Influence of growth hormone on the adult canine thymus

and evaluation of endocrine function in puppies from

immunodeficient dwarf parents

by

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EXPLANATION OF THESIS FORMAT

The thesis has been divided into 3 sections, each of which is to be submitted individually for publication. Section I is basically a review of the literature as a background for the research presented in sections II and III. At the beginning of sections II and III is an introduction with additional literature review appropriate for the research presented in that particular section. At the end of each individual section is a summary or discussion of the information and or research presented in that section.

SECTION I. HYPOTHALAMIC-ADENOHYPOPHYSEAL INFLUENCE ON THYMUS STRUCTURE

AND FUNCTION

Introduction

There is considerable experimental evidence of an association

between the immune system and the endocrine system, more specifically

the thymus and the endocrine system (12,24,41,72,97,118,). A proper

balance of hormones at a critical time in development is apparently

necessary for proper formation and function of the immune system as the

organism matures and later on in life (39,97). Hormones such as growth

hormone (GH), thyroxine and cortisol have effects upon the thymus (18)

and the thymus has effects upon endocrine glands such as the testes,

adrenal glands (23) and anterior pituitary. For example, thymectomy in

mice causes degranulation of the acidophils of the anterior hypophysis

(93), and thymosin fraction 5, an extract of bovine thymus, when

injected into monkeys causes increases in adrenocorticotrophic hormone,

cortisol and beta endorphin (64).

Thymus and Immune Function

The thymus gland plays an important role in immune function. It was

demonstrated in the early 1960s that neonatal thymectomy of mice and

rabbits led to immune deficiency (57,82). Thymectomized animals had a

decreased ability to produce antibodies to T-dependent antigens,

decreased ability to reject skin and tumor transplants, failure to

develop normal lymphoid structure in lymph nodes and spleen, and they exhibited poor growth and short life span (57,82). The thymus therefore is thought to be important in formation of the cellular arm of the immune system. It is important for normal development of the lymph node paracortical areas and periarteriolar areas of the splenic white pulp (111).

The Thymus as an Endocrine Gland

The experimental evidence which led to the concept of the thymus as an endocrine gland has recently been reviewed (58,66). Studies in which thymus transplants placed in millipore filters not permeable to cells had a beneficial effect on the development and function of the immune system in neonatally thymectomized mice support this concept. The demonstration that the injection of thymic extracts into mice would partially correct the effects of neonatal thymectomy also gives credence to the idea that the thymus exerts at least some of its effects upon the immune system through the production and release of chemical mediators. Substances shown to influence the development of lymphoid cells have subsequently been identified in serum or extracts of the thymus (58,66).

Thymosin fraction 5 is an extract of bovine thymus that contains many small peptides, some of which have been shown to have an effect upon the development and function of lymphoid cells. The function and structure of some of the peptides contained in this extract have been reviewed by Incefy (66) and Goldstein et al. (56). They are divided

into 3 groups, alpha, beta, and gamma, depending upon their isoelectric points (56,66). One such peptide, thymosin alpha 1, is made up of 28 amino acids. An identical peptide has been shown by radioimmunoassay to circulate in human blood (66). It is a strong inducer of helper T-cells in mice, and increases the response of murine lymphocytes to mitogens. Thymosin alpha 7, another hormone found in thymosin fraction 5, induces suppressor T-cells. Another peptide found in the same extract is thymosin beta 4. It contains 43 amino acids and stimulates T-cells at an early stage of differentiation, inducing the expression of terminal deoxynucleotidyl transferase. Thymosin beta 3, also in fraction 5, is very similar to beta 4 in structure and function (56,66).

Thymopoietin, another extract of bovine thymus, has been identified and its amino acids sequenced. It contains 49 amino acid residues, and exists in two forms, thymopoietin I and thymopoietin II, which differ by substitution of 2 amino acids. The active component of thymopoietin is made of 5 amino acids and has been synthesized in the laboratory. Thymopoietin induces the expression of T-cell alloantigens in the bone marrow cells of mice in vitro (66). It also stimulates T-cell functions in vivo, for example enhanced rejection of carcinomas in mice (3). Human plasma contains substances with activity like that of thymopoietin (66).

Thymic humoral factor (THF), an extract of bovine thymus, was isolated and characterized by Trainin et al. It has been recently reviewed by Bach (3) and Incefy (66). THF consists of 30 amino acids and causes differentiation and maturation of thymus-derived

lymphocytes. It will enhance the mixed lymphocyte reactions of lymphocytes from spleen and thymus of intact and thymectomized mice. THF also markedly increases the response of human peripheral blood lymphocytes to mitogens such as concanavalin A and phytohemagglutinin. Therapy with THF in immunodeficient human patients has improved cell mediated immunity (3,66).

Factor thymique serique (FTS) was isolated from the serum of swine and characterized by Bach et al. The literature on the structure and function of this thymic hormone has recently been reviewed (4). It contains 9 amino acids which have been sequenced, and the hormone in the serum of swine, humans and cattle is identical. The use of immunofluorescence has demonstrated that FTS is produced by epithelial cells in the thymus. The fact that an identical compound has been isolated from the extract of bovine thymus of Goldstein et al., thymosin fraction 5, helps confirm the thymic origin of FTS. The FTS peptide chelates zinc which is required for activity. The active form of the hormone containing zinc is called thymulin. Thymulin induces T-cell markers on spleen cells of adult thymectomized mice, normal bone marrow cells from mice and humans, nude (athymic) mouse spleen cells, and peripheral blood lymphocytes from patients with immunodeficiencies. The effect on any given parameter of T-cell function may depend upon the dose of thymulin given and the immune status of the subject. For example, delayed hypersensitivity to dinitrofluorobenzene is increased in adult thymectomized mice but decreased in normal mice treated with

thymulin. The clinical use of thymulin in human patients with immunodeficiency diseases has given positive results in some cases (4).

Hypothalamic-Adenohypophyseal Regulation of Thymus Function

Hypophysectomy in rats has been shown to cause atrophy of the thymus (18,115). Depression of immune responses has also been noted after hypophysectomy in mice and rats (11,32,53,90,107). Hypophysectomized rats had depressed antibody responses to sheep red blood cells (SRBC) (11,32,90) and Escherichia coli lipopolysaccharide (11), slowed allograft rejection (32), and decreased DNA synthesis in thymocytes (90). These depressed immune reactions could be restored to normal by GH (90,124) or prolactin therapy (11,85). According to Berczi et al., GH was not as effective as prolactin (11). Treatment of hypophysectomized rats with GH but not thyroxine, also caused an increase in thymic weight (46). Spleen cell natural killer activity against the mouse lymphoma cell line (YAC) tumor cells, and in vitro responsiveness of spleen cells to produce antibody to SRBC was suppressed in hypophysectomized mice. These could be restored to normal with GH therapy (53,107). Recovery of the ability to produce antibodies to SRBC, reject skin allografts and restore leukocyte numbers in irradiated adult rats was impaired if they were first hypophysectomized (32). Andenohypophyseal hormones therefore may play a role in maintenance of thymus and cell mediated immune function in adult life.

More evidence for the importance of hypophyseal hormones in the development of the thymus and cellular immunity is the fact that rabbit anti-mouse hypophysis serum caused thymus atrophy when given to young mice (93). A wasting disease like that seen with neonatal thymectomy was also observed. The anti-mouse hypophysis serum was given at the age when thymectomy causes wasting disease in mice (93). Anti-bovine somatotrophic hormone (growth hormone) serum (ASTH) also caused the same wasting syndrome in mice (94). Simultaneous treatment with GH prevented the disease (94). Rabbit anti-mouse hypophysis serum was shown to bind specifically with the acidophils of the anterior pituitary gland. The mechanism of action therefore in causing wasting was probably a decrease in production or release of GH or perhaps other adenohypophyseal hormones (96). Therapy with GH and thyroxine alone or together would not prevent wasting in neonatally thymectomized rats, indicating the effect of GH in preventing the wasting disease in anti-hypophysis treated animals is by its action on the thymus (35).

Experiments using nude mice have contributed to our knowledge of the importance of the hypophyseal hormones in the development of thymus function. Nude mice have a congenital, complete lack of a thymus and do not reject allogeneic skin grafts. The ability to reject skin grafts can be restored with thymic grafts. Thymic grafts however would not restore the ability to reject allogeneic skin grafts if anti-hypophysis serum was also given (98).

Growth hormone has been shown to cause increased uptake of radiolabeled metabolites by rat thymocytes in vitro (123,124). Human GH in vitro will stimulate proliferation of human peripheral blood lymphocytes (73). Mouse spleen cells responded in vitro to GH with the generation of cytotoxic T-cells (116). Growth hormone therefore appears to have a direct effect on thymocytes and lymphocytes. This effect appears to be mediated by specific GH receptors on those cells. Receptors have been identified on rat thymocytes (62), human lymphocytes (76), and calf and mouse lymphocytes and thymocytes (2).

The pituitary dwarf mouse has proven to be an excellent model for the study of the interaction of the hormones of the anterior pituitary and the thymus. The Ames and the Snell-Bagg dwarf mouse are both afflicted with a wasting syndrome starting at weaning and have a shortened life span (5,28,95). The immune system, especially the cell mediated arm, seems to be deficient. The thymus in dwarfs is like that of normal mice at 15 days of age, but it is markedly atrophied by 30 days of age (36). The peripheral lymphocyte count is low (31,36), T-cell dependent areas of the peripheral lymph nodes and spleen are poorly populated (5,31,36,95,128), and the thymus is small with a decreased number of lymphocytes (5,26,28,95).

Immune function studies in dwarf mice indicate a defect of cell mediated immunity. This is manifest as decreased antibody response to sheep red blood cells, which are thought to be T-dependent antigens

(5,30,31,95). The graft-versus-host reaction of spleen cells from dwarf
mice is also depressed (31). Dwarf mice have lower plasma levels of the
thymic hormone (FTS) than their normal littermates (40,91).

Pituitary dwarf mice are deficient in GH (28,36,95), thyroxine
(36,95), prolactin and adrenocorticotrophic hormone (28). Growth
hormone therapy alone improved the lymphoid structure of thymus and
spleen in these pituitary dwarf mice (128). Growth hormone with
thyroxine also reconstituted the lymphoid tissues including the thymus,
with a concomitant improvement in the antibody response to T-dependent
antigens, (SRBC) (6,95) and increased body growth (7). Growth hormone
and thyroxine therapy also increased the depressed level of serum thymic
factor (FTS) to normal (40). In other studies, GH and thyroxine
together prevented wasting and prolonged life, but the reversal of the
wasting disease and restoration of the immune system with hormone
therapy did not occur if the mice were first thymectomized (37,38).
Treatment of pituitary dwarf mice with lymphocytes from normal 40 day
old mice prevented the wasting disease and slowed premature aging
(38). The immune deficiency in dwarf mice therefore appears to be
caused by a lack of immunocompetent T-cells, because of a thymic
developmental defect, which in turn is caused by deficiency of GH and
possibly other pituitary hormones. Prolonged nursing or intraperitoneal
injections of mouse milk in Snell and Ames dwarf mice also prevented the
T-cell depletion, lymphopenia, thymic atrophy and depressed response to
sheep red blood cells (27,29). The factor in milk that restores

immunity in dwarf mice may be a pituitary hormone such as GH or prolactin.

A sex-linked dwarfism has been reported in chickens in which there is immunodeficiency. Treatment with GH increased humoral immunity and the size of the bursa of Fabricius. Therapy with thyroxine caused an increase in the size of the thymus (80).

Reports of human infants with panhypopituitarism and recurrent infectious diseases have been noted (67,114). Two of these were reported to have a small thymus or total lack of a thymus at autopsy (114,120). One baby was noted to have a small thymus and poor immune function. She had been maintained on cortisol and thyroxine therapy but did not show an improvement in immunocompetence until GH was added (13). Children with isolated GH deficiency have been noted to have T-cells that respond poorly in allogeneic mixed lymphocyte reactions (MLR) and non-T cells that are poor stimulators of MLR. They were also noted to have increased proportional numbers of OKT8+ (suppressor-cytotoxic) T-cells and B-cells (59).

Immunodeficient dwarfism has been reported in Weimaraner puppies (105). These puppies were afflicted with a wasting syndrome which began after weaning. There was severe hypoplasia of the thymus, but T-dependent areas of lymph nodes and peripheral lymphocyte counts were normal. There was a depression of the response of peripheral lymphocytes to phytohemagglutinin (PHA). They were confirmed to be GH deficient by measuring GH by radioimmunoassay before and after

clonidine stimulation. Administration of thymosin fraction five eliminated the wasting disease but did not restore the thymus to normal or improve the response of lymphocytes to PHA (105). Therapy with GH however did normalize the thymus and prevent the wasting syndrome (106).

The hypothalamus-adenohypophysis also affects the thymus through adrenocorticotrophic hormone (ACTH) and the adrenocortical hormones. The increase in cortisol associated with stress in humans causes severe atrophy of the thymus with reduction of lymphocyte numbers within 2 to 3 days, followed by regeneration within 8 to 10 days of the stressful event (14). Rats treated with ACTH or cortisone had severe destruction of their thymuses with many thymocytes destroyed within 48 hours (129). The elements of the thymus were decreased similar to that seen with old age atrophy, such that no differentiation between cortex and medulla could be made. Adrenalectomy on the other hand caused an increase in the size and cellularity of the thymic cortex and medulla (129). In rats, stress causes a decrease in GH (102). Dogs and humans with hyperadrenocorticism have decreased release of GH in response to normal stimulators of GH release. The response returns to normal when the hypercortisolemia is corrected (84,92,121). Corticosteroid therapy in humans also causes a lack of GH response to normal stimulators of release (50). A lack of the trophic effect of GH may then be at least partially responsible for the thymic atrophy associated with stress and increased adrenocortical hormones. In certain types of stress in

people however, plasma GH is increased (1,21). This increase in GH may then be a response to counteract the effects of cortisol as demonstrated in hypophysectomized mice in which GH restored immunity and prevented the immunosuppressive effects of corticosterone (53).

Aging and Thymus Involution

Part of normal aging is deterioration of immune function. This is evidenced by a decreased ability of mice and humans to produce antibodies to T-dependent antigens (20,54,103,130). Depression of T-helper activity and an increase in T-suppressor activity may be responsible for this decline in antibody formation (20,54,103). Aging in humans has been associated with a decreased ability to mount delayed type hypersensitivity reactions to antigens which normally cause such a reaction (103). The response of lymphocytes in vitro to mitogens such as phytohemagglutinin (PHA) is significantly decreased in aged humans and dogs when compared to younger controls (52,72,103). The ability of mice to resist challenge with syngeneic and allogeneic tumor cells in vivo also declines with age (79). All of this evidence then points to an age associated decline in immune function. Decreasing immune function has been associated with an increase in the incidence of autoimmunity and neoplasia in aged individuals (71).

The thymus peaks in size and activity at puberty or young adulthood and progressively declines with advancing age (72,88,113,126). In dogs,

there is a significant correlation between age and the number of
lymphocytes in the thymus (88). Studies in humans have also shown that
the thymic cortex involutes with age with a decrease in the number of
lymphocytes (72,113). This decrease in thymic cortical size appears, at
least in humans, to follow an exponential curve (126).

It is a popular belief that the age-related decline in immune
function is closely associated with or perhaps caused by thymic
involution (41,71,72,131). Plasma thymic hormone levels have been shown
to decrease with age along with atrophy and involution of the thymus
(55,77). Thymosin fraction 5, an extract of bovine thymus, has been
shown in vitro to enhance the mixed lymphocyte reaction (MLR) of
peripheral lymphocytes from aged and young humans. Lymphocytes from the
aged individuals had lower mixed lymphocyte reactions than lymphocytes
from the young individuals before treatment, but the reactions were
equivalent for both groups after treatment (19). Thymosin alpha 1 in
aged mice in vivo will restore helper T-cell activity and increase
antigen specific T-cell dependent proliferation of lymphocytes (51).
The fact that thymic hormone levels decrease with age and have been
shown to restore some of the impaired cellular immune functions that
also decline with age implies that a decline in the production of thymic
hormones by an involuting thymus gland is at least partially responsible
for immunologic senescence (41,72).

As the thymus and the cell mediated immune system involute with
age, GH secretion also declines. In aged men, the amplitudes of sleep

related GH peaks are significantly reduced when compared to young
controls (8,15,99,117). Growth hormone release, in response to normal
stimulators of its release is also reduced in aged men (8). Mean 24
hour plasma GH levels are reduced in older men as compared to young men
in some reports (8,47,81), but not in others (99). Some authors
indicate baseline GH levels in rats decrease with age (25), but others
indicate they do not (49). In dogs, plasma GH levels are significantly
higher in puppies than in adults (127).

Growth hormone then has a definite role in normal thymic and cell
mediated immune system development and function. This has been shown to
be important in young developing animals; GH and thyroxine therapy
prolonged life in immunodeficient dwarf mice (37,38). Endocrine
influence on the immune system in adulthood also appears to be
important; hypophysectomy of adult rats inhibited recovery of cell
mediated immunity after irradiation (32). Thymic involution and immune
senescence then may be due to or controlled by a concomitant reduction
in GH secretion. Other authors have suggested this association between
immune senescence and endocrine homeostasis (39,71).

The thymus appears to continue to have an effect upon immune
function in adult life as indicated by adult mice showing a decrease in
graft versus host reactions when thymectomized in adulthood (125).
Administration of GH to adult animals then may stimulate thymus function
and prevent or reverse senescence of the cell mediated immune system and
improve health in old age. This hypothesis has been tested in adult

dogs using bovine growth hormone (83a). The thymus was biopsied via

thoracotomy prior to therapy to determine that it was atrophied. Bovine

growth hormone was administered subcutaneously in 14 doses over a 28 day

period. Two weeks after the last dose the dogs were euthanatized and a

necropsy performed. All of the GH treated dogs responded well with a

marked increase in numbers of lymphocytes in the thymus with obvious

cortex and medulla compared to pretreatment thymus in which no

distinction could be made between cortex and medulla in most of the

dogs. This then indicates that GH may be useful in preventing or

reversing the senescence of the cell mediated immune system.

Summary

The endocrine system then is integrally involved in normal

development, function and aging of the immune system. This has been

shown experimentally by hypophysectomy, the use of antihypophysis serum,

and the demonstration of GH receptors on lymphocytes and thymocytes.

Natural models such as the immunodeficient pituitary dwarf mouse,

pituitary dwarf human patients, immunodeficient dogs with isolated GH

deficiency, and immunodeficient dwarf chickens have contributed greatly

to the idea of neuroendocrine regulation of immune function. The key to

immune senescence and aging may involve an imbalance or deficit of one

or many hormones including GH, prolactin, thyroxine and other

hypophyseal products. Further research is needed to assess the ability

of these hormones to restore the aged or deranged immune system, and to

evaluate immune function in patients with endocrinopathies. Compounds

which stimulate the release of these hormones should also be tested for

their ability to affect immune function. The use of hormones

therapeutically then may also prove useful for treating disease,

restoring immune function, and the prevention or reversal of aging.

The discovery and characterization of the structure and function

of the thymic hormones also has improved understanding of the function

and regulation of the immune system. The clinical use of these hormones

in primary and secondary immunodeficiencies, autoimmune disease,

neoplasia, and virus and fungal infections may prove to be of

considerable benefit to medicine and the overall health and longevity of

animals and man. They may also prove useful as a measure of thymic or

cell mediated immune function in a variety of pathologic and physiologic

conditions.

SECTION II. THE EFFECTS OF GROWTH HORMONE ON THE ADULT CANINE THYMUS

Introduction

The thymus is known to be very important in the development of the cell mediated immune system (111). This was shown dramatically by Good et al. and Miller when they observed the effects of thymectomy on mice and rabbits (57,82). The thymus normally involutes with advancing age in most mammals including man (113) and the dog (88), beginning shortly after puberty.

The proper function of the immune system, especially T-cell function, wanes with age (130). Depressed ability to mount delayed type hypersensitivity reactions, decreased responses to T-dependent antigens and lesser ability of lymphocytes to undergo mixed lymphocyte reactions and respond to mitogens in vitro all occur with aging (19,20,103). Thymic involution is thought to play a pivotal role in this immune senescence (71,79). In older humans, the levels in plasma of some thymic hormones are reduced (55,77), and administration of thymic hormones improves some age associated T-cell deficiencies (19,51). Thymic involution then is important in the decline of immune function with aging which is perhaps mediated by a decrease in thymic hormone production.

Growth hormone (GH) appears to have an effect upon the normal development and function of the immune system, particularly the thymus and cellular immunity. Pituitary dwarf mice are GH deficient and have

severe deficiencies in cellular immunity including decreased peripheral lymphocyte counts, depletion of T-dependent areas of lymph nodes and severe early atrophy of the thymus (5,29). These defects can be restored to normal by therapy with GH (5). Dwarfs with immunodeficiency that could be significantly improved with GH therapy have also been reported in humans (13) and dogs (106).

Further evidence for a role of the hypophysis and growth hormone (somatotrophic hormone) in cellular immunity is provided by the use of anti-somatotrophic hormone serum (ASTH) in mice. This serum caused the same immunodeficiency and wasting syndrome as that seen in dwarf mice, and could be prevented by administration of GH (94). Growth hormone therapy however could not prevent immunodeficiency when ASTH-treated rats were also thymectomized as neonates (35). This indicates the effect of GH in preventing the immunodeficiency in ASTH-treated animals is through its action on the thymus.

The concentration of GH in plasma of men has been shown to decrease with advancing age (8,47,81), especially the sleep related peaks of GH secretion (8,99,117). Plasma growth hormone in dogs is also significantly less in adults than in puppies (127).

Because GH has a definite effect on thymic development in young animals and plasma levels decline with age along with an involution of the thymus and the cell mediated immune system, it is hypothesized that this decrease in GH secretion is at least partially responsible for thymic involution. Administration of GH then to adult or aged animals

may prevent thymic involution or restore an involuted thymus and declining immune function to normal. Growth hormone therapy has been tested in old men and rats, however immune function and thymic morphology were not studied (34,104). The purpose of this experiment was to test the hypothesis that GH treatment of adult dogs with age atrophied thymuses would cause regeneration of the thymus gland, and thereby reverse the age associated decline in cell mediated immunity.

Materials and Methods

Animals

Eight young adult dogs (estimated to be approximately 2-5 years old) were obtained from the laboratory animal resources unit of the College of Veterinary Medicine, Iowa State University. Two dogs of known age and breeding were obtained from a commercial kennel, one 4.5 year old West Highland White Terrier female (dog 15) and one 3.5 year old Miniature Schnauzer female (dog 16). All dogs were evaluated by physical exam, complete blood count, urinalysis, fecal flotation for parasite ova, filtration test for heartworm microfilariae and serum chemistries including glucose, alanine transaminase, alkaline phosphatase, calcium, phosphorus, sodium, potassium, urea nitrogen, and albumin. Blood was also obtained for lymphocyte blast transformation (LBT) studies in response to non-specific mitogens performed as previously described (105,106). If fecal flotation was positive, the dogs were treated with pyrantel pamoate or dichlorvos depending upon the

parasite present, and a subsequent fecal flotation was performed to assure therapy was adequate.

Experimental design

If the dogs were found to be healthy, a thoracotomy was performed to obtain a biopsy of the thymus gland for histologic evaluation. If the biopsy results indicated thymus tissue was present and involuted, 5 dogs were randomly chosen to be treated with bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, Mo.), and 5 dogs were treated with pituitary derived bovine growth hormone (BGH) (Monsanto Co., St. Louis, Mo., Lot No. m-34-12162) at a dosage of 0.1 mg./kg. of body weight given subcutaneously daily for 5 doses, then every other day for 5 doses and then every third day for 4 doses. Therapy was begun approximately 10 days to 3 weeks after thoracotomy. A complete blood count and LBT were performed weekly on blood from each dog. Eight to 9 weeks after thoracotomy, and 2 weeks after the last dose of BGH or BSA, the dogs were euthanatized with sodium pentobarbitol intravenously and necropsied. Thymus was again evaluated histologically and all other major organs and all other endocrine glands evaluated for pathologic lesions.

Results

Thymic histology in BGH treated dogs (Table 1)

All dogs prior to therapy had moderately to severely atrophied thymus glands on histologic examination. They were characterized by

multiple small nodules of thymus consisting of small lymphocytes,
epithelial cells, Hassall's corpuscles, blood vessels, and cysts lined
by ciliated cells. In general, a distinction between cortex and medulla
could not be made.

After BGH treatment, all 5 treated dogs had a regenerated thymus
gland. The glands consisted of many lobules of thymus within a delicate
stroma of adipose tissue. There was a dense population of small, mature
and in some cases larger more immature lymphocytes in the cortex.
Approximately 5% of the cortical cells were large epithelial cells. A
clear distinction between cortex and medulla could be made and the
cortex was generally larger than before treatment. The medullary areas
contained small lymphocytes and some had large lymphoblasts as well.
There were epithelial cells and Hassall's corpuscles present. Some of
the glands contained low numbers of eosinophils and some contained
germinal centers, generally at the cortico-medullary junction
(Figure 1).

Thymic histology in BSA control dogs (Table 1)

Prior to therapy, the 5 BSA treated dogs had thymuses similar to
those of the BGH treated dogs before treatment. After the
administration of BSA, the thymus glands of the control dogs had not
regenerated as consistently or as markedly as the BGH treated dogs.
Dogs 11 and 19 did however have marked regeneration of the thymus

similar to the dogs that were injected with BGH. Dog 13 had an increase
in germinal centers, but did not show the typical growth in lobules and
expression of a cortex and medulla of the growth hormone treated
dogs. The thymus of dog 15 had a very slight increase in lymphocytes,
and that of dog 18 showed only a very mild increase in lymphocytes and
cortico-medullary distinction (Figure 2).

Table 1

Summary of histologic responses of thymuses of BGH and BSA treated dogs

Dog #	Breed	Sex	Weight Kg.	Trmt.	Histologic Response of Thymus
4	Poodle	F	3.0	BGH	Marked regeneration
5	Mix	M	3.2	BGH	Marked regeneration
9	Mix	M	4.8	BGH	Marked regeneration
10	Mix	F	3.0	BGH	Marked regeneration
16	Schnauzer	F	6.0	BGH	Marked to moderate regeneration
11	Shep mix	M	24.5	BSA	Marked regeneration
13	Mix	F	9.1	BSA	Slight change, germinal centers
15	Westie	F	8.2	BSA	Slight change
18	Mix	F	6.4	BSA	Mild regeneration
19	Mix	M	7.0	BSA	Marked regeneration

Lymphocyte blastogenesis studies
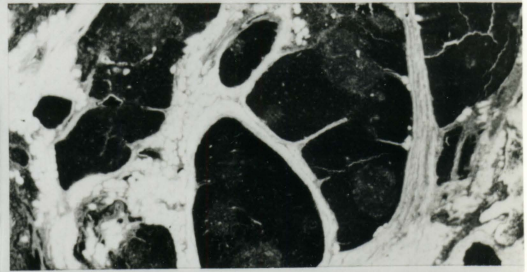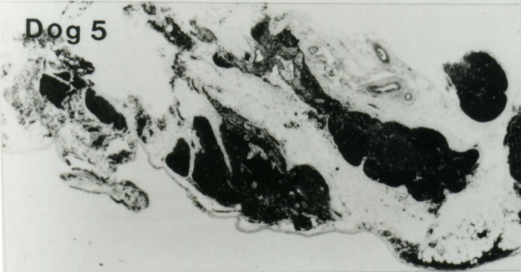
There were no consistent changes or trends in the response of
peripheral lymphocytes to mitogens in vitro in the BGH or BSA treated

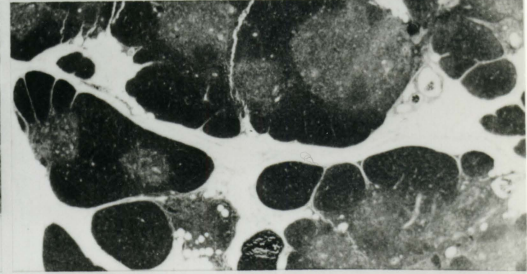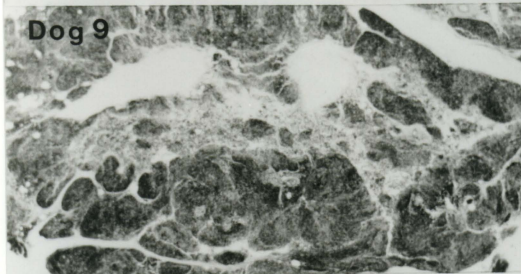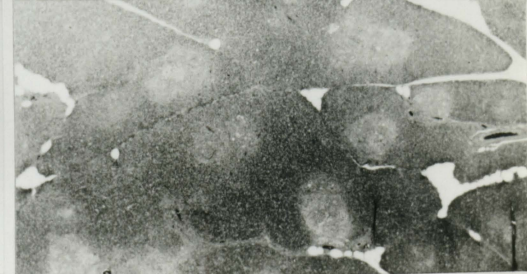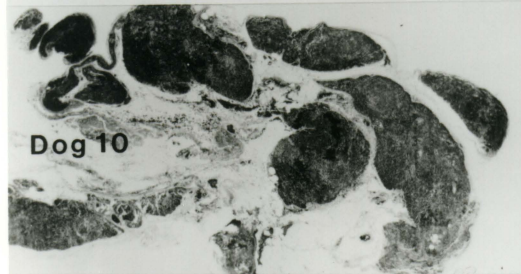Figure 1. Histologic sections, BGH treated dogs, 17.6 X

Dog 4
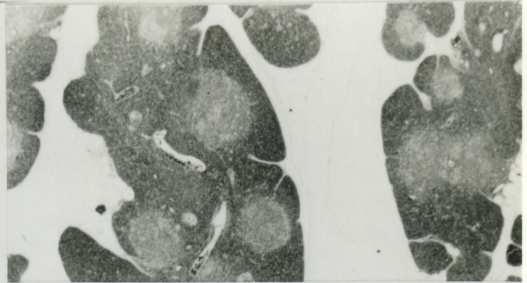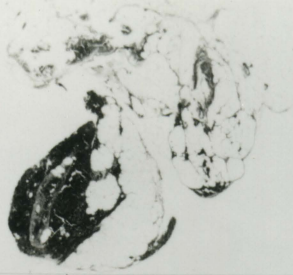
Dog 5

Dog 9

Dog 10

Dog 16
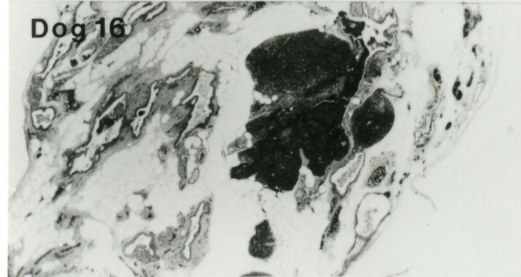
Before   After

Figure 2. Histologic sections, BSA treated controls, 17.6 X

Dog 11

Dog 13

Dog 15

Dog 18

Dog 19
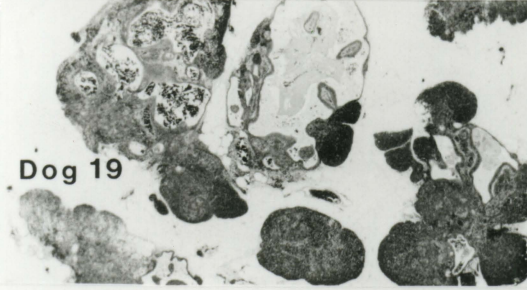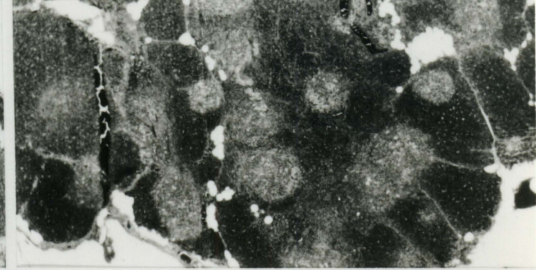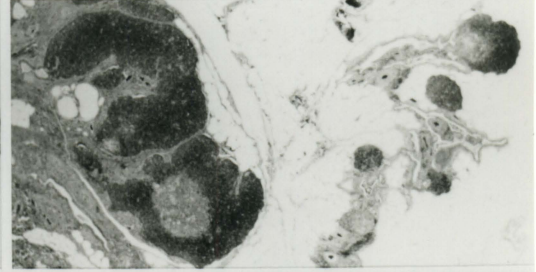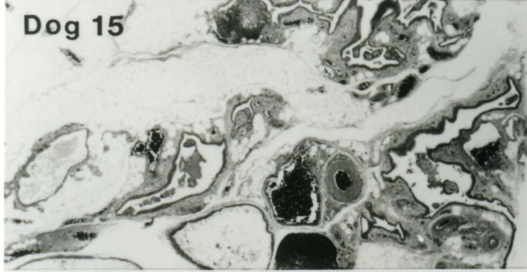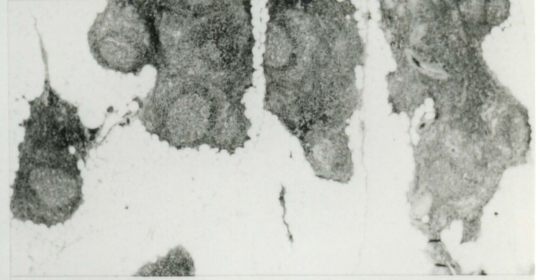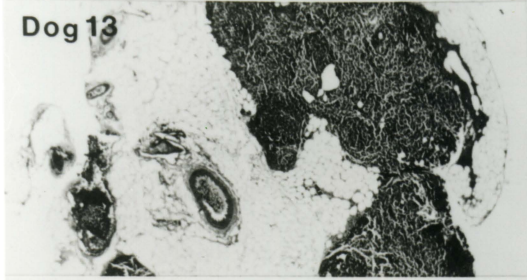
Before   After

dogs. There was marked variability of the response between dogs and within the same dog from week to week (data not shown).

The variability in the response of lymphocytes from the dogs in this study to non-specific mitogens is consistent with other reports of variability and inconsistency of the lymphocyte blastogenesis assay (75a,106). There was variability in the response of the lymphocytes from individual dogs from day to day, and variability among dogs on any given day. There was no reliable trend or change in the responsiveness of cells from the BGH treated dogs or the BSA treated control dogs. The lymphocyte blastogenesis assay therefore was not helpful in this study in determining the effects of growth hormone therapy on cell-mediated immune function.

Total white blood cell and lymphocyte counts

Total white blood cell counts in the BGH treated and BSA treated controls changed minimally from week to week, and there were no consistent trends of increasing or decreasing numbers. Likewise, lymphocyte numbers showed minimal variability between treatment groups, or week to week for individuals. There were no consistent changes in dogs from either group (data not shown).

Discussion

The GH treated dogs of this study regenerated thymus more consistently and markedly than the BSA treated control dogs, however, two of the controls responded just as well. Because of the unexpected positive response in some of the controls, one cannot conclude that growth hormone was the only factor stimulating thymic regeneration in these dogs. One can conclude that administration of growth hormone to adult dogs will cause or contribute to thymic regeneration. A decrease in growth hormone, therefore, along with many other factors may play a role in thymic involution and immune senescence.

The fact that the thymuses of some of the control dogs showed marked regeneration after surgery and BSA administration brings up many questions about the hormonal interactions with the immune system. The control dogs may have been exhibiting "accidental atrophy" induced by stress related increases in cortisol (14) when the thymus was first biopsied. In humans with accidental thymic atrophy there is severe reduction of thymic lymphocytes within 2 to 3 days of cortisol administration or a stressful event, and the thymus returns to normal within 8 to 10 days (14). Treatment of rats with ACTH or cortisol causes severe destruction of the thymus with destruction of thymic lymphocytes (129). Adrenalectomy however, was shown to cause an increase in the cellularity and size of the thymic medulla and cortex (129). In rats, stress has been shown to cause a decrease in GH (102).

In dogs and humans with hyperadrenocorticism, the release of GH in response to compounds which normally stimulate GH release is decreased (84,92,121). Corticosteroid therapy in humans also causes loss of normal GH release in response to stimulators (50). Thymic atrophy associated with increased adrenocortical hormones and stress may then be at least partially caused by a lack of the trophic effect of GH. Rebound release of endogenous GH may then be involved in thymic regeneration after atrophy due to stress. In humans, GH increases with stress (1,21), which may then counteract the effects of cortisol as shown in hypophysectomized mice in which GH prevented the immunosuppressive effects of corticosterone and restored immunity (53). The thymuses in the two control dogs that showed regeneration may have been atrophied due to stress and depressed GH release at the time of first biopsy. Subsequent rebound release of GH may then have caused the regeneration. Age may be an important factor concerning the ability of the thymus to regenerate after stress involution. Young men and dogs have higher plasma GH levels than aged men and dogs (8,47,81,127), and therefore may be able to release greater amounts of the hormone in response to stress induced atrophy of the thymus.

The effect of surgical manipulation upon a normally involuted thymus must also be considered. Manipulation of thymic tissue and adhesions which occurred in virtually all dogs in the study may have played a role in thymic regeneration in both the control and GH treated dogs. Perhaps inflammation with macrophage infiltration and release of monokines may

have stimulated the thymus. The stress of surgery may have stimulated release of endogenous GH which then caused the response. Wound healing may require endogenous GH release, implied by studies in rats in which GH therapy improved wound healing (74,100). Release of endogenous GH to promote wound healing may then have also stimulated thymic regeneration in the control dogs after surgery.

The question of what controls thymic involution then becomes very complex. Age related changes in the relative balance of many hormones may be involved in thymic involution and immune senescence (39). Immunodeficient dwarf mice are deficient not only in GH but also ACTH, TSH, thyroxine, and prolactin (28,36). Growth hormone and thyroxine together did a better job of restoring the lymphoid organs and immune reactivity in these mice than GH alone (6,7). Balb/c mice show a decrease in thyroxine with age along with age associated depression of immune responsiveness. Therapy of these animals with thyroxine increased serum thymic factor levels and T-cell responses to PHA (42).

Studies in rats have shown a relationship between prolactin and cellular immunity. In one study, prolactin was more effective than GH in restoring immunity in hypophysectomized rats (11). Other studies indicate prolactin, GH and human placental lactogen were equally capable of restoring immune function in hypophysectomized rats (85).

The immunodeficient pituitary dwarf dogs described by Roth et al. (105,106) appear to have isolated GH deficiency (83). Human and other canine cases of isolated GH deficiency however, have been reported

and apparently occur without impairment of immune function (87,109).

The sex steroids also appear to have an effect upon the immune system. Thymic cortical involution in women follows a biphasic curve, with an increase in thymic size just prior to menopause (112). Testosterone has been shown to decrease the plaque forming cell response of mice to sheep red blood cells (24).

The ability of thymus to respond to the various hormones that affect lymphoid function also decreases with age. In aged rats, the number of thymocytes with GH receptors is significantly lower than in young rats (62,122). The responsiveness of rat thymocytes incubated in vitro with GH also decreases with age (123).

Inhibition of GH and other pituitary hormones action or production and release may occur due to inhibitory substances produced by the hypophysis. This is implied in a study using middle-aged mice in which simultaneous hypophysectomy and replacement therapy with cortisol, thyroxine and GH caused thymic regeneration when compared to age-matched controls (63). The timing of hormone therapy may also affect the type of response obtained (119).

The cause for thymic involution and senescence of the cell mediated immune system is therefore probably very complex, involving the interaction of many hormones and chemical mediators. The study reported here does seem to indicate GH is involved in this involution process and that therapy with GH will at least play a role in stimulating thymic

regeneration in adult dogs. The use of dogs of known age and breeding, and a means of evaluating thymic size and function without surgery would improve ones ability to evaluate the effects of various hormones upon the thymus. Thymic hormones may prove useful for this purpose. Evaluation of the effects of hormones such as prolactin, thyroxine, somatostatin, somatomedins and growth hormone alone or in combinations may provide useful information on how to restore the aged immune system. Examination of hormone levels such as GH, thyroxine and prolactin after surgery may provide insight into why some of the control dogs also responded. This would help explain the apparent association between the endocrine system, thymic involution and immune senescence.

SECTION III.   EVALUATION OF ENDOCRINE FUNCTION IN PUPPIES FROM

IMMUNODEFICIENT DWARF PARENTS

Introduction

Immunodeficient dwarfism has been described in Weimaraner dogs by

Roth et al. (105).   All of the dogs in this colony are affected with

dwarfism and have lymphocytes which respond poorly to non-specific

mitogens in vitro.   These dogs have normal baseline levels of growth

hormone (GH) but lack a normal response to compounds which stimulate

GH release in normal dogs.   At weaning, some of the puppies develop a

wasting disease with poor growth, recurrent infections and death.   The

puppies with wasting syndrome have atrophied or hypoplastic thymus

glands.   Treatment of these puppies with thymosin fraction 5, an extract

of bovine thymus, caused marked clinical improvement in the wasting

disease, but had no effect on thymus morphology (105).   Growth hormone

therapy however not only prevented the wasting disease, but also caused

the thymus gland to regenerate to normal (106).

In puppies, it has been established that GH has an effect upon the

development of normal thymic size and activity (105,106).   Because

other pituitary hormones have been shown to have an effect upon the

thymus and other lymphoid tissues in other species (6,14,18,95,129),

puppies from immunodeficient dwarf parents were evaluated for thyroid

and adrenal cortical function and for GH.

Radioimmunoassay procedures for measuring plasma GH in the dog have

been shown to be sensitive and repeatable (33,60,127).   Because

baseline levels of GH in plasma are low, provocative tests are required to evaluate pituitary secretion. Clonidine has been shown to be reliable for stimulation of GH release, and therefore for evaluation of this aspect of pituitary function in the dog (61,109,127).

The thyroid stimulating hormone (TSH) response test has been shown to be a useful test for evaluation of thyroid gland function (9,65,69,70,78,89). Patients with a pituitary deficiency of TSH have a depressed response to exogenous TSH administration (10,86,110). Dogs with normal pituitary TSH production have a normal response to exogenous TSH administration. The TSH response test was therefore used in this study to evaluate TSH production indirectly. Measurement of plasma TSH in the dog with the use of a human TSH specific radioimmunoassay procedure has not proven useful for the evaluation of thyroid (16,75b) or pituitary function (75b).

For evaluation of adrenal cortical function in the dog, the use of exogenous adrenocorticotrophic hormone (ACTH) has proven to be useful (17,43,68,108). Dogs with hypopituitarism, and therefore lacking ACTH would be expected to show little increased release of cortisol in response to exogenous ACTH administration due to atrophy of the adrenal cortex (86,109). The measurement of plasma ACTH in the dog is possible, however there is considerable overlap of the range of values in normal dogs and dogs with endocrine dysfunction (44,45). The ACTH response test was used in the puppies in this study in order to indirectly evaluate pituitary function in terms of ACTH production.

Materials and Methods

Animals

Two litters of 3 puppies each, 6 months of age, born to immunodeficient dwarf Weimaraner parents were the subjects of the study. Litter 21 was the offspring of dogs 1 and 3, and litter 23 was the offspring of dogs 1 and 6 described previously by Roth et al. Dog 1 had been affected by the wasting disease described therein and dogs 3 and 6 (females) had not been affected with wasting, but were siblings of dog 1 and lacked the normal release of GH in response to clonidine administration (105). The puppies in the present study were not apparently affected with the wasting syndrome, but were smaller than normal for the breed and considered to be dwarfs. Three puppies in a litter (litter 24) born to normal mixed breed parents were used as controls for the growth hormone evaluation.

Clonidine stimulation and determination of growth hormone

Clonidine was used to stimulate GH release. Two baseline blood samples were drawn, then clonidine hydrochloride (Boehringer Ingelheim Ltd., Elmsford, New York, supplied by Dr. E. Socolow and Dr. S. Garbus) was injected IV at a dosage of 16.5 ug./kg. of body weight. Additional blood samples were taken at post-injection minutes 15, 30, and 60. All samples were collected in heparinized tubes and placed immediately into an ice bath. Plasma was separated by centrifugation at 4 C and frozen at −70 C. The samples were then packed in dry ice and shipped frozen to

the New York University Medical Center for performance of the canine GH specific radioimmunoassay which has been validated in the dog (60). The upper and lower limits of the assay were 30 ng./ml. and 1 ng./ml. respectively. For purposes of statistical calculations, plasma samples with undetectable levels of growth hormone were assigned a value of 1, and samples with levels above the upper limits of the assay were assigned a value of 30. A t-test was used to determine the significance of differences between experimental and control dogs.

TSH response test

The puppies were fasted for 12 hours prior to testing. A resting blood sample was drawn. Ten units of TSH (Dermathycin, Burroughs Wellcome Co., Kansas City, Missouri) was then given IV and a second sample was drawn 8 hours later. The samples were allowed to clot and serum separated by centrifugation and sent to a commercial laboratory (SmithKline Clinical Laboratories, Inc., St. Louis, Missouri) for analysis by radioimmunoassay for T4. Reference values for T4 in the dog were established by the laboratory at 1.2 to 4.2 ug./dl. for resting samples. Radioimmunoassays developed for use in humans have been validated for T4 in canine serum (101).

ACTH response test

After a 12 hour fast, a resting blood sample was taken from each puppy. ACTH (Adrenamone, Burns-Biotec Laboratories, Inc., Omaha, Nebraska) was then given IM at a dosage of 1 IU./lb. and a second blood sample was taken 2 hours later. All blood samples were drawn into EDTA tubes and centrifuged to separate the plasma. Samples were sent out to a laboratory for analysis of canine cortisol by a radioimmunoassay technique which has been validated in the dog (V. K. Ganjam, University of Missouri College of Veterinary Medicine, Columbia, Missouri).

Results

Growth hormone

The results of growth hormone assays are summarized in Table 1. A t-test for two sample means indicated growth hormone levels in the experimental dogs were significantly lower than in the control dogs at 15 (P <0.01), 30 (P <0.01), and 60 (P <0.01) minutes post-clonidine injection.

TSH response test

The results of thyroid hormone assays are summarized in Table 2. Resting levels and post-TSH responses were normal for all puppies. Normal resting values were provided by the laboratory (1.2 to 4.2 ug./dl.). Two times the baseline value or greater than 4 ug./dl. was considered to be the normal response to TSH (9,65,69,70,78,89).

Table 1

Growth Hormone Concentration in Plasma ng./ml.

Immunodeficient Dwarf Dogs

Dog #	0 Min.	0 Min.	15 Min.	30 Min.	60 Min.
21-2	1.7	1.6	13.9	14.4	1.8
21-6	5.2	5.8	28.2	28.6	6.7
21-3	10.1	6.8	14.0	15.1	4.0
23-1	4.9	7.2	22.3	25.1	7.9
23-3	10.5	10.2	16.5	15.5	7.6
23-5	8.8	6.0	23.0	23.6	4.7
Mean	6.87	6.27	19.65	20.38	5.45
SEM	1.42	1.14	2.35	2.50	0.97

Normal Control Dogs

Dog #	0 Min.	0 Min.	15 Min.	30 Min.	60 Min.
24-1	18.6	11.8	>30.0	>30.0	22.6
24-2	1.5	1.4	29.2	>30.0	20.7
24-3	<1.0	<1.0	>30.0	>30.0	19.5
Mean	7.03	4.73	29.73	30.0	20.93
SEM	5.79	3.54	0.27	0.0	0.90

Upper and lower limits of assay, 30.0 and 1.0 ng./ml.

Table 2

Thyroxine, T4 in Serum, Resting and Post-TSH, ug./dl.

Dog #	0 hour	8 hours
21.2	1.6	6.4
21.3	1.8	7.7
21.6	1.6	7.6
23.1	2.0	7.9
23.3	1.9	7.5
23.5	2.8	7.2

Normal levels, 1.2 to 4.2 resting, over 4.0 post-TSH

Table 3

Plasma Cortisol, Resting and Post-ACTH, ug./dl.

Dog #	0 Hour	2 Hours
21-2	1.5	13.1
21-3	1.8	11.2
21-6	2.5	11.3
23-1	1.4	13.9
23-3	2.4	13.1
23-5	2.1	12.1

Normal levels, 0.5 to 2.5 resting, 11.0 to 20.0 post-ACTH

ACTH <u>response</u> <u>test</u>

The results of cortisol hormone assays are summarized in Table 3.
Resting values and post-ACTH respones were normal for all puppies.
Normal values were provided by the laboratory (0.5 to 2.5 ug./dl.
resting, 11.0 to 20.0 ug./dl. post ACTH).

Discussion

There has been a great deal of experimental evidence which indicates
there is a close association between the immune system and the endocrine
system (12,18,24,41,72,97,118). Immunodeficient dwarf dogs which have
severe thymic hypoplasia or atrophy and develop a wasting syndrome at
weaning have been shown to be GH deficient (105). Therapy with GH in
puppies affected with the wasting disease causes clinical improvement
and regeneration of the thymus (106). This is similar to
Immunodeficient dwarfism in mice which have thymus atrophy, lack of
lymphoid development, poor cell mediated immunity and develop a wasting
syndrome that leads to early death. Treatment of these mice with GH
improves immune function and restores lymphoid tissues to normal (118).

In pituitary dwarfism, GH is deficient, and TSH and ACTH may be
deficient as well (109,110). Evaluation of pituitary dwarfs for TSH and
ACTH function is then also important. With prolonged deficiency of TSH
and ACTH, the target glands of these hormones atrophy and will not
respond normally to stimulation (10,86,109,110). The puppies in this

study had normal responses to TSH and ACTH and therefore probably produce normal amounts of these hormones. When compared to normal mixed breed control puppies, the puppies of this study from immunodeficient dwarf parents had significantly less release of GH in response to clonidine administration. Immunodeficient dwarfism in Weimaraner dogs then appears to be an isolated GH deficiency, however prolactin and the gonadotrophic hormones were not evaluated. This deficiency may be a lack of production of GH or an inability of the adenohypophysis to release it in response to clonidine.

All of the dogs in the immunodeficient dwarf colony are deficient in cell mediated immunity, but only some of them develop the wasting disease with severe thymic atrophy or hypoplasia. All of the dogs, however, fail to respond normally to clonidine administration with release of GH demonstrated in this study and that of Roth et al. (105). There may be a threshold level of GH production required to prevent wasting disease. The puppies which develop the wasting syndrome then may produce even less GH than the non-wasting, but yet GH and immune deficient puppies.

Puppies thymectomized at birth do not show any appreciable depression of cell mediated immunity or alterations of lymphoid structure (48). Thymectomy of 48 day old dog fetuses however caused depression of the ability to reject allografts and decreased blood lymphocytes at 24 hours and 3 to 5 months of age respectively (22).

Hormone levels in utero then may be important in normal development of the thymus and cell mediated immune system. Maternally derived hormones such as GH or prolactin may be affecting this development. Growth hormone deficient puppies which become affected with wasting after birth may have been in a position in utero where they were deprived of maternally derived hormones, as opposed to GH deficient puppies which do not develop wasting after birth.

Immunodeficient dwarf puppies that develop wasting, versus puppies that do not develop wasting may be deficient in other hormones not assayed in the present study. Hypophysectomy in rats led to depression of cell-mediated immunity which was improved toward normal more effectively by prolactin therapy than by GH therapy (11). The immunodeficient dwarf puppies that develop wasting may then be deficient in prolactin as well as GH and a combined defect in the production of these two hormones may then be necessary to cause the wasting syndrome. Replacement of one of these hormones, GH, may be enough to prevent the disease as demonstrated by Roth et al. (106). A lack of somatomedin activity in the puppies that develop wasting as compared to puppies that do not show wasting may also be involved.

The hypothalamic-adenohypophyseal defect in immunodeficient dwarf dogs then appears to be a deficiency in GH but not TSH or ACTH, at least in the dogs which do not develop the wasting syndrome. The reason why some immunodeficient dwarf puppies develop wasting disease and some do not is unknown. Wasting that develops after weaning may be brought

about by in utero events that affect thymic development or deficiencies
of other hormones besides GH.

SUMMARY

The endocrine system and the immune system are interdependent. Normal function and structure of the immune system in the developing animal as well as the adult appears to be influenced by the endocrine hormones, particularly growth hormone.

Immune function normally declines with age. Immune senescence may be associated with or caused by age-involution of the thymus gland. Growth hormone also declines with age in most animals. This decline in growth hormone may have an important influence upon thymic atrophy and immune senescence. It was demonstrated by the research presented in this thesis that the administration of growth hormone to adult dogs with age-atrophied thymuses would cause or at least contribute to regeneration of the thymus. Growth hormone therapy may therefore be useful for restoring or improving immune function in aging animals.

Immunodeficient dwarf Weimaraner puppies have been shown to be growth hormone deficient. The hypophyseal hormones adrenocorticotrophic hormone and thyroid stimulating hormone were evaluated in these puppies in the study presented in this thesis and found to be normal. Based on this evaluation, immunodeficient dwarf Weimaraner puppies appear to have an isolated growth hormone deficiency, although prolactin and the gonadotrophic hormones were not evaluated.

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APPENDIX: INFORMATION ON THE USE OF ANIMALS IN RESEARCH AND TEACHING

This research was conducted according to the rules and regulations of the Animal Welfare Act.