The growth hormone secretagogue activity and thymotrophic effects of clonidine in dogs of varying age

by

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EXPLANATION OF THESIS FORMAT

This thesis is divided into two sections, each of which is to be submitted for publication. The literature review is meant to provide a context for the research presented in Sections I and II. At the end of Sections I and II is a discussion of the results of the research presented in those sections. A summary of the research and conclusions drawn from the data generated by the research is provided at the conclusion of the text.

LITERATURE REVIEW

Introduction

Conditions in dogs and humans such as neoplasia and immune mediated illness are more common in older populations and occur, in part, through a failure of one or more components of the immune system (41,43,47,50). The decline of immune function and increased susceptibility to disease seem to be unavoidable consequences of aging (14,19,50,56). A reversal, however, of the aging associated decline of immune function could lead to a decrease in some "old age" illnesses attributable to immune senescence. The objective of this study is to evaluate several non-toxic agents that when supplementing the daily ration of dog food will mitigate the increased susceptibility of different age populations of dogs to diseases linked to immune senescence.

Neuroendocrine Influences on the Thymus Gland and Changes Associated with Aging

The thymus gland has consistently been identified as an organ that has enormous control over the ontogeny and competence of the immune system (14,17,19,25,28). Thymic epithelial cells provide the microenvironment through which thymocytes migrate during their maturation and differentiation into T-lymphocytes (34). Under a light

microscope, the epithelial cells of the thymus are seen as an extensive network of cells with long projections of cytoplasm extending between thymocytes. The anatomy and fine structure of human and rodent thymus epithelial cells has recently been reviewed (33).

Thymic epithelial cells produce hormone substances that control many processes related to the immune system (25,28,34). The best described of the many thymic hormones that participate in the development of T-lymphocytes are thymosin fraction 5, thymopoietin and thymulin (35). The structure and some of the functions of the various fractions have recently been reviewed by Incefy (28) and Goldstein et al. (25). Thymosin fraction 5 is an impure extract from bovine thymus glands. When added to bone marrow cells in vitro, it causes their differentiation into cells biochemically and functionally indistinguishable from T-lymphocytes (34). Thymopoietin is another extract from the bovine thymus. It exists in two forms, which differ by two amino acids. Thymopoietin induces expression of some T-lymphocyte alloantigens in stem cells and stimulates many T-lymphocyte functions (41). Thymulin is a zinc containing serum thymic factor (also known as facteur thymique serique or FTS) that was first isolated from swine serum (41). Thymulin stimulates most T-lymphocyte functions (41). The function of B-lymphocytes is not dependent upon the thymus although the function of B-lymphocytes is regulated by helper or suppressor subsets of T-lymphocytes (47).

The thymus gland of humans and dogs normally begins a process of progressive age related involution and decline of thymic hormone secretion starting shortly after puberty (41,43,47-49). An age associated decline in the thymic dependent (cell-mediated) immune system begins at approximately the same point in life (50). Secretion of growth hormone (GH), a product of the adenohypophysis, also declines with age from peak secretion at puberty (14,42,53). The events of age related thymic involution and the decline in the thymus dependent immune system and the decline in GH secretion approximately parallel each other over time (24,45,47,56). It is becoming evident that these events are highly interrelated.

When neonatal mice are surgically thymectomized they become immunoincompetent and they develop a wasting syndrome that is characterized by failure to grow, then weight loss and death (45). A similar clinical syndrome is seen when normal neonatal mice are innoculated with heterologous anti-mouse hypophysis or heterologous anti-bovine growth hormone immunoglobulin (45). In both instances the mice have inhibition of body growth, involution of the thymus gland, reduced or absent lymphocytes in the thymus dependent areas of the spleen, a diminished response to mitogens and a wasting syndrome that is similar to that observed after neonatal thymectomy (40,47). The actions of heterologous antisera in these experiments suggested an intimate relationship between GH which is secreted by the

adenohypophysis, the thymus, and immune competence.

Other investigations with autosomal recessive Snell-Bagg pituitary dwarf mice have further characterized the relationship between the adenohypophysis and the thymus gland (pituitary-thymic axis) (17,18,55). These mice are almost totally GH deficient and they develop a severe immunodeficiency (hypoplasia of the thymus gland and peripheral lymphoid tissue, lymphopenia and failure to reject allogenic skin grafts) and a wasting syndrome similar to that observed in thymectomized neonatal mice (49). Treatment of these mice with bovine growth hormone (bGH) alone or in combination with Thyroxine (T_4) restores the normal morphologic features of the thymus and the impaired immune function and prevents the fatal wasting syndrome (45). The enhanced effectiveness seen in several of the combined bGH and T_4 treatments is consistent with the concept that thyroid hormone functions in a "permissive" manner, sensitizing target tissue for increased responsiveness to other hormones (39). Normalization of immune function was not observed when dwarf mice were thymectomized as adults and treated with bGH. This suggests that the thymus and not bGH was responsible for the reconstitution of impaired cellular immunity (45).

A series of experiments have further clarified certain aspects of the rodent pituitary-thymic axis (13,31,32). In one experiment, Kelly et al. found that is was possible to regenerate normal thymic structure

and T-cell function in 18 and 24 month old Wister-Furth rats that had been implanted with GH_3 cells (pituitary adenoma cells that secete GH and prolactin) (13,32). The thymuses of the 18 month old GH_3 implanted rats had distinct cortical thymocytes and medullary epithelial cells and were histologically indistinguishable from those of 3 month old controls (13,32). The responses of T-cells from the 18 month old GH_3 implanted rats to standard mitogens were 2 to 5 times higher than comparable control rats (32). The thymus glands of the 24 month old GH_3 implanted rats had increased cellularity and fewer adipocytes than controls, but they were not fully reconstituted (32). Responses of T-cells from the 24 month old GH_3 implanted rats to standard mitogens and interleukin 2(IL-2) production were greatly enhanced when compared with controls (32).

In another experiment with 27 month old female Fischer 344 rats that were treated with GH, prolactin or GH plus prolactin, prolactin was found to augment the proliferative responses of splenocytes to standard mitogens (31). In contrast to the experiment with GH_3 implanted Wistar-Furth rats, exogenous GH did not affect the size or fat content of the thymus glands but GH did increase the numbers of epithelial cells and thymic corpuscles (31). Prolactin had no detectable effect on the thymus glands and neither hormone affected IL-2 synthesis from mitogen activated splenocytes (31).

When Davila et al. gave ovine GH to 26 month old Fisher 344 rats for five weeks they found that mitogenic responses and natural killer (NK) activity of splenocytes were enhanced even though the morphology of their thymuses were unchanged (13). Enhanced thymic size is apparently not required for the reconstitutive effects of GH.

Thymus glands from six week old transgenic mice (mice containing an exogenous metallothionein-rat GH gene that express high rat GH concentrations in serum) were found to have more epithelial cells and thymic corpuscles and less adipose tissue than thymus glands from normal littermates (13). Splenocyte proliferation at suboptimal mitogen concentrations were greater in transgenic than in control littermate mice, but neither IL-2 synthesis or antibody synthesis to sheep erythrocytes were affected (13).

Davila et al. also implanted GH_3 cells into Rowett nude (congenitally athymic) rats and responses to T-cell mitogens, IL-2 synthesis, antibody synthesis to sheep erythrocytes, and NK activity were found to be unaffected which suggests that the thymus gland is the unifying link between GH and immune enhancement (13).

Collectively, these experiments show that GH can regenerate the thymus glands of old rodents and that T-cells and NK cells from aged rats are not inherently defective because their mitogenic responses and cytolytic activity can be augmented by exogenous GH. Growth hormone can also augment T-cell proliferative responses in young rodents but

the immunoenhancing effects are not as apparent as those observed in immunocompromised aged animals (13).

Bovine GH has been shown to be active in immunodeficient sex-linked dwarf White Leghorn chickens. When 200 mg/kg of bGH was given subcutaneously to day old chicks once a day for 3 weeks it resulted in enhancement of the humoral immune system and bursal growth in a manner analogus to the thymotrophic and cell-mediated immune system enhancement seen in mammals (39).

The effect of thymectomy on puppies is unclear and has recently been reviewed (49). In one study, dog fetuses thymectomized after 48 days of gestation showed no evidence of a wasting syndrome, had normal lymphoid tissue development but defective humoral and cell-mediated immune response (14). Other studies of thymectomized puppies report normal humoral and cell-mediated immune response (normal allograft rejection) and no signs of a wasting syndrome (58). A different author reported that thymectomy of one month old puppies resulted in inappropriate survival of allografts which indicates defective cell-mediated immunity (54). Still another author reported that 18 of 18 thymectomized puppies 3 to 6 weeks of age died of a wasting syndrome while only one of 9 puppies thymectomized between 10-12 weeks of age died of a wasting syndrome (55). Differences in surgical techniques (complete vs incomplete thymectomy), age at thymectomy, breed of dog, nutritional factors, and environmental infectious agents are likely

among the factors contributing to the conflicting reports on the effect of thymectomy in puppies (49).

Immunodeficient pituitary dwarf Weimaraner dogs also exhibit a lack of thymus cortex, growth hormone deficiency and a wasting syndrome similar to that of Snell-Bagg mice (47-49). When affected pups were treated with either thymosin fraction 5 (an impure thymic hormone containing many thymus derived peptides and polypeptides) or bGH, clinical improvement and a marked increase in the thickness and cellularity of the cortex of the thymus was observed (48,49). Improved mitogen responsiveness (cell-mediated immunity) in these dogs, however, was variable (49).

When young (2-5 year old) adult dogs with the normal age related involution of the thymus were given bGH subcutaneously (0.1 mg/kg; 14 doses in 30 days) they show more consistent regeneration of their thymus glands than did comparable controls who were given bovine serum albumin (BSA) (42). Mitogen responsiveness of lymphocytes from these dogs given bGH was quite variable but this variability was consistent with other reports of dogs similarily treated (49).

Growth Hormone

In the normal animal, GH is spontaneously and episodically secreted by cells of the adenohypophysis in response to a variety of stimuli (20). The actions of GH are mediated by at least two

somatomedins, A and C (20,44).

Somatomedin A and C are peptides with molecular weights of 7500 daltons. Because of their similarity to proinsulin, they are also known as proinsulin-like growth factors (IGF) I and II. Growth hormone stimulates the secretion of somatomedin C (IGF-I) by the liver. Somatomedin A (IGF-II) is only weakly GH dependent (20).

The effects of GH on carbohydrate metabolism occur primarily via the promotion of hyperglycemia through insulin antagonism (20,23). Secondary hyperinsulinemia and diabetes mellitus may follow prolonged or sustained increases in plasma GH concentrations (20).

The secretion of GH is regulated by endogenous neural rhythms (sleep rhythms, stress and exercise promote secretion), neurotransmitters (B-adrenergic inhibit while alpha-adrenergic and dopaminergic stimulate secretion) somatostatin (inhibits secretion) and hormonal feedback loops acting on the pituitary and hypothalamus (9,20,23,44). The majority of stimuli affect GH release by influencing hypothalamic secretion of growth hormone releasing hormone (GHRH) or somatostatin (7,9,20). However, the dominant influence of the hypothalamus on GH secretion is stimulatory (20).

Variations exist between and among species in the secretion of GH in response to stimuli. For example, moderate exercise will stimulate GH release in humans (30) but not in dogs (20). Arginine is a potent stimulant to GH secretions in humans (2) and rats (11), but has given

inconsistent results in the dog (20). The GH response to insulin-induced hypoglycemia in dogs has also been variable (20). In humans, several studies have shown a diminished response of GH secretion to clonidine in patients with depression or panic when compared with controls (15,56).

Secretagogues of Growth Hormone

A variety of agents including arginine (3), ornithine (16), L-dopa (17), insulin (37), glucose (20) and clonidine (46) have been used in humans and rodents to induce the secretion of GH under a variety of clinical and experimental conditions.

Clonidine

Clonidine is a specific vasomotor center alpha-2 adrenoceptor agonist that is inhibitory to sympathetic central nervous system (CNS) outflow activity (36). Clonidine has effects on the CNS similar to those of chlorpromazine. It produces a marked, but dose-dependent, sedation in humans and in laboratory animals (44). Its major clinical use in humans is in the treatment of essential hypertension (spontaneous elevation of blood pressure) and in the provocative testing of the status of endogenous GH secretion (27,30).

Intravenous or oral clonidine has been shown to be a safe and reliable test of GH secretion in normal children and adolescent and adult humans (8,27). Evidence of some neonatal and adult rat models indicate that clonidine stimulates GH secretions via stimulation of the hypothalamic neurons that synthesize or store GHRH (9,46). In children (4 to 12 years) however, clonidine induced GH secretion is not preceded by elevations in GHRH (7). Clonidine does not increase GH secretion in adult humans with essential hypertension or during exercise (8,30). Other studies indicate that GH release during periods of exercise following clonidine administration may be dependent upon endogenous opiate stimulation (23). The effects of clonidine on GH secretion are delayed in opiate addicted humans and blunted in depressed (15) or panicked humans (56) or when given with amphetamine (51).

The use of clonidine in humans as a screening test for GH deficiency in short children is not universally accepted (37). A subset of short statured children (3.5 to 20.6 years) who had normal GH secretion after provocative testing but markedly reduced 24 hour endogenous GH output has been identified (6). In addition, marked variation in GH responsiveness of children (3 to 13 years) have been reported after oral clonidine (37).

Clonidine has effects on other endocrine hormones besides GH (8,35,46). The effects of clonidine on plasma cortisol are inconsistent and vary with the test population (35,27). Oral clonidine

in some studies is reported to depress plasma cortisol concentrations by 50% from 90 to 120 minutes after a single dose of clonidine in normal and in GH deficient children and in healthy adults (8). The decreased cortisol values are accompanied by a 50% drop in plasma ACTH concentrations. Other authors have reported no difference in plasma cortisol concentration following clonidine administration (46). In a different study of GH response during exercise, clonidine blunted plasma catecholamine increase by more than 60% of the control responses without significantly altering the normal exercise induced rise of plasma cortisol or ACTH (30).

In opiate addicted adult humans, clonidine did not induced the 50% decrease of plasma cortisol concentrations that was observed in non-addicted controls (22). Clonidine was also found to raise the opiate depressed plasma B-endorphin concentrations in addicted humans and it was capable of blocking acute opiate withdrawal symptoms (22). Clonidine had no effect on the plasma B-endorphin concentrations of non-opiate addicted controls (22).

Clonidine is reported to have no effect on plasma concentrations of follicle-stimulating hormone, thyrotropin, glucose, lutenizing hormone, prolactin, testosterone or estradiol B-17 (8,35,36,46).

Arginine

Arginine is an amino acid that is essential for dogs during growth and during adult life (38). All tissues utilize arginine for

cytoplasmic and nuclear protein biosynthesis (2). Intestinal absorption of arginine occurs via a transport system shared with lysine, ornithine and cystine (2). Since arginine is a constituent of the urea cycle, dietary deficiency suppresses hepatic conversion of ammonia to urea and symptoms of hepatoencephalopathy can develop (2). Dietary arginine deficiency has lead to cataract formation in dog and wolf pups raised on commercially available milk replacer (38).

Old Balb/c mice given dietary supplementation with arginine had recovery of age related decline of mitogen responsiveness and expression of T-cell markers (19). Endocrine activity of the thymus in these mice, as measured by thymulin production, was also reactivated. When arginine was combined with lysine the beneficial effects on thymic and immune function were further enhanced (19).

Supplemental dietary arginine has been demonstrated to promote wound healing, improve thymic weight and size and to abrogate post-traumatic weight loss and immune suppression in rats (3,4,5). Supplemental dietary arginine will also increase thymic weights in uninjured rats and it can lessen or abrogate the thymolytic effects of stress (2).

The exact mechanism of arginines thymotrophism is unkwown but it appears to require an intact hypothalamic-hypophyseal axis (2). None of the beneficial effects of supplemental dietary arginine of enhanced wound healing and restoration of thymic and immune function are

observed in hypophysectomized rats or in hypophysectomized rats given bGH (2). In other experiments with hypophysectomized rats supplemented with saline, thyroxine, and testosterone, arginine failed to improve wound healing (2).

Arginine is considered safe in normally nourished animals (2). In rats the LD_{50} (intraperitoneal, after a 24 hour fast) for arginine is 3.8 g/kg (2). Humans can tolerate intravenous doses of 0.5 g/kg up to 30 g total over 20-30 minutes without side effects (2). Only a single case of anaphylaxis to arginine infusion has been reported (2). Caution is urged in the use of arginine in humans with severe metabolic alkalosis, renal insufficiency, and hepatic insufficiency (2).

Recently, arginine infusions have been shown to lead to hypophosphatemia in normal and insulin-dependent human diabetics (2). The hypophosphatemic effect of arginine may be due to displacement of intracellular potassium by the cationic amino acid arginine (2).

Arginine, when perfused through isolated canine pancreas glands, stimulates both glucagon and insulin secretion which are also highly influenced by the glucose concentration of the perfusion medium (29).

Ornithine

Ornithine is a non-essential amino acid for growing and adult dogs. Ornithine also possesses strong secretagogue activity for human GH that can be higher and more sustained than that induced by arginine (2).

SECTION I. ORAL CLONIDINE AS A SECRETAGOGUE OF GROWTH HORMONE IN YOUNG AND OLD DOGS

Introduction

The objective of this study was to evaluate, in dogs, the effectiveness of various oral agents that could be mixed with the daily ration of food in promoting GH secretion so that later studies could assess effects on thymic morphology and function and immune function. Various agents were evaluated and the accuracy of the radioimmunoassay (RIA) procedures for measuring GH concentrations in plasma was established.

The efficacy of oral clonidine as a secretagogue for GH in humans is well established. A second objective of this experiment has to define a dose of oral clonidine as a dietary supplement that will reliably induce GH secretion in young and old dogs.

Materials and Methods

Experiment I - Animals and Experimental Design

Five 16-18 month old (young dogs) and five 6-8 year old (old dogs) female beagles of known age and medical background were used in this study.

Each of the following dietary supplements were evaluated in both young and old dogs: arginine at 0.5%, 1.0% and 1.5% of the total diet and arginine plus ornithine, each at 1.5% of the total diet. The

effects of food alone on GH secretion was also evaluated in both young and old dogs. The food used in this experiment was Purina Hi Pro (Ralston Purina, St. Louis, MO). Each dog was fed one cup of food at approximately 8:30 AM and 4:30 PM. This provided each dog with between 60 and 80 calories/kg of body weight. The dietary supplements were mixed with a small amount of water and allowed to soak into the food prior to feeding. Once on different days, arginine (25 mg/kg and 100 mg/kg) and arginine plus ornithine (25 mg/kg each) were given intravenously to each of the young and old dogs. On different days bovine growth hormone releasing hormone (bGHRH) (1 ug/kg) and clonidine (16.5 ug/kg) were given intravenously to all of the dogs in the study. There was a minimum of 48 hours between each of the challenge stimuli.

Venous blood samples were collected into EDTA starting between 8:30 and 9:30 AM immediately prior to (time zero) and at 15, 30 and 60 minutes after each of the challenge stimuli. Blood samples were immediately put into crushed ice. The plasma was separated by centrifugation at $4^{o}C$ and stored frozen at $-70^{o}C$.

Growth hormone was determined using a canine validated RIA procedure developed by Dr. A. R. Parlow, Harbor UCLA Medical Center, Torrance, CA. Twenty micrograms of lyophilized canine GH was solubilized in 0.01M $NaHCO_3$ at 100ug/ml of buffer. A cold standard was prepared by further diluting this solution with 1% BSA-phosphate buffered saline (PBS) to 10 ug/ml. The GH was iodinated to low

specific activity using Iodo-Beads (Pierce Chemical Company, Rockford, IL). The stock solution of 1:100 monkey anti-canine GH gamma globulin was diluted with sterile distilled water to a final concentration of 1:31,250. Nine hundred microliters of 0.05M PBS, 100 ul of cold standard or unknown sample, 100 ul of iodinated canine GH and 100 ul of diluted monkey anti-canine GH gamma globulin were mixed together and incubated in tubes for 18-24 hours at room temperature. One-hundred microliters of goat anti-monkey gamma globulin diluted 1:12 with sterile distilled water, 100 ul of normal monkey serum reconstituted to 2 ml with sterile distilled water and diluted 1:40 with 0.05 M PBS (both from Antibodies Inc., Davis, CA) and 2 ml of polyethylene glycol were added to each sample. The tubes were incubated for one hour at room temperature and centrifuged at 1500 x g at 4^{o} C. The supernatant was removed and the pellet counted using a gamma counter.

Values for the young and old dogs for each blood collection period were, within each group, analyzed and compared with the time zero values (least significant difference).

Experiment II - Animals and Experimental Design

Five 20-24 month old (young dogs) and four 6.5-8.5 year old female beagles (old dogs) of known age and medical background were used in this study.

The young and old groups of dogs were, on different days, each given 16.5 ug/kg, 50 ug/kg, 150 ug/kg and 450 ug/kg of clonidine mixed with their normal food ration as described above. Venous blood samples were collected into EDTA immediately prior to (time zero) and at 30, 60, 90 and 120 minutes after feeding. The dogs were fed twice each day. The morning feedings were always between 8:30 AM and 9:30 AM while the afternoon feedings were always between 4:30 PM and 5:30 PM. Blood samples were handled in the identical fashion as described in experiment I. Growth hormone was determined using the same RIA procedure as in experiment I.

Values for the young and old dogs were, for each collection period, averaged and compared with the time zero values (least significant difference).

Results

Experiment I

Food alone, oral arginine, oral arginine and ornithine, intravenous arginine, and intravenous arginine and ornithine in the concentrations of each that were tested failed to induce any significant release of GH in either the young or old dogs (Table 1).

Intravenous bGHRH (1 ug/kg) induced significant ($P < 0.05$) GH release at 15 minutes (10.4 $\pm$ 2.3 ng/ml) in the young dogs and at 15 minutes (5.2 $\pm$ 1.7 ng/ml) and 30 minutes (4.3 $\pm$ 1.0 ng/ml) in the old dogs (Table 2).

Intravenous clonidine (16.5 ug/kg) induced significant ($P<0.05$) GH release at 15 minutes (47.8 $\pm$ 13.4 ng/ml) and 30 minutes (24.5 $\pm$ 9.5 ng/ml) in the young dogs and at 15 minutes (13.0 $\pm$ 7.9 ng/ml) in the old dogs (Table 3).

Experiment II

The food ration supplemented with clonidine at 16.5 ug/kg produced significant ($P<0.05$) GH secretion in young dogs (13.6 $\pm$ 8.1 ng/ml). No significant amounts of growth hormone were secreted in response to clonidine at this dose in the old dogs (Figures 1, 2).

When the food ration was supplemented with clonidine at 50.0 ug/kg significant plasma ($P<0.05$) elevation of GH occurred in young dogs at 30 minutes (24.3 $\pm$ 12.0 ng/ml) and at 30 minutes (12.3 + 4.3 ng/ml) and 60 minutes (13.0 $\pm$ 2.5 ng/ml) in the old dogs (Figures 1, 2).

Clonidine supplemented at 150 ug/kg in the normal food ration produced significant ($P<0.05$) GH secretion at 30 minutes in young (30.5 $\pm$ 8.0 ng/ml) and old (31.1 $\pm$ 9.3 ng/ml) dogs (Figures 1, 2).

With clonidine supplementing the daily food ration at 450 ug/kg significant ($P<0.05$) GH secretion occurred at 30 minutes in the young dogs (31.5 $\pm$ 12.1 ng/ml) and at 30 minutes (41.0 $\pm$ 13.5 ng/ml) and 60 minutes (25.5 $\pm$ 8.4 ng/ml) in the old dogs (Figures 1, 2).

Clonidine supplementation at 16.5 ug/kg and 50.0 ug/kg had no clinically detectable side effects in either the young or old dogs.

Mild sedation and slightly pale oral mucous membranes were observed at the 150 ug/kg dose of clonidine. With the 450 ug/kg dose of clonidine marked sedation and very pale oral mucous membranes were noted.

Table 1

Growth Hormone Release in Response to Various Agents

Agent	Dose	Route	GH Release
Food		Oral	-
Arginine	0.5%	Oral	-
Arginine	1.0%	Oral	-
Arginine	1.5%	Oral	-
Arginine	25 mg/kg	IV	-
GHRH	1 ug/kg	IV	+
Clonidine	16.5 ug/kg	IV	+
Arginine & Ornithine	1.5% each	Oral	-
Arginine & Ornithine	25 mg/kg each	IV	-
Arginine	100 mg/kg	IV	-

+ = Significant ($P<0.05$) GH release

- = Failure to release GH

Table 2

Growth Hormone Release in Response to Intravenous bGHRH (1 ug/kg)

Number of Samples Y/O[a]	Time Post Treatment (minutes)	Young (Mean ± SEM) GH (ng/ml)	Old (Mean GH ± SEM) GH (ng/ml)
5/5	0	2.4 ± 0.2	2.2 ± 0.2
5/5	15	10.4*± 2.3	5.2*± 1.7
5/5	30	4.2 ± 0.8	4.3*± 1.0
5/5	60	2.4 ± 0.2	2.3 ± 0.3

[a] Y = Young; O = Old
* $P < 0.05$

Table 3

Growth Hormone Release in Response to Intravenous Clonidine (16.5 ug/kg)

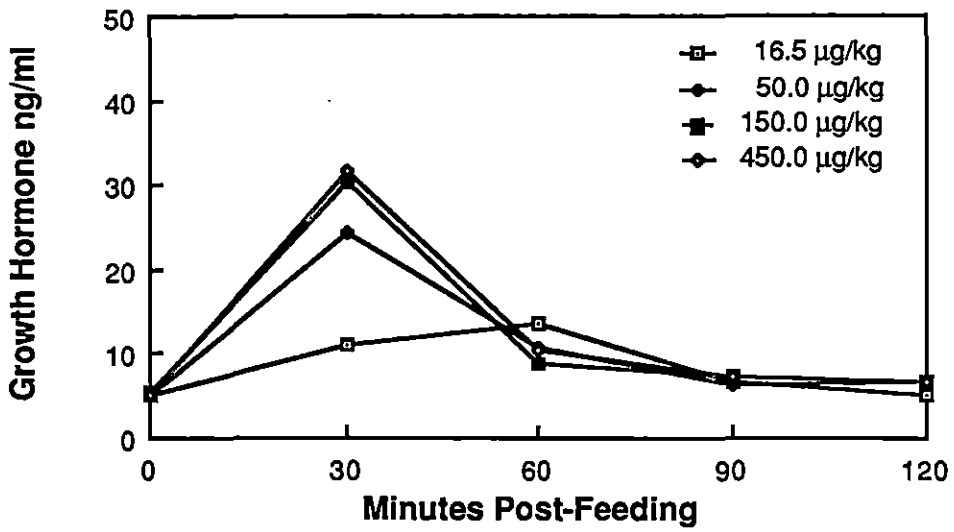
Number of Samples Y/O[a]	Time Post Treatment (minutes)	Young (Mean ± SEM) GH (ng/ml)	Old (Mean GH ± SEM) GH (ng/ml)
5/5	0	2.9 ± 0.3	2.2 ± 0.2
5/5	15	47.8*± 13.4	13.0*± 7.9
5/5	30	24.5*± 9.5	5.4*± 3.3
5/5	60	5.6 ± 1.9	4.4 ± 1.3

[a] Y = Young; O = Old
* $P < 0.05$

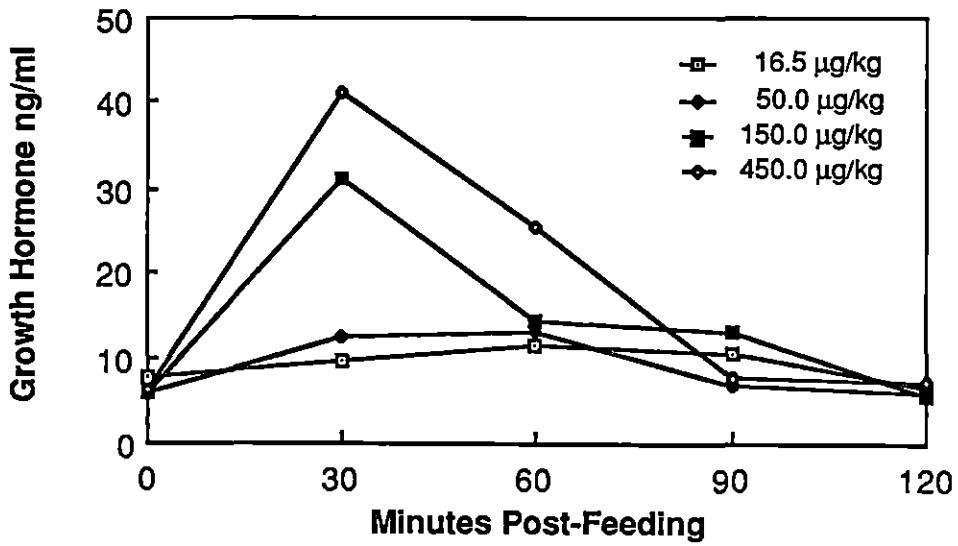
Figure 1. Growth Hormone Secretion in Young Dogs in Response to Oral Clonidine Supplementation of the Daily Ration of Dry Food

Figure 2. Growth Hormone Secretion in Old Dogs in Response to Oral Clonidine Supplementation the Daily Ration of Dog Food

Growth Hormone Response to Clonidine in Young Dogs



Growth Hormone Response to Clonidine in Old Dogs



Discussion

Food alone did not stimulate GH secretion in the dogs tested. This is important since excitement (stress) is a factor known to stimulate GH secretion in some species (23). Judged subjectively by the author, the dogs in this study were always very excited by food preparation and feeding.

Arginine and arginine combined with ornithine failed to induce GH secretion in any of the dogs. The failure of arginine and ornithine to induce GH secretion in the dogs tested is consistent with some of the prior observations in dogs (20). This was disappointing since arginine is known to be a potent secretagogue for GH in humans and rodents (2,20). Likewise, ornithine possesses strong secretagogue activity for human pituitary GH (2,20). Differences in species response to various GH secretagogues apparently do exist. The mechanism of arginine GH secretagogue activity is unknown but possibly involves insulin-induced hypoglycemia (2). Intravenous, oral or intraduodenal arginine will induce a marked insulin release in humans (2). Hyperinsulinemia can cause hypoglycemia and insulin-induced hypoglycemia is a factor known to stimulate GH release in young and adolescent humans (23). Insulin-induced hypoglycemic stimulation of GH secretion in dogs is inconsistent with some investigators reporting significant elevations and others reporting minimal responses (10,20). Interestingly, ornithine does not share the insulin secretagogue activity of arginine

and its mechanism of action on GH secretion, although unknown, may be different from arginine (2).

Growth hormone releasing hormone is a known secretagogue of GH in the dog (20). Species differences among the GHRHs are believed to exist and the sequencing and full characterization of canine GHRH has not yet been completed (20). Apparently bGHRH is similar enough to canine GHRH to induce pituitary GH secretion in the dogs tested.

Intravenous clonidine is also a known secretagogue for GH in dogs (26). Oral clonidine has not been established as an effective secretagogue of GH in dogs although oral clonidine is known to induce GH secretion in humans (20,27). The known GH secretagogue activity of bGHRH and intravenous clonidine established these agents as the positive controls among the other stimuli tested. The RIA procedure used was concluded to be a reliable method of measuring canine GH concentration in plasma.

Clonidine, given orally by mixing it with dog food was determined to be a reliable secretagogue for GH at all doses tested in young dogs and in all doses except the 16.5 ug/kg dose in the old dogs. When given the lowest two doses of oral clonidine, GH secretion by the old dogs was poor when compared with the young dogs. However, at the highest two doses of oral clonidine given, GH secretion by the old dogs exceeded that observed in the young dogs. This observation is very significant in that it establishes that given an adequate stimulus

(higher clonidine dose in this case), GH secretion by dogs is not limited by, and may even be enhanced by, increased age.

Although the animals were not instrumented for definitive measurements of blood pressure, the pale oral mucous membranes observed when the highest two doses were given probably indicated hypotension. From the concentrations of GH produced by the various doses and the observed side effects of clonidine, it is estimated that the optimum oral dose of clonidine needed to achieve safe and reliable GH secretion is 100 ug/kg.

SECTION II. ORAL CLONIDINE AS A THYMOTROPHIC AGENT IN MIDDLE AGED DOGS

Introduction

The morphologic restoration of the thymus glands of normal adult dogs and immunodeficient dwarf Weimaraner dogs in response to exogenously administered bGH has previously been established by Roth et al. (49) and Monroe et al. (42). The improvement of canine thymus endocrine function in response to exogenously administered bGH was established recently by Goff and others (24). Goff et al. demonstrated that bGH would consistently restore the age associated decline of plasma thymulin concentrations in middle age (33-55 months) and old (63-83 months) beagle dogs when compared with dogs given BSA (24). Morphologic features of a young (prior to age associated involution) thymus gland were restored in the middle age but not the old dogs who were given bGH (24).

The experiments described in the previous sections established a dose (100 ug/kg) of oral clonidine that can be mixed with the daily food ration that will safely and reliably induce GH secretion of either young or old dogs. It is now possible to test the hypothesis that clonidine, mixed with the food ration of dogs on a daily basis will induce endogenous GH secretion that will in turn restore thymus gland morphology and function and the thymus-dependent immune system. Immune system restoration may ultimately decrease the incidence of diseases

associated with aging and immune senescence.

Materials and Methods

Animals and Experimental Design

Twelve four-year-old female beagle dogs of known medical history were used in this part of the study.

The twelve dogs were segregated into two groups of six individuals that approximated each other by the additional characteristic of weight. One group (experimental) had their daily food ration supplemented with 100 ug/kg of clonidine for 30 days. The other group (control) had their daily food ration supplemented with distilled water (1 ml/kg of body weight) for 30 days. All dogs were fed twice a day, once between 8:30 AM and 9:30 AM and again between 4:30 PM and 5:30 PM.

Venous blood samples were collected into EDTA on the first (day 1) and last (day 30) days of the feeding trial prior to feeding, and at 30, 60 and 90 minutes after feeding for evaluation of plasma GH concentrations. Plasma GH concentrations were measured with the same RIA procedure as was used in section I of this thesis.

Venous blood was also collected from each dog into acid citrate dextrose (ACD) once each week (beginning one week before the start of the experiment) following the morning meal to assess lymphocyte responsiveness to standard mitogens (concanavalin-A, pokeweed mitogen, phytohemagglutinin) by a method previously reported (49). The weekly

values for each group were compared and expressed as percent of control values. Percent of control values were calculated by averaging the percents of the average control values for each experimental dog.

On the last day of the feeding trial and after the morning meal was consumed, venous blood was drawn into EDTA for determination of plasma thymulin titers. Plasma thymulin titers were determined in the laboratory of Dr. Genevieve Incefy, Memorial Sloan-Kettering Cancer Center, New York, NY. The plasma thymulin assay is based upon rosette inhibition of mouse spleen cells incubated for 75 minutes at 37^{0} C with azathioprine. Rosette forming cells from spleens of thymectomized mice are less sensitive to azothioprine than are cells from normal mice. Plasma with thymulin activity restores to normal the sensitivity to azathioprine of rosette-forming cells from adult thymectomized mice resulting in inhibition of rosette formation (24,28). Data were converted to the $\log_2$ of the reciprocal of the titer for comparison.

One week after the start of the feeding trial, each dog was immunized with heat-killed strain 19 of Brucella abortus. Antibody responses were determined with a standard tube agglutination test on serum collected prior to immunization on day 1 and again on day 30.

On day 30 each dog had an intravenous glucose tolerance test performed using 600 mg/kg of glucose. Venous blood samples were collected prior to glucose administration, and at 5, 10 15, 25, 35, 45 and 60 minutes after glucose administration. Glucose values were

determined on an automated system (Rotachem IIa, American Instrument Company, Silver Spring, MD).

Approximately one week after the conclusion of the feeding trial, each dog was euthanatized using an intravenous injection of a barbiturate substance (Sleepaway, Fort Dodge Laboratories, Inc., Fort Dodge, IA). Each dog was necropsied within 5 minutes of death. Thymus tissue and samples of other major organs were collected from each dog and fixed in 10% formalin.

Results

Significant ($P < 0.01$) amounts of GH were secreted by the experimental group at 30, 60 and 90 minutes on day 1 when compared with controls (Figure 3). There was no significant difference between the experimental and control group in the amount of GH secreted on day 30 (Figure 3). Significantly more GH was secreted by the experimental group on day 1 at 30 minutes ($P < 0.05$), 60 minutes ($P = 0.07$) and 90 minutes ($P < 0.01$) than at the corresponding times on day 30 (Figure 3).

The average mitogen responsiveness was significantly ($P < 0.05$) higher in the experimental group when compared with controls. The results of the blastogenic response to the mitogens used are summarized in Table 4. However, blastogenic resonses were highly variable. Phytohemagglutinin enhanced blastogenic responses during the two weeks following the start of the experiment but enhancement diminished during the third and fourth week (Figure 4). Blastogenic responses to

concanavalin-A and pokeweed mitogen were enhanced during the first and third weeks of the experiment but diminished during the second and fourth week (Figures 5, 6).

No significant difference was detected between the plasma thymulin titers of the experimental and control dogs on day 30 of the study (Figure 7). Concurrent with the determination of plasma thymulin titers from the dogs of this study, plasma thymulin titers were determined on three four-month-old pups to serve as positive controls for the assay (young pups have higher titers than adults).

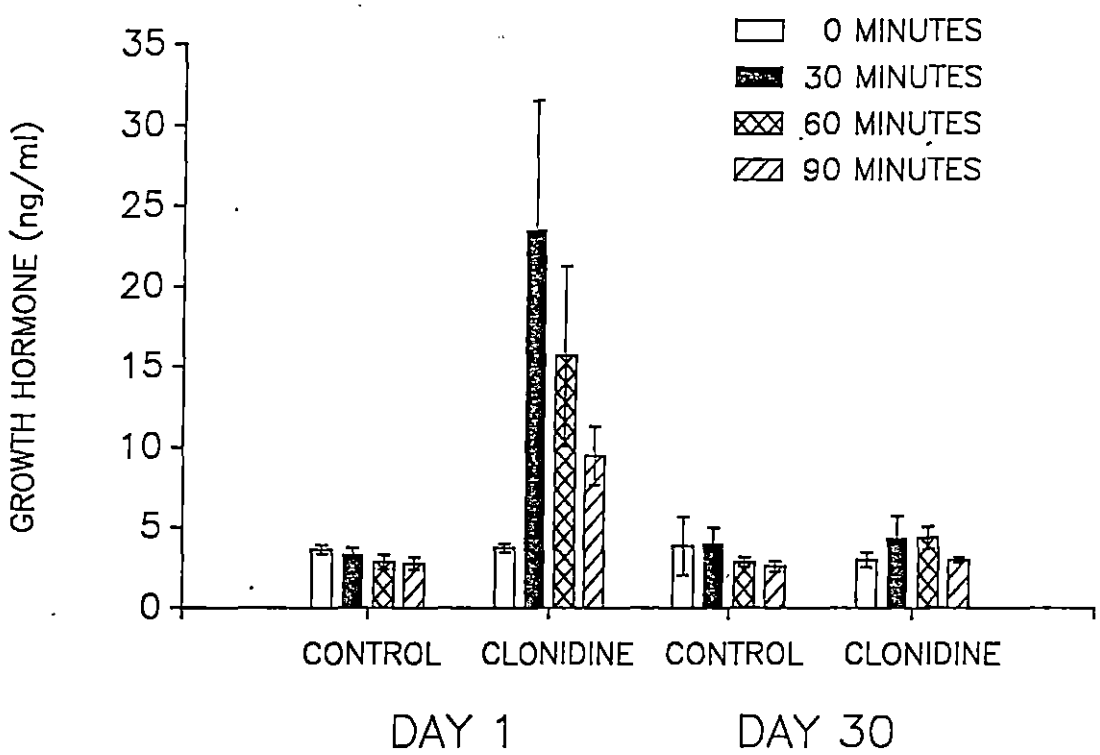


Figure 3. Growth hormone secretion in middle age dogs in response to clonidine supplemented dog food for 30 days

Table 4

Lymphocyte Blastogenesis in the Clonidine and Control Fed Dogs During the Four Week Treatment Period

Mitogen	Clonidine[a]	Controls[a]
None	382 ± 82	495 ± 134
PHA SI	129.3 ± 22.3[b]	64.8 ± 9.3
PHA dcpm	41,810 ± 8,190	26,120 ± 5,420
Con A SI	129.6 ± 21.4[b]	74.2 ± 10.9
Con A dcpm	41,530 ± 8,030	28,590 ± 5,710
PWM SI	75.9 ± 13.0[b]	41.0 ± 6.5
PWM dcpm	25,029 ± 5,410	15,390 ± 3,240

[a] Each value represents the mean (± SEM) of four weekly determinations on six animals per group during the clonidine feeding period.

[b] $P < 0.05$ when compared to the value for control animals.

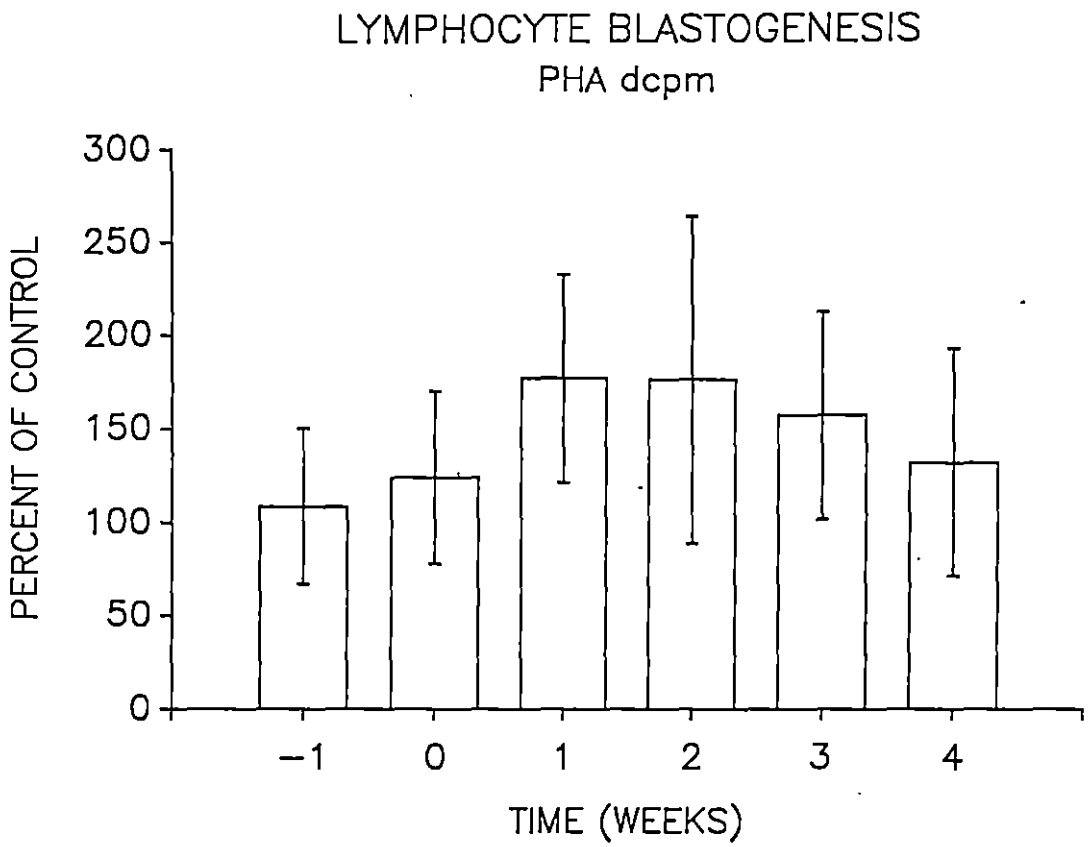


Figure 4. Blastogenic responses of lymphocytes to phytohemagglutinin

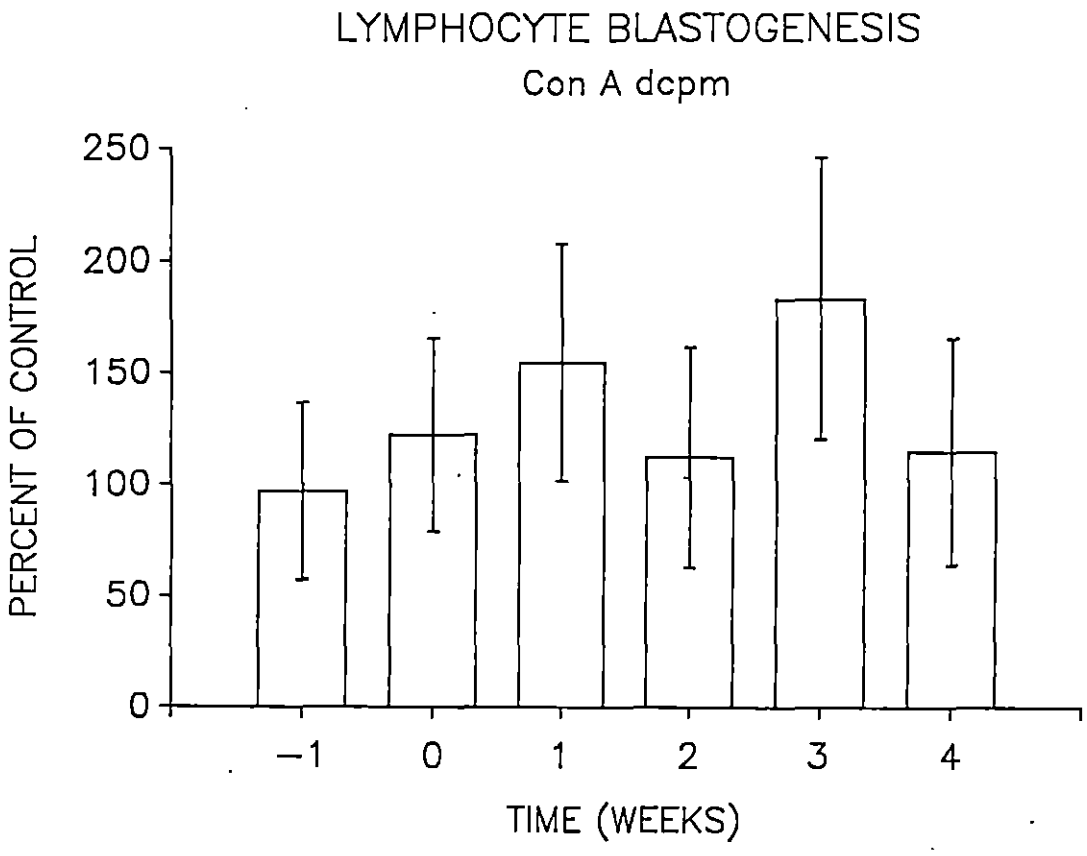


Figure 5. Blastogenic responses of lymphocytes to concanavalin-A

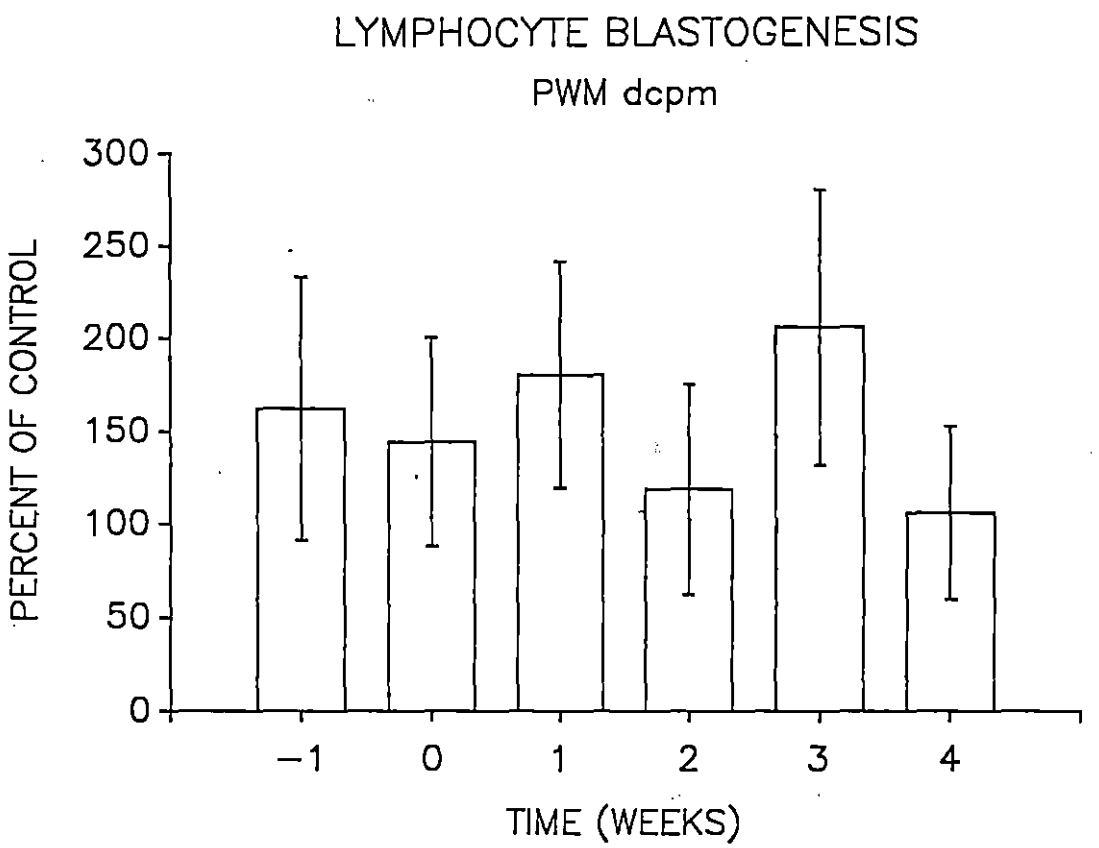


Figure 6. Blastogenic responses of lymphocytes to pokeweed mitogen

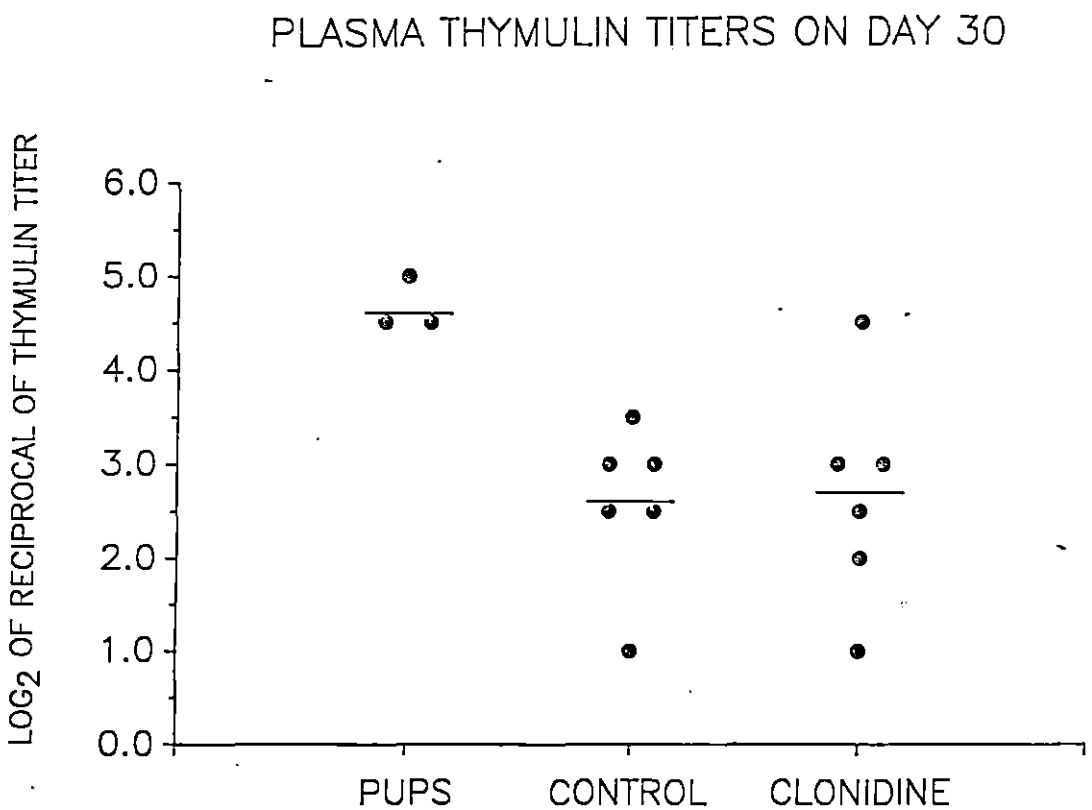
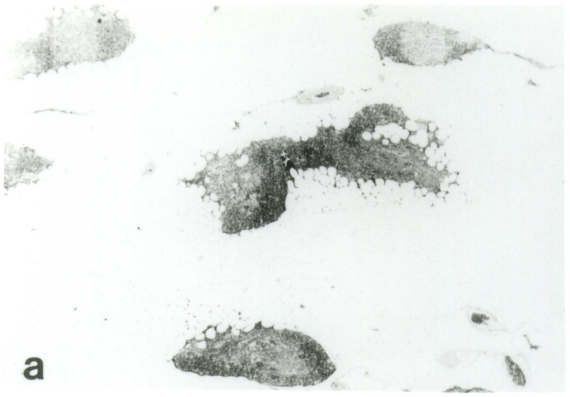


Figure 7. Plasma thymulin titers of three normal pups (four-months-old), experimental dogs fed clonidine, and control dogs on day 30 of the feeding trial

Figure 8. Histologic sections of thymus glands of dogs fed a distilled water supplemented diet in Figures b and c have greater cellularity and less adipose tissue than in Figures a, d, e, and f, 17.6X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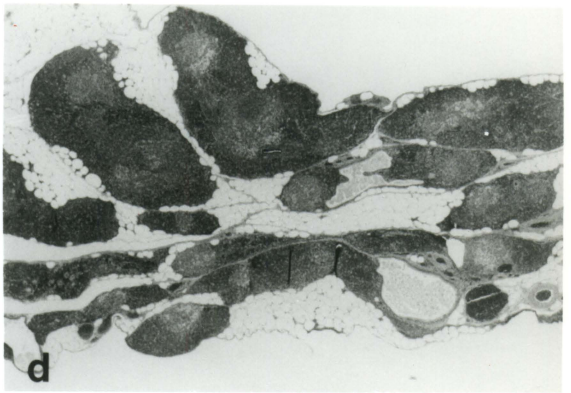
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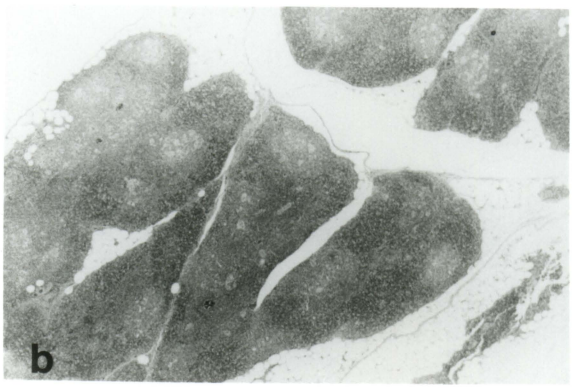
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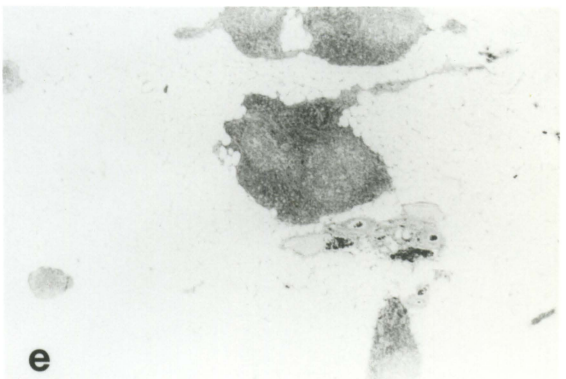
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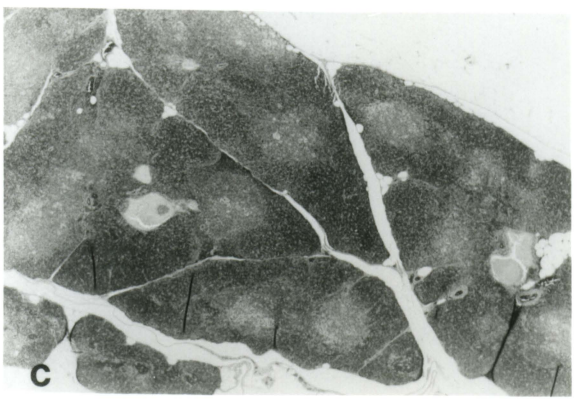
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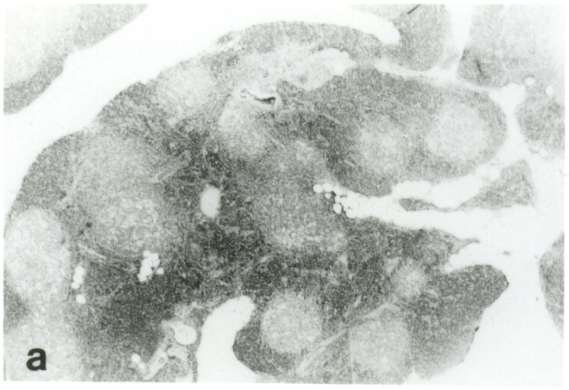
Figure 9. Histologic sections of thymus glands of dogs fed a clonidine supplemented diet in Figures a, d, e, and f have greater cellularity and less adipose tissue than in Figures b and c, 17.6X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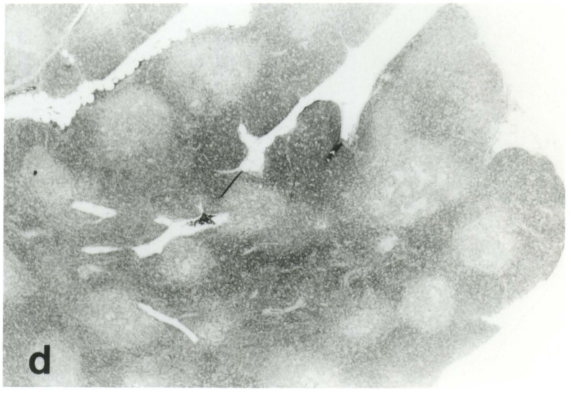
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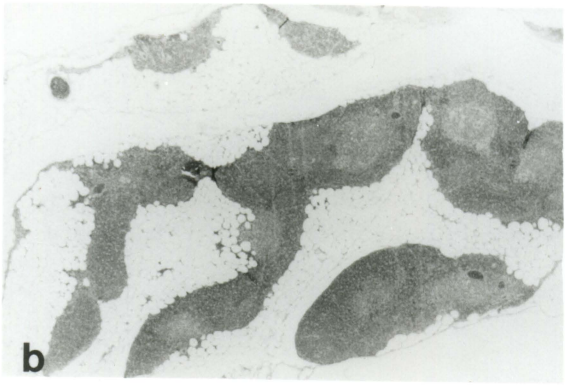
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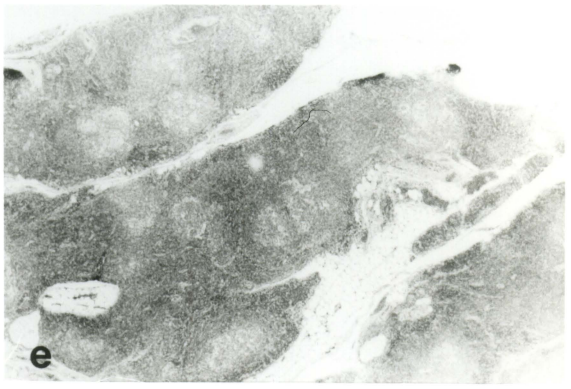
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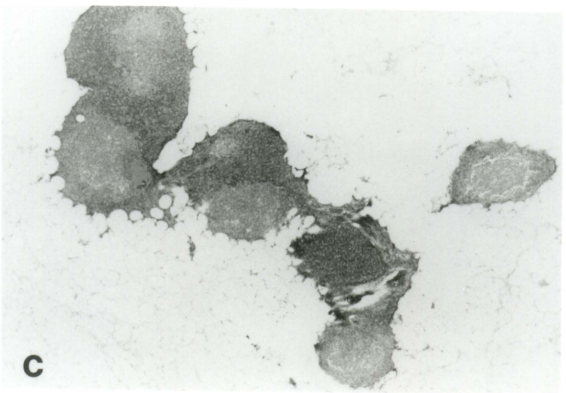
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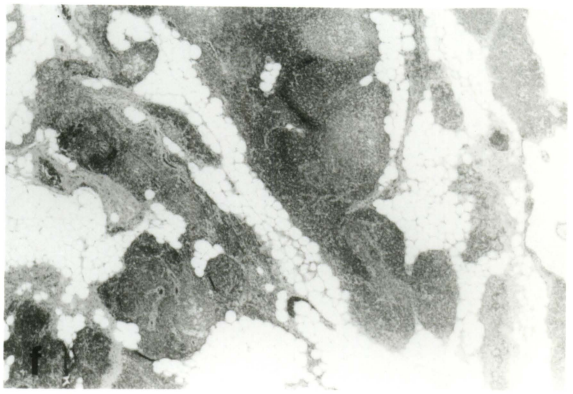
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The morphologic data were assembled from the unbiased observation of a pathologist who was unaware of which treatment group each dog had been assigned to. The thymus morphology was judged to be normal (age related involution) in four of the six control animals (Figures 8 a, d, e, f). Surprisingly, two control dogs had better developed thymus tissue than expected for dogs of this age (Figures 8 b, c). Four of six experimental dogs had improvement in their thymus morphology (Figures 9 a, d, e, f). Two of six experimental dogs had appropriate age associated thymic involution (Figures 9 b, c).

There were no significant differences between the experimental and control groups in their antibody response to Brucella abortus immunization (data not shown).

Glucose tolerance tests in all dogs done at the end of the experiment were normal (data not shown).

Discussion

The excellent secretagogue activity of oral clonidine (100 ug/kg mixed with food) for GH was clearly apparent on day 1 but absent on day 30. This may have occurred because of the phenomenon of tolerance, also known as tachyphylaxis, desensitization and down regulation. Down regulation is recognized in many clinical situations. Down regulation simply means that with chronic administration of a drug it takes larger doses to produce effects that are apparent at lower doses in naive

subjects (52). For example, human asthmatics have a decreased responsiveness to epiniphrine after multiple administrations (52). Both metabolic and receptor changes are involved in the altered sensitivity of some drugs. Metabolic tolerance to alcohol and barbiturates in humans and barbiturates in dogs are derived, in part, from increased metabolism of these substances with chronic use (20,51). Circulating antibody to insulin receptors is a known cause of insulin resistance in diabetic humans and is a suspected cause of insulin resistance in diabetic dogs (20).

Other possible mechanisms to explain down regulation involve changes in the drug receptors themselves (52). A reduction in the number of receptors or a proliferation of "inactive" receptors have also been cited as explanations for down regulation (52). Certain models propose that biochemical changes other than the drug receptors are responsible for down regulation through mechanism like second-messenger (cyclic AMP/adenyl cyclase) modulation (52).

It is unknown by what mechanism or how quickly these beagle dogs become tolerant to the secretagogue effects of clonidine. All that can be concluded is that down regulation to oral clonidine occurs in dogs within 30 days when administered twice each day at a dosage of 100 ug/kg with food.

The responses to standard mitogens were highly variable. When the data from each week were averaged together the experimental group had

significantly ($P < 0.05$) greater response to the mitogens tested than did the control group. The reasons for high variability of the responses to standard mitogens are not fully understood but they have been observed previously (42,49). The data do suggest that oral clonidine can, at least transiently, enhance immune function as determined by lymphocyte responses to mitogens. Whether this enhanced immune function is the result of the GH secretion induced by clonidine before the dogs stopped responding to it or via another mechanism is unknown.

Plasma thymulin titers in the dogs of the experimental and control groups were comparable on day 30. It is unknown if clonidine had any beneficial effect on thymic endocrine function as determined by a rise in plasma thymulin titers prior to day 30. From the experiments of Goff et al. (24) who were able to consistently induce a rise in plasma thymulin titers in beagle dogs given exogenous GH, it would be expected that had the dog not become unresponsive to oral clonidine, a rise in plasma thymulin titers should have followed the endogenous GH secretion induced by clonidine. Since tolerance to clonidine apparently occured and since plasma thymulin titers were not determined at frequent intervals, the true relationship between oral clonidine, growth hormone and plasma thymulin titers remains speculative.

Antibody response to challenge with B. abortus reflect primarily B-lymphocyte function rather than T-lymphocyte function although some

subsets of T-lymphocytes modulate the activity of B lymphocytes. The antibody responses in both groups of dogs to B. abortus were comparable and do not directly reveal any measure of immune function enhancement following clonidine administration.

Since the plasma thymulin titers and B. abortus antibody titers were comparable to control values and lymphocyte responses to mitogens were so variable it is unlikely that clonidine, as given, has any clinically relevent benefit to immune function.

The morphology of the thymus glands was judged to be normal (appropriately involuted for four year old dogs) in four of the six control dogs. The finding of two of six dogs with better than anticipated thymus morphology was unexpected. However, many authors have recorded the persistence of epithelial cells after thymic atrophy in rodents and regional differences in cell proliferation within the thymus are recognized in other species (33). The relationship between the numbers of thymocytes and epithelial cell nuclei is not age related and, in some individuals, differ in different parts of the same gland (33). The size of the adult human thymus gland is very variable and even in glands much reduced in size, there are often compact aggregates of thymocytes bulging into the adipose tissue around the medulla (33). Based upon these observations in other species, it is possible that the natural variability of adult thymus tissue could account for the observed differences in thymic morphology of the control group.

The morphology of the thymus (increased cellularity of the cortex, decreased width of the interlobular septae and decreased amounts of adipose tissue) was judged to be enhanced in four of the six dogs in the experimental group. Since none of these dogs had gross differences in their thymus glands and enhancement was judged upon microscopic appearance, it is possible that natural variability of adult thymus tissue accounted for the increased cellularity of the thymic morphology noted. Although four of six experimental dogs showed improved microscopic appearance, the sample sizes in this study were too small to conclude that thymuses from the experimental dogs benefited by increased cellularity from clonidine. The possibility that the observations of improved thymic morphology reflect natural variability cannot be dismissed.

There was no clinically apparent effect on glucose metabolism in the experimental group when compared with controls. All dogs had normal intravenous glucose tolerance tests on day 30. Growth hormone excess is known to produce glucose antagonism and a depressed glucose tolerance test (prolonged hyperglycemia) (1,20). Since tolerance to clonidine developed, growth hormone was not being secreted in significant amounts on day 30 when the glucose tolerance tests were conducted. Had glucose tolerance tests been conducted more frequently, effects of clonidine induced GH secretion on glucose metabolism may have been noted.

SUMMARY AND CONCLUSIONS

This study was conducted in the hope of identifying a non-toxic oral substance that could be given to dogs mixed with their food with the ultimate hope of decreasing the incidence of age-related diseases that are linked to immune senescence. Based upon previous investigations in dogs by Roth et al. (48,49), Monroe et al. (40,42) and Goff et al. (24) an attempt to reverse the age associated decline of immune competence was made by evaluating several secretagogues of GH. Arginine and ornithine although effective secretagogues of GH in some species were shown here to be ineffective in beagle dogs.

Oral clonidine (100 ug/kg), when mixed with dog food was found to be a safe and reliable secretagogue of GH. Old dogs were found to be capable of GH secretion in response to oral clonidine that exceeded that of young dogs. This established that, in beagle dogs at least, GH secretion is not obtunded by, and may be enhanced by, advanced age if the proper stimulus is provided. However, the release of GH by oral clonidine is diminished with daily administration. Humans who chronically take clonidine for control of essential hypertension are not reported to suffer from an increased risk of diabetes mellitus or acromegaly. Although the total adult human dose (0.2 to 0.8 mg/day given in divided doses) is far less than that given to the dogs in this study, humans might also experience down regulation with respect to GH secretion or there would likely be a higher incidence of side effects

referable to GH excess. It is not known how soon down regulation to oral clonidine develops in dogs but it apparently does occur within 30 days.

Likewise, immune enhancement seems to be blunted by the daily administration of clonidine. The apparent transient beneficial effects of phytohemagglutinin on lymphocyte blastogenesis is, however, encouraging.

Thymus endocrine function (as reflected by plasma thymulin titers) was not enhanced by the addition of clonidine to dogs diets. Insufficient evidence exists in this study to conclude that there was any benefit on thymus morphology from feeding clonidine supplemented food to dogs. The natural variability of adult thymus glands and the induction of tolerance to clonidine precluded a clear outcome from this study.

Future investigations giving clonidine less frequently might avoid the induction of down regulation and clear benefits of clonidine supplemented food might become evident.

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APPENDIX: INFORMATION ON THE USE OF ANIMALS IN RESEARCH

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