.S.	13 ± 10	0.05
Old GH-treated	10 277 ± 626		480 ± 200		64 ± 7		16 ± 5		68 ± 41		24 ± 8	

[a]No reference standard for IL-1 or IL-2 was available to use at the time culture samples were titrated; therefore, the values are expressed in terms of CPM.
[b]Values represent mean percent killing of four samples of each group ± S.E.
[c]Values represent mean ng/ml of four samples of each group ± S.E.
[d]Values represented mean SI of four samples of each group ± S.E.
*Not significant.

TABLE II

EFFECT OF GH TREATMENT ON MOUSE SPLENOCYTE FUNCTIONS (SECOND TREATMENT)

Mice group	*Cytokine activity*								*Proliferation assay*			
	IL-1[a] *(cpm)*	*P<*	*IL-2*[a] *(cpm)*	*P<*	*TNF*[b]	*P<*	*IgG*[c] *(ng/ml)*	*P<*	*Con-A*[d] *(SI)*	*P<*	*PWM*[d] *(SI)*	*P<*
Young	8427 ± 1114	N.S.*	1572 ± 1133	N.S.	80 ± 8	N.S.	6 ± 3	N.S.	216 ± 61	0.05	22 ± 7	0.05
Old GH-treated	8188 ± 1289		1081 ± 1162		71 ± 9		10 ± 11		85 ± 51		10 ± 4	

[a]Values represent mean CPM of nine samples (young group) and 15 samples (old group) ± S.E.
[b]Values represent mean percent killing of nine samples (young group) and 15 samples (old group) ± S.E.
[c]Values represent mean ng/ml of nine samples (young group) and 15 samples (old group) ± S.E.
[d]Values represent mean SI of nine samples (young group) and 15 samples (old group) ± S.E.
*Not significant.

After the first months of GH therapy we observed gross physical differences between the hormone-treated group and the saline-treated group. The mortality curve (Fig. 1) showed striking differences in longevity between experimental and control groups. Since our primary objectives, in the beginning, were to look at immune functions, we did not include other strains of mice. Whether this effect is specific for strains and/or sex needs to be investigated. Administration of GH was begun when the mice were showing signs of senescence and deterioration of their immune system (neoplasia, infection and casual death). Thus the observed prolongation of the mean life expectancy in the hormone-treated group seems to be due to a delay or prevention of age-associated disorders.

The effect of GH therapy on immune functions was determined by splenocyte proliferative response to Con-A and PWM. We found no restoration in Con-A response even after a second period of GH therapy (Table I). In the case of PWM some improvement was observed but the response was still lower than that of young mice. This was not surprising to us since 30 μg GH/mouse was not sufficient for activating lymphocytes *in vivo*. A similar finding for antibody production by mitogen and prolactin therapy has also been reported [62].

We have also looked at various cytokines production following GH therapy. Cytokines have a central role in all aspects of immune response. In addition they are a means of communication (regulatory) between the central nervous system and immune system [63]. The IL-2 production was increased by hormone therapy (Table I) so that by the end of the second treatment period we did not observe a significant difference between IL-2 production in young and old mice (Table II). This is consistent with others who reported that IL-2 synthesis could be restored by implantation of GH_3 pituitary adenoma cells in rats [64]. This also supports the assumption that T-cells in aged animals are not inherently defective and their function can be augmented by exogenous GH [44]. Whether the longevity of GH treated mice is correlated to IL-2 production enhancement need to be investigated. The increased production of IL-2, however, may explain at least, part of GH mode of action as an immunomodulator. Goff *et al.* [65] found that GH treatment stimulates thymic peptides production. The general functions of those peptides is to induce differentiation of pre-T cells and to modulate T-cell functions [66].

We found levels of total IgG higher in old saline-treated mice than in young mice; growth hormone therapy appeared to reduce the level of IgG. This finding agrees with other investigators who reported that B-cell response to PWM declines with advancing age, but the level of immunoglobulin, mostly autoantibodies, increases [reviewed in 67]. We did not find any decline in production of IL-1 or TNF in aged mice nor any enhancement following GH therapy (following second period of GH therapy, see Table II). In case of IL-1, our findings contrast that of Inamizu *et al.* [68] who found IL-1 production in aged Balb/c is reduced by 40%; however, the same report indicated that addition of indomethacine into culture increased the production of IL-1 considerably. We incorporated indomethacine in our culture media

for blocking PGE_2 production. This may be a contributing factor for detecting higher IL-1 levels in aged mice.

ACKNOWLEDGEMENTS

This research was supported by funds in part from the North Dakota Agricultural Experiment Station and the USDA, Section 1433, Animal Health Grant; Authors wish to thank Dr. J. Colville for editorial help and Ms. S. Erickson for typing this manuscript.

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