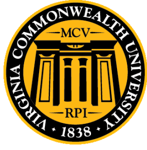

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30 April 1990

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IMMUNOMODULATORS IN SENESCENT MICE

A dissertation submitted in partial fulfillment
of the requirements for the degree of

Doctor of Philosophy

at Virginia Commonwealth University

By

Kevin R. McCormick

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Medical College of Virginia
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Richmond, Virginia
December 1989

DEDICATION

It is with deep love and respect that I dedicate this work to my grandmother, Mildred S. Lyons, "Meme". I shall always strive to emulate her example of service to others and graceful resistance to the forces of aging.

Acknowledgements

It is a rare work that is entirely the result of a single person's efforts. It is only fitting therefore that before the reader examines the work which follows that the author give credit to those who have contributed to this project. And so it is with pleasure and gratitude that I acknowledge the following people for their contribution to my Ph.D..

With friendship and respect I thank my classmates Claudia, John, Kathi, Margarita, and Tom. It is a fortunate student whose classmates become such close friends.

I thank my wife Kitty for her support and encouragement during this project, which often called for large measures of both. Special thanks to my friend and housemate, Andrew Hall, for his interest, support, and patience throughout this project and to Dr. Jeffrey Taubenberger for his support and assistance during all phases of this project.

The Anatomy Department office staff has been extremely helpful and kind to me and I gratefully acknowledge their assistance. Likewise, I am indebted to the reference staff of Tomkins-McCaw Library for their generous assistance. I am indebted to Ms. Leigh Doane for teaching me the various immunological assays used in this project and to Dr. Brian Susskind and the members of his laboratory for their advice and the cells necessary for the cytotoxicity assays.

Several faculty members have made a significant contribution to my graduate education and so I wish to thank Drs. Caroline Jackson, George Leichnetz, and Robert Spencer. I am indebted to Dr. Richard Krieg for his contribution to the growth hormone studies in this project.

Fran White's assistance was critical to this project and I am grateful to her for her skill and patience.

I thank my parents for teaching me the value of education and for their efforts to mitigate the penury of graduate student life. I thank my godparents for their interest and encouragement in this and all aspects of my life.

It is with gratitude and friendship that I acknowledge the assistance, support, and patience of my advisor Jack L. Haar. We have endured drama, comedy, farce, and a few minor tragedies in the last four years. Through the many trials, I have never regretted my decision to train in *your lab*. Thank you.

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IMMUNOMODULATORS IN SENESCENT MICE

ABSTRACT

A dissertation submitted in partial fulfillment of the requirements for the degree of doctor of philosophy.

by Kevin R. McCormick

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Virginia Commonwealth University

Advisor: Jack L. Haar

The suppression of the immune response in senescent animals, including humans, is not complete and may be ameliorated by specific interventions. Previous studies have suggested that the replacement of thymus factors lost with age may rejuvenate senescent immune function. Similarly, exogenous growth hormone has been reported to improve senescent immune function in certain mammals. Other studies with the immunomodulator PSK claim to restore tumor-induced immunosuppression even in aged mice. This project investigated the abilities of thymus supernatant, ovine growth hormone, and PSK to rejuvenate different parameters of the senescent immune response.

In the first series of experiments, erythroid depleted bone marrow cells from 3 month and 24 month old CBA (Thy 1.2) mice were given to irradiated AKR (Thy1.1) mice and allowed to repopulate for 30 days. Flow cytometry analysis using

mAb Thy 1.1 and Thy 1.2 revealed that the old bone marrow was deficient in its ability to repopulate the thymus. Subsequent experiments revealed that treatment of the old bone marrow with thymus supernatant, made from neonatal thymus cultures, could restore the thymus repopulating ability of these cells.

The second part of this project investigated the reported ability of growth hormone to rejuvenate the age-involuted thymus and senescent immune response. Limited success was achieved using subcutaneous timed-release pellets containing ovine growth hormone. Twenty-four month old mice treated in this manner for 8 weeks demonstrated larger thymuses with nearly normal thymus morphology, i.e. distinct cortical and medullary regions. Various assays of cellular immune function exhibited no improvement.

PSK injections every other day injections of 18 month old mice, for one month, resulted in an increase in splenic mass when compared to saline treated age-matched controls. There was no improvement in the thymus morphology or in the cellular immune function of the treated animals.

GENERAL INTRODUCTION

This dissertation examines the function of the senescent thymus in mice as it relates to bone marrow stem cells and cellular immune function. These systems are significantly impaired with age and result in serious health problems in elderly individuals. Motivated by the possible health-care benefits for a rapidly aging society and a desire to understand basic principles of immune system ontogeny, numerous investigators have turned their attention to this topic over the last two decades. What follows is a brief description of normal thymus status and senescence related changes. Specific background related to the experiments of this dissertation is given in the introductions to each of the chapters.

Normal Thymus Morphology

The thymus is a primary lymphoid organ that is located just posterior to the sternum, slightly superior to the heart and anterior to the aorta and superior vena cava in the anterior mediastinum. Embryologically the thymus is derived from the epithelium of the third and the ventral portion of the fourth pharyngeal pouches. It is a bilobed structure that is flattened in its anterior-posterior aspect. Each

lobe is incompletely divided into lobules by connective tissue septae which project inward from the thin capsule of the lobes. Within each lobule, the thymus is divided into a cortex and medulla. The cortical area is densely populated with immature or developing lymphocytes and for this reason is dark staining in routine H&E histological preparations. The less densely packed medulla is lighter staining and the thymic epithelial cells are clearly distinguishable. The medulla also contains concentric whorls of epithelial cells that sometimes contain keratohyalin granules. These structures are called Hassall's or thymic corpuscles. The thymus has efferent lymphatics which drain the organ but there are no afferent lymphatics, thus there is no lymphatic drainage through the thymus. Blood is supplied to the thymus by capillaries of the inferior thyroid and internal thoracic arteries. These capillaries enter the cortex and form anastomosing arcades along the cortico-medullary border. Blood drains via medullary venules into internal thoracic, inferior thyroid, and left brachiocephalic veins.

Normal Thymus Function

In order to understand the role of the thymus in normal immune function one must understand the ontogeny of lymphopoiesis. Three populations of cells represent the major cell types in this process. The first is multipotential hematopoietic stem cells. These first

develop in the blood islands of the yolk sac from mesenchymal cells (Moore and Metcalf, 1970; Haar and Ackerman, 1971). Hematopoietic stem cells from the yolk sac seed the fetal liver. The fetal liver in turn seeds the spleen and the bone marrow with stem cells. After birth, red bone marrow is the chief site of hematopoiesis. Each of these sites has a pool of self-renewing multipotent or pluripotent stem cells capable of differentiating into all of the mature functional blood cells (Abramson et al., 1977; Suda et al., 1983). The second population of hematopoietic cells is composed of several types of differentiating progenitor cells. These cells have a strong, but probably limited, ability to renew themselves and exhibit varying degrees of commitment to specific lineages (Abramson et al., 1977; Wendling et al., 1985). The prothymocyte or pre-thymic stem cell is an example of one of these cell types. These yet to be isolated cells have differentiated from pluripotent stem cells and are developmentally committed to becoming thymocytes. The third population of hematopoietic cells is represented by the fully functional mature blood cells (Cormack, 1987).

Researchers attempting to isolate pluripotent stem cells have done so by seeking cell markers that are unique to this first population of hematopoietic cells. Monoclonal antibodies (mAbs) have been a major tool in these studies. Two such mAbs are Sca-1 and Sca-2 (stem cell antigens 1 and 2). Spangrude et al. (1988a) reported that Sca-1$^+$ bone

marrow cells with the following characteristics are pluripotent hematopoietic stem cells: 1.expression of low but significant levels of cell differentiation antigen Thy-1 (Thy-$1^{lo)}$; 2.are negative for cell surface markers characteristic of B cells, granulocytes, and myelomonocytic cell lineages ($Lin^{-)}$. These Sca-1^{+}, Thy-$1^{lo,}$ and Lin- cells represent the first population of hematopoietic cells, pluripotent stem cells. Sca-1 antigen is expressed throughout T cell development, including mature peripheral T cells and is identical to the previously known Ly-6A.2 molecule. Sca-2 is lost during maturation and is therefore only found on immature thymocytes. In addition, Sca-2 is expressed on germinal center B cells (Spangrude et al., 1988b).

Attempts to isolate the second population of hematopoietic cells, committed precursor cells, have also used cell markers that are specific for a given lineage. One such marker of differentiated stem cells is terminal deoxynucleotidyl transferase (TdT). TdT is an intracellular enzyme which marks the initial differentiation of the pluripotent stem cells of the yolk sac, bone marrow, and liver into lymphocyte precursors. TdT is also found on cortical, Thy 1+, thymocytes. TdT is lost during intrathymic maturation and is not found in the medullary T cell populations or in peripheral lymphoid organs (Bollum, 1975; Goldschneider et al., 1977). TdT+ bone marrow cells represent the second group of

hematopoietic cells, committed but undifferentiated progenitors. As such, TdT represents the earliest marker for stem cells committed to the lymphocyte lineage.

Lymphopoiesis begins at 11-14 days of gestation in the mouse, 8 to 9 weeks in humans, when prothymocytes colonize the thymus. At this stage of development, the liver is the site of proliferation and early differentiation of pluripotent hematopoietic stem cells. At day 13 the earliest known thymocyte (T cell) surface antigen, Thy 1 (CD2 in man), is expressed on the lymphoid cells which have seeded the thymus. Thy 1 is the earliest intrathymic T cell marker, while ontogenetically TdT is a marker for stem cells committed to lymphocyte lineages and Sca-1 is found on pluripotent stem cells.

The thymus is necessary for the development of normal immune function (Miller, 1961; Stutman, 1977). Bone marrow derived T cell progenitors mature in the thymus, a process commonly referred to as "thymic education". While the maturation of T-lymphocytes is known to take place in the thymus, the exact mechanism of this "education" is unknown. Current theories of T cell maturation state that positive and negative selection events occur in the thymus (von Boehmer et al., 1989). The overwhelming majority of thymus immigrants do not survive this selection process and die intrathymically. Negative selection involves the removal of all cells that react too well (i.e. as if foreign) with

self-MHC. Negative selection is the mechanism by which tolerance to self antigens is induced. Kappler et al. (1987) have shown that T cells which react with MHC class II proteins are depleted during intrathymic maturation and are therefore eliminated from the mature thymocyte and peripheral T cell populations. Positive selection is the maturation of the cells that recognize foreign antigen in association with self-MHC. A recent study (Marrack et al., 1989) indicates that positive selection may occur through cell-cell contact with a unique form of MHC present on thymic epithelial cells. Thymic nurse cells, which completely enclose developing thymocytes, are also believed to play a role in the selection process, although what that role is has yet to be determined (Kyewski, 1986). The relationship between these thymic epithelial cells and developing thymocytes is unique and may provide a critical microenvironment for T cell development.

Recent work on the ontogeny of the T cell receptor (TCR) has shed some light on what happens genetically to the developing thymocyte in the thymus but what role the various cell types play and how the selection occurs remains unclear. The T cell receptor is composed of one of two heterodimeric proteins, either $\alpha\beta$ or $\gamma\delta$. The $\gamma\delta$ TCR appears first ontogenetically but decreases with the approach of birth and only accounts for 1-10% of the peripheral blood T cells in man and mouse. TCR $\alpha\beta$ is the form found on most mature T cells. Studies of TCR

development indicate that the thymus is the principal site of the gene rearrangements responsible for the antigen recognition and self-tolerance of the mature TCR (review: Strominger, 1989). Still, the relationship between these events and the various constituents which compose the thymic microenvironment remains unelucidated.

The general pattern of development is as follows. T cell progenitors leave the bone marrow or fetal liver and migrate through the blood to the thymus. The cells leave the blood stream and enter the subcapsular space of the thymus. Eighty-five to ninety percent of the thymus lymphocytes are located in the cortex. In addition, it is in the cortex of the thymus that the majority of intrathymic proliferation takes place. Cortical thymocytes are sensitive to cortisone and the ability to survive cortisone treatment is often used to distinguish between mature and immature thymocytes. The maturing T cell is believed to move progressively into the cortex and eventually into the medulla of the thymus before it is exported to the peripheral lymphoid organs. There is conflicting evidence as to the status of recent thymus emigrants. Some report that the T cell is fully mature when it leaves the thymus, while others claim that there is post-thymic maturation in secondary lymphoid organs (Stutman, 1978; Piquet et al., 1981; Scollay, 1982). The weight of evidence favors the view that recent thymus

emigrants are fully mature T cells from the medulla of the thymus (Scollay et al., 1984; Shortman et al., 1987).

Humoral factors derived from thymic epithelial cells are believed to be involved in the maturation of T cells and the maintenance of cellular immunity but their exact role is unclear. There are three recognized and relatively well characterized thymic hormones: thymosin, thymopoietin, and thymulin (originally called facteur thymic serique, FTS). These and other more crude preparations have been reported to promote the differentiation of T cells as indicated by cell surface antigens (Good, 1983; Stutman, 1983). However, due to contradictory findings with varying doses and the ability of non-thymus factors to mimic some of these effects the role of thymic humoral factors is still unclear (Stutman, 1983). While these humoral factors have been shown to be sufficient for alteration of T cell phenotype, the ability of non-thymus products to mimic these effects has complicated the explanation of their role in T cell maturation and differentiation. Stutman (1983) maintains that cAMP changes induced by thymic hormones is the necessary event in T cell maturation.

Other experiments have suggested that the precursors need to come in contact with thymic epithelial cells in order to become fully functional (Potworoski et al., 1986; LaRocelle and Jones, 1989). As discussed above, the exact mechanisms of T cell differentiation are unclear but the

thymic microenvironment appears to be critical for the production of mature functional T cells. According to Stutman's model of T cell differentiation (Stutman, 1983), the necessary components of the thymic microenvironment needed for the production of mature and functional T cells are the thymic humoral factors, thymic stromal cells, and possibly the resident mature T cells of the thymus.

Thymus Morphology In Senescence

The first manifestation of the senescence of the immune system, thymic involution, was known to anatomist centuries before an even rudimentary understanding of immune function was obtained. In this century, nearly three decades before the role of the thymus in lymphocyte maturation would be elucidated, Boyd (1932) published his paper on the weight of the thymus in health and disease. Boyd noted that the overall size of the human thymus was decreased with advancing age and that connective tissue, particularly adipose tissue, composed 90% of the organ after 40 years of age. The thymic cortex, which accounted for 45% of the thymic mass during childhood and adolescence, was reduced to 7-8% of the total mass. The medulla, which had accounted for over 20% of the original mass, only comprised 2-3% of the thymus after the age of forty.

Textbooks and even journal articles describing thymus morphology are replete with references to a puberty initiated involution (Cormack, 1987; Weiss, 1983;

Graudecker, 1978; Sztein and A. Goldstein, 1986; Frasca et al., 1982). Yet examination of human thymus from sudden death autopsies reveals steady rate of involution beginning at one year of age (Steinmann et al., 1985). Others have described thymus size, weight and degree of involution as so variable as to make mean values by age worthless (Sloan, 1943; Kendall et al., 1980). Sloan (1943) gives lengthy descriptions of the thymuses he examined. His descriptions are of a gradual infiltration of fat between thymus lobules such that in many older individuals the thymic epithelium is reduced to thin plates sandwiched between fatty connective tissue. Gaudecker (1978) also describes the thymus of an older person as "islands" of thymus tissue within adipose tissue and adds that these islands are no different than that of a child. A more recent review of human thymic involution (Kraft et al., 1988) has reaffirmed these findings. Kraft et al. referred to the organ as the "thymic fat body" and described the involution as a "terminal disorganization" of the entire organ in which the lobular structure is lost and the resulting thin branching system of epithelial plates lack Hassall's corpuscles. The above descriptions are further developed in a report by Steinmann et al. (1985) which describes extensive adipose replacement of connective tissue and perivascular space. Their report also supports a puberty-independent thymic involution.

Regardless of whether thymic involution is initiated by the hormonal events of puberty, it is clear that steroid compounds have a significant and deleterious effect on the thymus and cellular immune function (Claman, 1972; Arya et al., 1984). It is also clear that the thymus does undergo an involution with age that includes extensive infiltration of adipose tissue into the organ. The rate and extent of this involution is highly variable and is affected by many factors including disease states (Boyd, 1932; Sloan, 1943; Kendall et al., 1980).

Senescent Thymus Function

Contrary to traditional clinical thought, the thymus does continue to function throughout adult life (Kendall,1984).T cell precursors continue to enter the thymus from the bone marrow and differentiate into phenotypically mature T cells throughout adult life (Steinmann and Muller-Hermelink, 1984). However, the senescent thymus functions at a greatly reduced level, reflecting its morphological state. The production of thymus factors is decreased in later life (Bach et al., 1975a,b; Lewis et al., 1978; Oosterom and Kater, 1981; Goff et al., 1987). The decrease in at least some thymic hormones may be due in part to inhibiting factors in the blood (Bach and Beaurain, 1979). Thymus factors have been postulated to act both intrathymically, as discussed above, and peripherally, including the bone marrow. The loss of thymic hormones with age has been hypothesized to be a contributing factor to the overall

senescence of the immune system (Kruisbeek, 1981). In addition, the number of T cells leaving the thymus is believed to decrease with age. This is supported by the observation that thymuses from old mice have a decreased ability to repopulate the T cell dependent areas of lymph nodes (Hirokawa and Makinodan, 1975). The decrease in thymus function with age may very well be the primary event in the senescence of the immune system.

Senescent Immune Function

The decline in immune function with aging is widely known and well documented (reviews: Cinader, 1982; Doggett et al., 1981; Jones and Ennist, 1985; Szewczuk and Wade, 1983; Walford, 1980; Weksler, 1982,1983). This decline in immunocompetence is primarily the result of deficiencies in cellular immune function. Declines in humoral immunity are only slight and most likely due to dysfunction of regulatory T cells.

The changes in humoral immunity with age are slight and generally do not contribute greatly to the clinically expressed pathologies of immunosenescence. Total immunoglobulin titers do not change significantly with age, although the distribution of different classes does change. IgA and IgG concentrations are increased, while IgM concentration is decreased (Hallgren et al., 1973). Paradoxically, antibody to foreign antigens decreases with advanced age (Roberts-Thomson et al., 1974), while auto-

antibodies increase (Axelsson et al., 1966; Rowley et al., 1968). In spite of the increased incidence of auto-antibodies, there is no increase in the incidence of autoimmunity with age. There is a significant increase in the incidence of benign monoclonal gammopathies in later life (Axelson, 1966). This dysregulation of humoral immunity appears to be thymus related since neonatal thymectomy results in an earlier onset and an increased incidence of monoclonal immunoglobulins (Radl et al., 1980).

The changes in cellular immune function with age are much more profound than those of the humoral component. Studies of cellular immunosenescence in humans have reported that the total number of T cells is decreased with age (Lighart et al.,1985; Nagel et al.,1981; O'Leary et al.,1983). Other studies in humans have found no decrease or an insignificant decrease in the absolute number of peripheral T cells (Cowan et al., 1981; Nagel et al., 1981; Kruisbeek, 1981). These discrepancies may be a product of the assays used. Early studies used the sheep erythrocyte rosette forming assay while more recent studies have utilized monoclonal antibody labelling techniques. It should also be realized that total numbers of T cells may exhibit great variability among individuals and may not reflect immunological status. More important than cell numbers is the functional status of the cells. The functional state is greatly reduced as determined in numerous assay systems

which use equal numbers of cells from young and aged subjects, thereby controlling for any differences in cell numbers (see reviews above). Studies in mice have found that the changes in T cell number vary according to the tissue source but in general are unchanged with age (Makinodan et al.,1986). There is however, a significant decrease with age in the percentage of T cells that respond to antigenic challenge (Cowan et al., 1981) and an overall decrease in nearly all T cell functions.

Various assay systems have been devised to assess normal cellular immune function. The immune system of aged humans and laboratory animals are seriously impaired as measured by assays of T cell function. The results of these assays reflect the increased incidence of disease, particularly infectious diseases and cancer, seen in the elderly population. T cells have a decrease in their response to plant lectins, both Con A (Hirokawa and Makinodan, 1975; Gillis et al., 1981; Thoman and Weigle, 1981 & 1982) and PHA (Cowan et al., 1981; Gillis et al.,1981; Mysliwska et al., 1985) . T cell populations from aged donors also have a decreased level of IL-2 production and IL-2 receptors (Chang et al., 1982; Gillis et al.,1981; Gilman et al., 1982; Miller and Stutman, 1981; Thoman et al., 1981; Thoman and Weigle, 1982). The failure of the IL-2 system is one of the critical failures in the senescent immune response since IL-2 is necessary for the proliferation of activated lymphocytes. Cytotoxic T-lymphocytes (CTLs) are

responsible for immune surveillance and elimination of virally infected and neoplastic cells. CTL activity is greatly decreased with age (Becker et al., 1979; Haar et al.,1988) and corresponds to the increased incidence of cancer and serious viral illnesses among the elderly. The rejection of foreign tissue grafts is another example of the body's immune system fighting foreign invasion. The alloantibody response of aged animals is also greatly reduced with age (Makinodan et al., 1976). Another generalized example of a vigorous immune response is delayed-type hypersensitivity (DTH). DTH is diminished in elderly individuals and exhibits an inexplicably high correlation with mortality (Roberts-Thomson et al., 1974).

Interventions in the Aging Process

The suppression of the immune response with age is not complete and exhibits some reversibility, at least under certain experimental conditions. These attempts at immunological rejuvenation generally fall into one of three categories: 1. Attempts to replace physiologically active factors which are lost or diminished with age; 2. Pharmacological treatments; and 3. Nutritional manipulations. Limited success in boosting immune response has been achieved with each of these forms of treatment. Still no lasting and clinically applicable treatment has been found.

Attempts to rejuvenate the senescent immune response by replacing thymus tissue and/or thymus-derived factors have been numerous and variably successful. Bach (1977) found that thymic factor could prevent the depression of lymphocyte cytotoxicity in adult thymectomized mice but depressed the cytotoxic response in young and aged mice (Bach, 1977). In one study (Cowan et al.,1981), thymosin fraction 5 in vitro was found to increase the mixed lymphocyte reaction of human peripheral blood lymphocytes. However, other studies found no improvement in the immune response of peripheral blood lymphocytes of elderly human subjects (Quinti et al., 1981; Ershler et al., 1985). Frasca et al. (1982) found that injections of synthetic thymosin-α1 in aged mice increased helper cell activity as measured by anti-TNP antibody production and T cell proliferation. Two later studies (Frasca et al., 1986; Frasca et al., 1987) found that it was the N-terminal half of thymosin-α1 (N_{14} fragment) which was responsible for the augmented immune response in aged mice. These later studies also found that the N_{14} fragment was responsible for increasing the frequency of mitogen responsive cells in the spleens of old but not young mice.

The mechanism by which thymic factors may stimulate the senescent immune response remains to be elucidated but it is an encouraging line of inquiry. A recently reported clinical study (Ershler et al., 1989) indicates that

thymosin-$\alpha 1$ may act as an effective adjuvant for prophylactic vaccinations in the elderly.

Other researchers have attempted to rejuvenate the senescent immune response through manipulations of the endocrine system. Fabris et al. (1986) examined the immunological effects of a specific dietary supplement known to act on the endocrine system. It is known that lysine is essential for body growth and that arginine can augment the secretion of growth hormone. Fabris and colleagues treated aged mice with a commercially available lysine-arginine supplement (Neoiodoarsolo^{R}) for fifteen days. Their results indicate a restoration of thymic production of thymulin and an increased responsiveness of spleen cells to PHA. Con A, LPS, and natural killer cell responses were unchanged. Levels of circulating thymulin in aged human subjects were found to be increased after 30-40 days of treatment. It was noted that there was no direct effect of the lysine-arginine on spleen cells in vitro. This lack of direct effect on splenocytes indicates that the improvement is probably due to thymic or other endocrine changes.

Recently two groups of investigators have attempted to restore immune function in senescent animals using anterior pituitary hormones (Goff et al., 1987; Roth et al., 1984; Kelley et al., 1986). These studies in rats and dogs indicate that pituitary hormones may be able to improve the

senescent immune response. This work may contribute significantly to our understanding of the interactions of the neuroendocrine and immune systems in aging and other life events. The design and results of these studies are reviewed in greater detail in the introduction to Chapter 2.

Still other researchers have attacked the immunosenescent state armed with pharmacological agents believed to be immunopotentiators. Bruley-Rosset et al. (1986) achieved improved immune responses in aged mice using a four month treatment of sodium dithiocarbamate, DTC (Imuthiol^{R}). Treatment with this synthetic agent restored Con A response, CTL activity, and serum thymic factor levels. PHA response and delayed-type hypersensitivity were also improved. Natural killer cell activity and graft versus host mortality were not changed by the treatment. The authors believe that the improvement is due to restoration of thymus endocrine activities.

Another chemical that has been shown to have immunopotentiating activity is isoprinosine. Isoprinosine is a chemical immunopotentiator that has been shown to be capable of stimulating immune function in cancer patients. A study with hamsters has shown that isoprinosine is capable of restoring certain immunological functions to near youthful levels (Tsang et al., 1983). They found that a single IP injection was capable of restoring natural

killer cell activity, suppressor cell function, and PHA response. Furthermore, they found that weekly injections maintained these immunological parameters at or near the levels found in young hamsters. The mechanism of action is unknown but the authors speculate that it may be due to increases in interleukin production.

Caloric restriction in aged mice has been shown to improve the senescent immune response, increase survival time, and decrease the incidence of tumors (Gerbase-Delima et al., 1975; Weindruch et al., 1982; Weindruch et al., 1983). While these studies may help to unravel the mysteries of immunosenescence, the clinical applications of caloric restriction are obviously limited.

These studies indicate that rejuvenation of the senescent immune response is a promising endeavor. The current body of knowledge is encouraging but the basic mechanisms of the impairment and restitution of immune function are still unresolved. In addition, the clinical applications of these immunotherapies has yet to be determined.

Chapter One

The Bone Marrow - Thymus Axis in Senescence

The necessity of the thymus for the development of normal immune function is well documented (Miller, 1961; Stutman, 1977), and its involution with age has long been known (Boyd, 1932). In spite of this, all of the functions of the thymus are still not understood including the mechanism of thymocyte maturation. It has been postulated that the thymic hormones may act on lymphocyte precursors in the bone marrow and that the loss of thymic factors during senescence may be a contributing factor to the decreased cellular immune function (Kruisbeek, 1981).

There is a significant amount of experimental evidence to support the hypothesis that thymic factors influence T cell precursors in the bone marrow. Bone marrow from thymus deprived mice have a greatly reduced capacity to produce colony forming units in the spleen (CFU-S) (Zipori and Trainin, 1973; Zipori and Trainin, 1975 a,b). Tyan (1977) reported that aged mice, whose thymuses were involuted, exhibited an age-related decrease in T cell progenitors.

Haar et al. (1988a) have shown that the ability of bone marrow cells of senescent mice to migrate to thymus supernatant in vitro is greatly reduced. This migration was restored to youthful levels by the engraftment of thymic epithelial cells from neonatal mice into 24 month old mice. Similarly, athymic nude mice (AKR/J-nu^{str}/nu^{str}) have a depressed in vitro migration of bone marrow cells to thymus supernatant which can be restored by the grafting of thymic epithelial cells into the nude mice (Haar et al., 1988b). Koninkx et al. (1986) found that thymus supernatant fraction 5 could induce the expression of TdT, an immature lymphoid cell marker, on the bone marrow cells of athymic rats. These results strongly support the contention that thymic factors act on bone marrow cells and may be necessary for the differentiation of prothymocytes in the bone marrow.

A deficiency in the hemopoietic stem cell compartment of the bone marrow has been postulated as a possible cause of the decline in immune function with age (Tyan, 1977; Gozes et al., 1982). There have been conflicting findings as to the status of hemopoietic cells in senescent animals. There are several reports of studies measuring colony forming units in the spleen (CFU-S) in which no difference between young and old mice was found (Lajtha et al., 1971; Coggle et al., 1975; Harrison et al., 1975; and Scholfield et al., 1986). Gozes et al. (1982) found no differences between young and old bone marrow in CFU-S

ability. However, they did detect a significant decline in mixed lymphocyte reactions and Con A response, at 12 months, in recipients of aged bone marrow. Another study found that aged and young bone marrow were equally capable of curing the stem cell deficiency of W/W^v mice (Boggs et al., 1984). Work with bone marrow cultures showed a pattern of growth similar to Hayflick's work with fibroblasts, i.e. decreased cumulative cell recovery and culture survival time as donor age increased (Lipschitz and Udupa, 1984). Bone marrow cultures have also been used to reconstitute lethally irradiated mice. In one study young and aged bone marrow were compared for their ability to produce CFU-S. During the first four weeks of culture, the aged bone marrow actually produced more CFU-S than the young bone marrow. This was reversed after four weeks and cumulative CFU-S over a 13 week period were greater in cultures of young bone marrow (Mauch et al., 1982).

Bone marrow chimeras produced between young and old mice using Thy1.1 and Thy1.2 congenic mice have been used to investigate the ability of senescent bone marrow cells to repopulate thymus and splenic T cells (Hirokawa et al., 1986). Analysis by flow cytometry revealed fewer donor cells were present in the thymuses and spleens of the mice given old bone marrow than in mice given young bone marrow. The ability to accurately follow donor bone marrow makes this a good method for examining the effects of thymus factors on senescent bone marrow. Also, the ability to

treat donor bone marrow with thymus factors prior to intravenous injection makes this an attractive method for evaluating thymus - bone marrow interactions.

The goal of this experiment was to evaluate what effect thymus supernatant has on the ability of bone marrow cells from senescent mice to repopulate the thymus of an irradiated host. We found that the bone marrow from old mice had a greatly reduced ability to repopulate the thymus of an irradiated host. Further, we found that a brief treatment of the old bone marrow with thymus supernatant significantly improved its thymus repopulating ability.

Materials & Methods

Animals

Aged CBA male mice 24 months of age were obtained from Charles River Laboratories (Kingston, NY) through the National Institute on Aging. AKR/J male mice 6-8 weeks old and CBA/J male mice 2-6 months old were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were housed three per cage. The cages were kept in laminar flow hoods on a 12 hour light/dark cycle. Mice were provided with water and autoclaved Purina mouse chow ad libitum.

Preparation of Supernatant

Thymus supernatant was prepared by placing 3 minced neonatal thymuses from CBA/J mice in 3 ml of Iscove's serum free-media (GIBCO, Grand Island, NY) in 35 mm petri dishes (Falcon, Oxnard, CA). The tissue was cultured for 48 hours at 37°C in a humid atmosphere of 5% CO_2. After the cells and debris were pelleted by centrifugation, the supernatants were collected, filtered through 0.22μm filters (Gelman, Ann Arbor, MI) and stored at -70°C until used for incubating aged bone marrow cells.

Preparation of Bone Marrow Cells for In Vitro Migration Assay

Young and aged CBA male mice were killed by cervical dislocation and the tibias and femurs were removed. The bones were crushed with a sterile mortar and pestle in cold Hank's balanced salt solution (GIBCO, Grand Island, NY) with 5% BSA. After transferring to a test tube, the large debris was allowed to settle for 5 minutes. The remaining cell suspension was pipetted off, centrifuged, and the erythroid cells were lysed with Gey's lysing buffer. The cells were then washed twice. Following this the cells were counted and viability determined by trypan blue exclusion. Aged bone marrow cells were placed in thymus supernatant or Iscove's media (GIBCO, Grand Isle, NY) at 37°C for one hour after which they were resuspended in fresh Iscove's media at a concentration of $5x10^6$/ml.

In Vitro Migration Assay

Blind well chambers (Nuclepore, Pleasonton, CA) were used containing two chambers each with a volume of 200µl, separated by a 5µm pore size filter. Two hundred µl of thymus supernatant or Iscove's media was placed in the lower chamber and 200µl of bone marrow cells were placed in the upper chamber. Multiple wells were set up for each experimental and control sample. The wells were incubated for 90 minutes at 37°C. Then the bottom chambers for each sample type were collected, counted, and percent migration calculated.

Preparation of Bone Marrow Cells for In Vivo Migration

The bone marrow cells were prepared as for the in vitro assay (described above) with the following additions. Following lysis of the erythroid cells, the remaining bone marrow cells were treated with mAb Thy 1.2 (ICN, Lisle, IL) and incubated for 60 minutes at 4°C. The cells were then washed and treated with Low-Tox-M rabbit complement (Cedarlane Laboratories, Ontario, Canada) for 60 minutes at 37°C to lyse the mature T cells. The cells were washed and then resuspended in the appropriate experimental or control media. Aged bone marrow was placed in thymus supernatant or Iscove's media at 37°C for one hour. Untreated control bone marrow cells were incubated in normal saline on ice until injected. Young bone marrow cells were also resuspended in normal saline and held on ice. Supernatant and Iscove's treated cells were washed after treatment and

all cells were counted on a hemacytometer and viability determined by trypan blue exclusion. Cells were adjusted to a concentration of $2x10^7$/ml.

Recipient Mice

Six to ten week old AKR male mice were given 600 or 850 rads (R) of radiation at a rate of 100R/minute in a Mark I-Cesium 137 Irradiator (J.L. Sheperd & Associates, San Fernando, CA). After irradiation, the mice were injected via the tail vein with $4x10^6$ bone marrow cells while confined in a rodent restraint (Plas Labs, Lansing, MI).

Examination of Thymus and Spleen for Donor Cells

At the end of four weeks the recipient mice (AKR) were sacrificed by cervical dislocation after which thymuses and spleens were quickly removed and weighed. Cell suspensions were then prepared by passing the tissue through 100 mesh nylon cloth, and treated with Gey's lysing buffer to remove the erythrocytes. Aliquots of cells from both thymus and spleen were subsequently treated with either Thy 1.1 or Thy 1.2 FITC conjugated monoclonal antibodies (ICN, Lisle, IL). After labelling, the cells were washed and resuspended in PBS for analysis by flow cytometry to determine the percentage of donor positive cells (Thy 1.2^+) and radiation resistant host cells (Thy 1.1^+) in each organ.

RESULTS

In Vitro

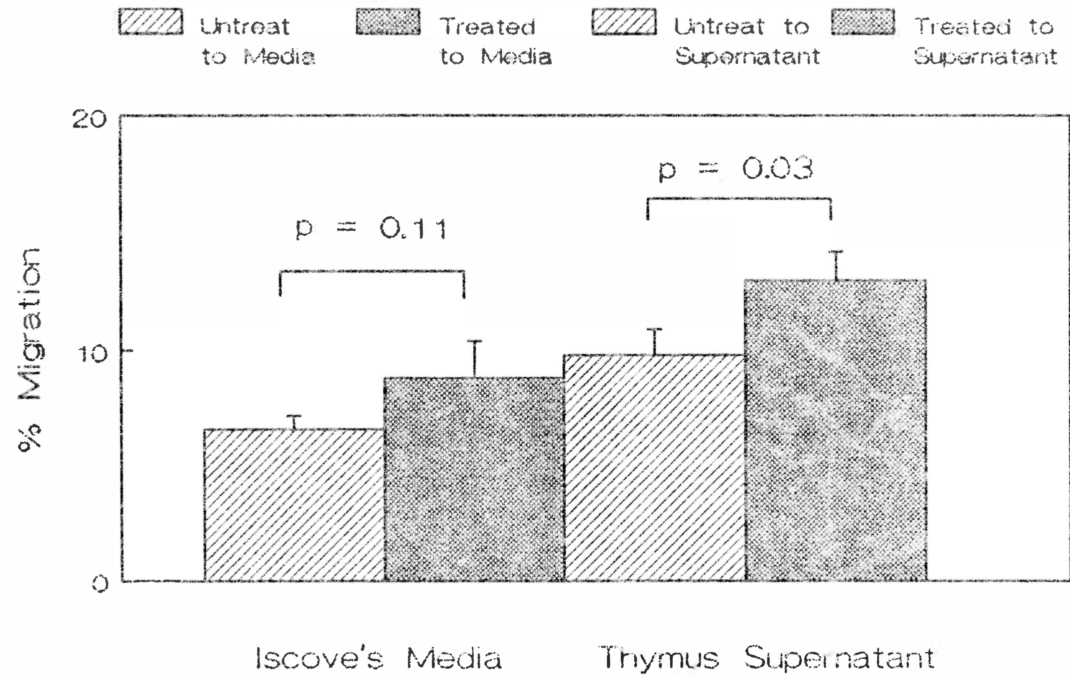
In the in vitro assay of bone marrow chemotaxis it was found that incubation of bone marrow cells in thymus supernatant for one hour increased the migration of the cells. Migration to thymus supernatant was increased by 32% ($p = 0.03$) but the control migration to Iscove's media was not significantly ($p = 0.11$) increased by the thymus supernatant pre-treatment (Figure 1.1).

In Vivo

In the in vivo assay of thymus homing ability it was determined that bone marrow from senescent mice was impaired in its ability to repopulate the thymus of an irradiated host when examined at 4 weeks post transplant ($p = 0.027$) (Figure 1.2). Our assay did not show a significant difference between bone marrow from young and aged mice in their ability to repopulate the spleen with mature T cells at this same time interval (Figure 1.3).

Treatment of the aged bone marrow cells with a one hour incubation in thymus supernatant prior to injection resulted in a dramatic improvement in the ability of senescent bone marrow to repopulate the thymus of an irradiated host ($p=0.031$) (Figure 1.4). The ability of the thymus supernatant treated bone marrow to reconstitute the thymus was improved to a level that was not significantly

Figure 1.1 In vitro Migration of Aged BM Before & After Supernatant Treatment



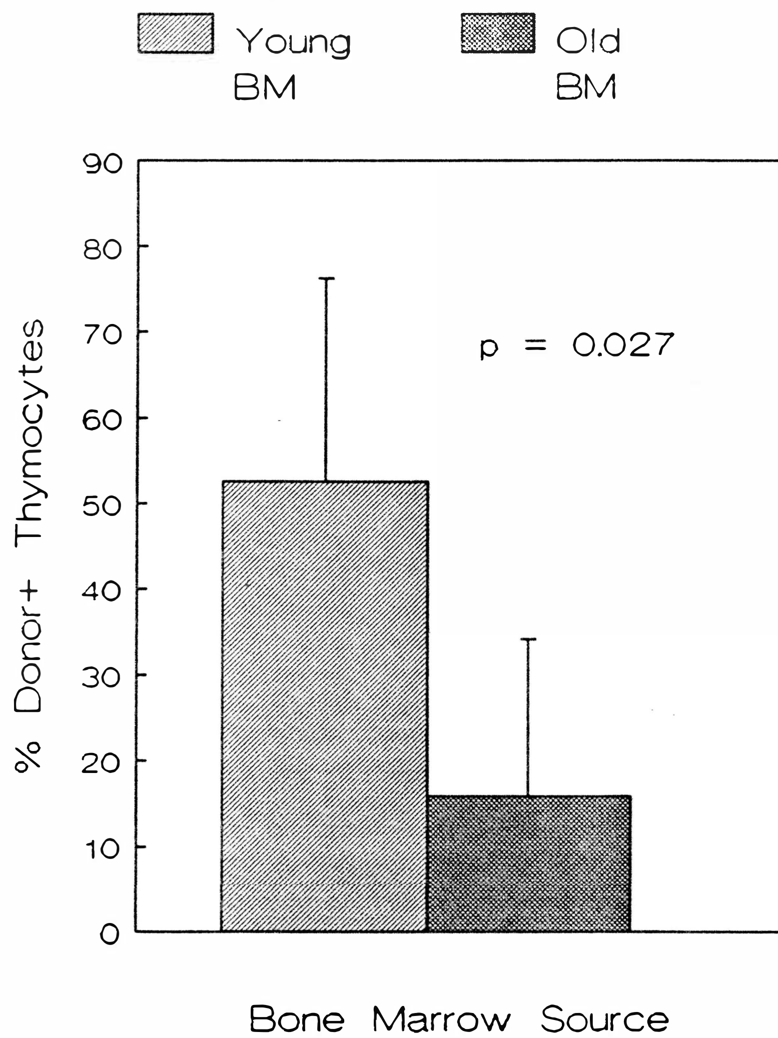


Figure 1.2 Thymus Repopulation
Young vs. Old Bone Marrow
Young BM
Old BM
90
80
70
60
50
40
30
20
10
0
% Donor+ Thymocytes
p = 0.027
Bone Marrow Source

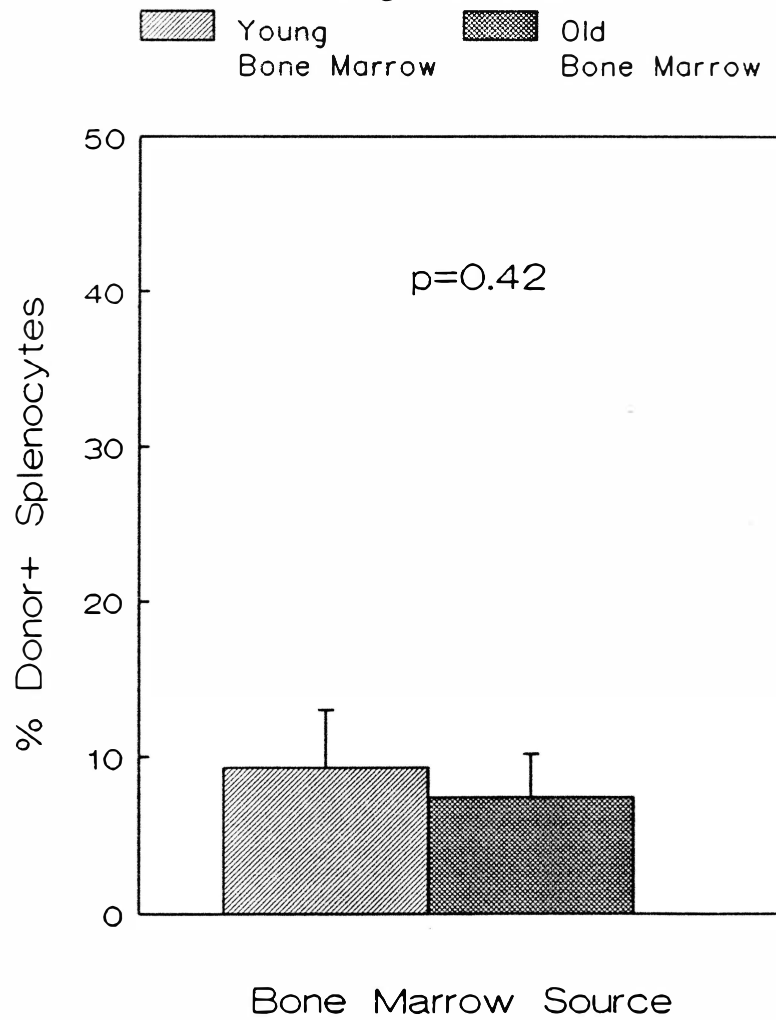


Figure 1.3 Spleen Repopulation
Young vs Old BM
Young
Bone Marrow
Old
Bone Marrow
50
40
30
20
10
0
% Donor+ Splenocytes
p=0.42
Bone Marrow Source

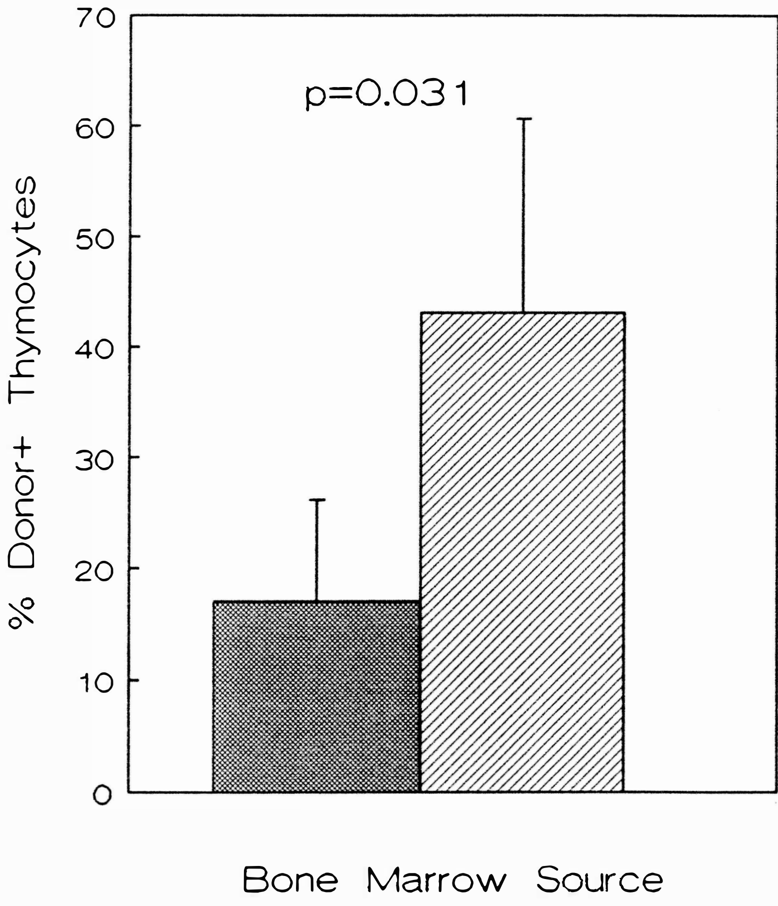


Figure 1.4 Thymus Repopulation
Old vs. Old Treated BM
Old
Bone Marrow
Old Treated
Bone Marrow
p=0.031
% Donor+ Thymocytes
0
10
20
30
40
50
60
70
Bone Marrow Source

different from that of young bone marrow (Figure 1.5). From these data it appears that the senescent bone marrow has been restored to youthful levels of function as regards thymus seeding abilities.

Treatment of aged bone marrow cells with Iscove's media, the media base for the supernatant, did not improve their thymus repopulating ability over that of untreated aged bone marrow (p=0.26) (Figure 1.6). There was no difference between young and aged bone marrow cells in their ability to seed the spleen with mature T cells at this time In addition, treatment of aged bone marrow cells with thymus supernatant prior to injection had no effect on their ability to repopulate the spleen with mature T cells (Figure 1.7).

There was significant variability in the thymus repopulating ability of the bone marrow from young mice as well as from aged mice. One of the factors which was found to affect the thymus repopulating ability was the number of radiation resistant host cells in the thymus. Typical results are shown in Table 1.1. As these figures clearly demonstrate, high numbers of host cells in the thymus preclude repopulation regardless of the source of the bone marrow. However, the inferiority of the aged bone marrow compared to young bone marrow was consistent as was the improvement with thymus supernatant treatment. The consistency of the thymus repopulating ability of the

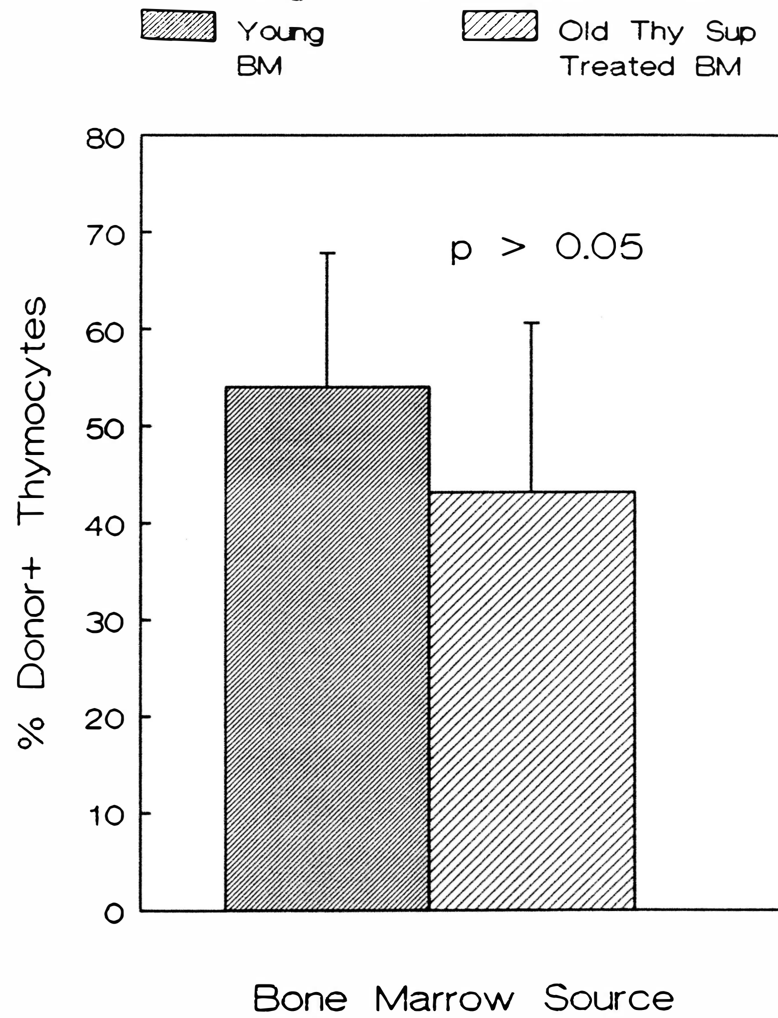


Figure 1.5 Thymus Repopulation
Young vs Old Treated BM
Young BM
Old Thy Sup Treated BM
p > 0.05
% Donor+ Thymocytes
80
70
60
50
40
30
20
10
0
Bone Marrow Source

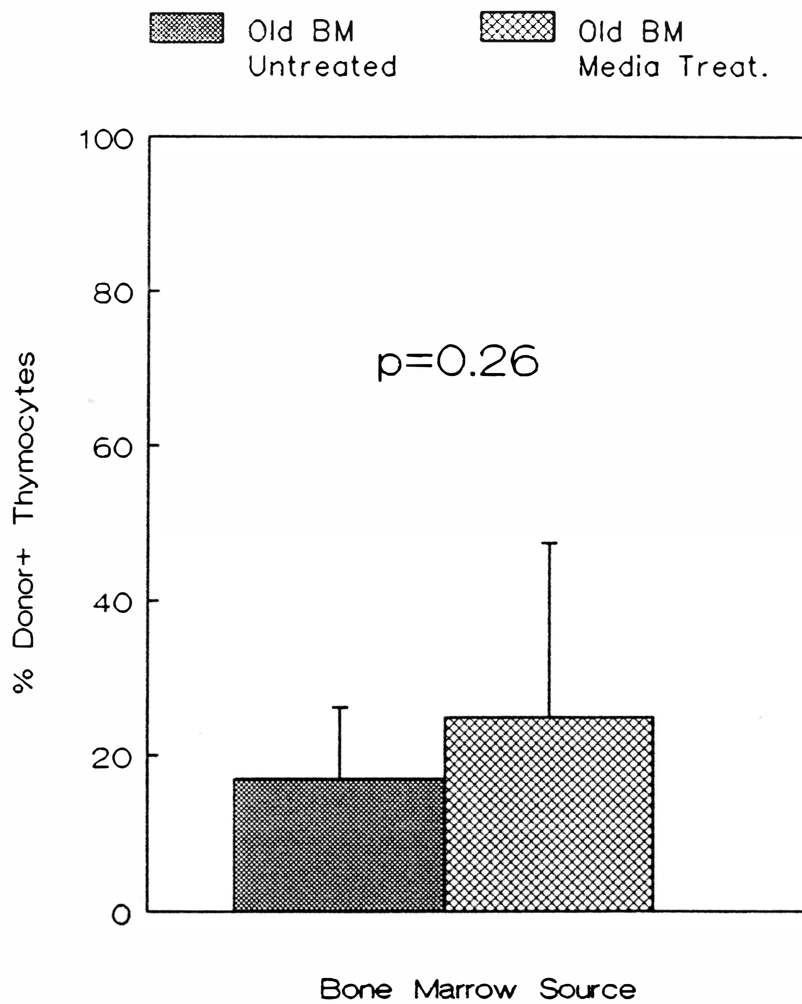


Figure 1.6 Thymus Repopulation
Old BM: Untreated vs Media
Old BM
Untreated
Old BM
Media Treat.
100
80
60
40
20
0
p=0.26
% Donor+ Thymocytes
Bone Marrow Source

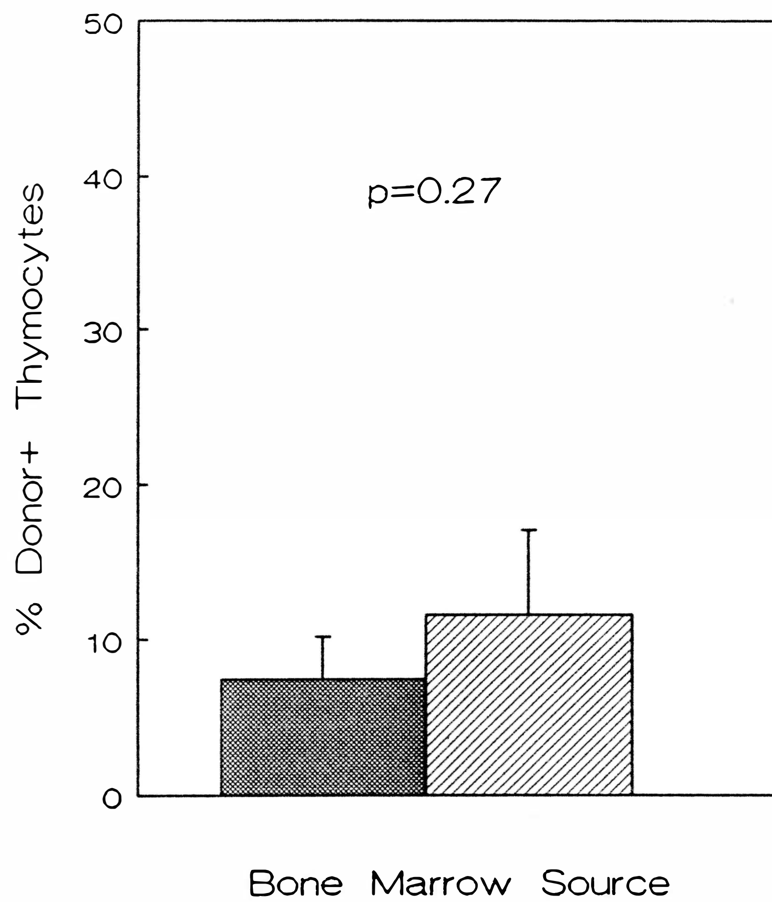


Figure 1.7 Spleen Repopulation
Old vs Old Treated BM
Old
Bone Marrow
Old Treated
Bone
% Donor+ Thymocytes
50
40
30
20
10
0
p=0.27
Bone Marrow Source

Table 1.1. Radiation Resistant Host Cells (Thy 1.1) and Donor Cells (Thy 1.2) as % of Thymocytes at Four Weeks Post-Irradiation. Repopulation is greater in mice where host cells are few regardless of the source of donor cells.

Rads	Thy	Young	Old	Old Treated
600	1.1	34.2	71.8	63.8
	1.2	24.9	0.68	2.69
850	1.1	1.51	1.35	0.64
	1.2	85.43	43.52	89.55

Table 1.2. Thymus Repopulating Ability for All Experiments Expressed as a Ratios of Percentage of Donor+ Thymocytes.

	Young : Old	Old Treated:Old
x	28.20	3.96
σ_n	24.86	2.67

different bone marrow populations is clear when one expresses the results of all the experiments, regardless of radiation exposure and number of radiation resistant host cells in the thymus, as a ratio (Table 1.2). If the groups are similar the ratio obviously would be equal to one or nearly so. As can be seen in Table 1.2, this is not the case. Both the ratio of young:old and old treated:old are significantly greater than one.

DISCUSSION

Our results indicate that there is some factor in the thymus supernatant that improves the thymus homing ability of senescent bone marrow cells. Treatment of bone marrow from senescent mice with thymus supernatant restored the ability of these cells to migrate to both thymus supernatant in vitro and to the thymus in vivo. The fact that Iscove's media, the media base for the thymus supernatant, did not improve the thymus repopulating ability of the aged bone marrow indicates that the improvement is due to a factor produced by the thymus cultures.

It is known that the radiation resistant host thymocytes proliferate in the thymus and reseed the peripheral lymphoid organs after irradiation (Hirokawa et al., 1985). In a previous study it was determined that in bone marrow

chimeras the donor-type T cells first repopulate the thymus and then emigrate to peripheral lymphoid tissues (Hirokawa et al., 1986). The donor-type T cells were first detected in the spleen at 14 days and the difference in the number of donor cells from young and aged donor cells was not determined until 28 days. The percentage of donor-type T cells in the spleens of irradiated mice was not significantly different between young and aged donors until 56 days. Similarly, in the present study it was not possible to detect a difference in the percentage of donor-type cells from young and old donors in the spleen at 28 days. Nor could we detect any difference in the ability of old bone marrow and thymus supernatant treated old bone marrow to generate splenic repopulation. This is probably due to the low numbers of donor derived T cells present in the spleen at this time. The differences that are seen in the thymus have yet to manifest themselves in the spleen since the number of mature T cells that have been exported to the peripheral lymphoid tissues is still too low at this time after irradiation.

The present study alone does not reveal whether the factor responsible for maintaining T cell precursors in the bone marrow is a previously unidentified factor produced by the thymus. It is possible that we have discovered another function of previously isolated thymus products such as thymulin, thymopoietin, or one of the thymosins. Since the production of these thymus factors is decreased in

later life, (Lewis et al., 1978; Goff et al., 1987; Oosterom and Kater, 1981; Bach et al., 1975a,b) this would explain the decreased function of bone marrow T cell precursors cells from senescent mice. Thymus cultures produce nonunique products (e.g. ubiquitin and general metabolic products) which need to be excluded before a definitive statement can be made. Further studies will be necessary to determine what factor is responsible for the thymus's apparent modulation of T cell progenitors in the bone marrow.

The role of the thymus in the development of the immune system has yet to be fully elucidated but the decreases in known thymus factors with age has been clearly documented as has their beneficial effect on the senescent immune system (Bach et al 1975a,b; Bach, 1977; Cowan et al.,1981; Erschler et al., 1985; Frasca et al., 1986; Frasca et al.,1987; Goff et al., 1987; Erschler et al., 1989). The recognition of the thymus's influence on bone marrow cells may be one step toward a more thorough understanding of the development of the immune system as well as its senescence.

It has been shown here and previously (Tyan, 1977; Hirokawa, 1986) that the T cell precursors in the bone marrow of senescent mice have a decreased ability to seed the thymus. Previous work in this laboratory with immunodeficient mice (athymic nude mice and 24 month old aged CBA mice) has demonstrated that there is an

impairment in the in vitro migration of the bone marrow cells in response to thymus factors (Haar et al., 1988a,b). In addition, these studies showed that thymic epithelial cell grafts were capable of restoring the in vitro migration of bone marrow cells to thymus supernatant to normal levels as well as improving cellular immune function. Those results alone indicated that the thymus is to some extent responsible for the maintenance of bone marrow cells in addition to its role in the development of a competent immune system. In the context of the present study, those previous experiments lend further support to our conclusion that thymus derived factors are responsible for the maintenance of T cell precursors in the bone marrow.

Previous work on the effects of thymus derived factors have sometimes overstated the unique properties of these factors. Therefore we are cautious in our interpretation of the above findings. However, since the T cell progenitors in the bone marrow of senescent mice are impaired, it is only logical to attribute the impairment and its rejuvenation to a factor known to be reduced in advanced age. We therefore claim that it is both possible and likely that the decreased ability of T cell precursors from the bone marrow of senescent mice to repopulate the thymus is the result of a loss of necessary tonic thymus factors acting on the bone marrow cells.

While there have been several studies on the effects of irradiation on the status of bone marrow cells (Boggs et al., 1967; Harrison and Astle, 1982), the effect of whole body irradiation on the thymus has not received such attention. From these results it appears that in order for significant repopulation of the thymus to occur the resident thymocyte population must be eliminated. It remains to be determined what the specific characteristics of the radiation resistant population of thymocytes are. Are they more mature? Are the radiation resistant cells the same population as the cortisone insensitive cells? Are they localized in the cortex or the medulla? The answers to these questions might help us to more fully understand what happens to thymocytes during their residence in the thymus.

The status of bone marrow stem cells in senescent animals is a matter on which there is some disagreement. One of the reasons for disagreement among investigators is the incomplete knowledge of hematopoietic differentiation of bone marrow stem cells even in non-senescent animals. Since it is not possible to isolate hematopoietic stem cells, numerous indirect assays of varying specificity have been employed. The specificity of different assays for pluripotent versus committed progenitor cells is unclear. A recent review by Spangrude (1989) discusses this problem and the significant advances that been made toward isolation of murine hemopoietic stem cells. The

difficulties involved in the specificity of stem cell assays have led to a second problem in the interpretation of such experiments in senescent animals, a failure to distinguish among different progenitor cells. Totipotent stem cells may be unimpaired with age but progenitor cells for specific lineages may not be as well preserved. Failure to distinguish between these different categories of cells has led to apparent contradictions. For example, Harrison has shown through some excellent studies that erythrocyte progenitors are not impaired with age (Harrison and Astle, 1982; Harrison et al., 1984). It would be incorrect, however, to extend these results to all progenitor cells or pluripotent hematopoietic stem cells. Erythrocytes must be replenished constantly due to the brief lifespan of the fully differentiated red blood cells. Exhausted red blood cells must be replaced through cells less differentiated than mature erythrocytes since the fully differentiated red blood cell is incapable of replication. T cells are known, however, to have lifespans that may be as long as a decade or more. In addition, fully mature T cells are capable of mitotic division. Therefore, because the T cell progenitor is not necessary for maintaining the normal population of mature cells, it would not be surprising to learn that the T cell progenitor is impaired or even lost in advanced age. Since the progenitor cells would not be called upon after the immune system had been fully established, such an impairment might be insignificant for normal immune function. For this

reason, we consider it unlikely that a deficiency in the T cell progenitor is a major factor in the decline of cellular immune function with age; we do consider this to be a possible contributing factor. However, the major benefit derived from investigating the status of the T cell progenitor in senescence is to more fully understand the variety of stem cells in the bone marrow and to more completely comprehend the senescent state.

Chapter Two

The Effects of Anterior Pituitary Hormones in Senescent Mice

Interactions between the endocrine system and the immune system have been reported with increasing frequency over the last two and a half decades. One of the earliest endocrine-immune system interactions was noted at the same time that the role of the thymus in immune function was being elucidated. Takemoto et al. (1962) reported that growth hormone causes an enlargement of the thymus. Another significant insight into the endocrine - immune relationship occurred when Pierpaoli and Sorkin (1967) reported that pituitary deficiency caused wasting disease, a syndrome normally associated with neonatal thymectomy. Subsequent to making this serendipitous finding, they treated young mice with "anti-hypophysis serum" which resulted in thymic involution and premature death.

As the following studies illustrate, experimental evidence indicates that the immune system is closely linked to the endocrine system via the thymus. The life span of short-lived dwarf mice can be extended by the injection of growth

factors and thyroxine but not if the thymus has been removed (Piantanelli and Fabris, 1977). Several studies have indicated that the thymus or thymus products can modulate hormone levels including luteinizing hormone, triiodo- thyroxine, and insulin (Fabris et al, 1972; Piantanelli et al., 1978; Rebar et al., 1981). However, the mechanism of such regulation is not understood. It has also been observed that neonatal thymectomy results in the degranulation of the acidophils of the anterior pituitary; these are the cells responsible for the production of growth hormone and prolactin (Pierpaoli and Sorkin, 1967).

More recent work indicates that anterior pituitary hormones may be able to reverse, at least partially, some of the immune dysfunction seen in senescent animals (Roth et al., 1984; Kelley et al., 1986; and Goff et al., 1987; Monroe et al., 1987). By treating various aged dogs with bovine growth hormone, it was found that growth hormone resulted in a rejuvenation of the thymic morphology of middle-aged (33-35 months) dogs. Thymus mass was increased, cortical and medullary regions were clearly defined, and fatty infiltration was minimal (Goff et al., 1987). It was also determined that serum levels of thymulin are decreased with age and that growth hormone treatment increased the level of thymulin in aged dogs. Earlier, Fabris and Mocchegiani (1985) had reported decreased levels of thymulin with age and variations in thymulin levels with endocrine imbalances.

Kelley et al. (1986) have reported similar restoration of thymus structure and cellular immune function in 18 and 24 month old rats given GH3 pituitary adenoma cells which secrete both growth hormone and prolactin. The GH3 cells were implanted subcutaneously and the animals sacrificed approximately two months later. While the 18 month old rats achieved a greater degree of rejuvenation, both 18 and 24 month old GH3 treated rats showed an improvement in thymus morphology and cellular immune response.

The current study investigated the ability of growth hormone to rejuvenate the age-involuted thymus and to restore the senescent immune response in 24 month old mice. Three means of delivering growth hormone to senescent mice were employed. The first method used GH3 cells enclosed in Millipore[R] diffusion chambers. The second method was daily injections of ovine growth hormone. Timed release pellets of ovine growth hormone implanted subcutaneously was the third method of hormone treatment. At the end of the treatment period, thymus mass and morphology were evaluated and assays of cellular immune function were performed. All animals were examined for gross pathologies at the end of the treatment period. Aberrant organs were removed and pathological evaluation was performed by qualified personnel.

Materials and Methods

Animals

CBA male mice 24 months of age were obtained from Charles River Laboratories (Kingston, NY) through the National Institute on Aging. Mice were housed 3 per cage. The cages were kept in laminar flow hoods on a 12 hour light/dark cycle. Mice were provided with water and autoclaved Purina mouse chow ad libitum.

GH3 Cells

GH3 cells are derived from rat pituitary adenoma and they secrete both growth hormone and prolactin. Kelley et al. (1986) injected these cells into the peritoneal cavity of senescent rats as a source of anterior pituitary hormones. Since the present study was conducted in mice, this technique was not feasible. Rat MHC antigens would be recognized as foreign by the mouse. By placing the GH3 cells in diffusion chambers (Millipore, Bedford, MA) the cells were protected from immunological attack by the host (mouse). At the same time this technique allowed GH3 cell products to diffuse into the peritoneal cavity and be absorbed by the mouse. The GH3 cells used in this experiment were the generous gift of Dr.Robert Adler.

Diffusion Chambers

Materials required for the assembly of diffusion chambers (DC) were obtained from Millipore Corporation (Bedford, MA). The DC were composed of lucite rings 13mm in diameter, 0.22μm pore membrane filters, and cement for attaching the membranes to the rings. The membranes were placed in boiling water for 5 minutes prior to use to remove any surfactants. After drying, a membrane was attached to one side of the lucite ring. When the glue had dried, the half completed DC and unattached membranes were sterilized by irradiation. Under sterile conditions GH3 cells were added to the partially assembled DC. Some of the media was allowed to diffuse out of the chamber, after which glue was applied to the lucite ring and the second filter was attached to seal the chamber. When the glue had dried (approximately 10 minutes) the DC containing the GH3 cells were ready for implantation in the mouse. The DC were cultured in 2 ml of media for 24 hours after which the media was examined for cells to further confirm the integrity of the seal of the membranes. An acidic change in the pH of the media was taken to indicate viable cells in the DC. The DC were washed with serum free media prior to implantation.

Surgery

Mice were anesthetized with tribromoethanol (0.2ml/10g body weight, 20mg/ml). DC were inserted in the superior aspect of the peritoneal cavity via a 13-15mm incision. The

incision was then closed with two layers of sutures (peritoneal and dermal). At the end of the 4 week treatment period, the mice were sacrificed by cervical dislocation.

Antigen Priming

P815 tumor cells were irradiated with 1000 rads and an inoculum of $1x10^7$ was administered I.P. in 0.5 ml of PBS. The mice were sacrificed by cervical dislocation 10 days after P815 priming.

Histology

Thymus glands fixed in neutral buffered formalin were dehydrated in graded alcohols, infiltrated and embedded in JB-4 embedding plastic. Sections 3-5μm thick were cut and stained with hematoxylin and eosin for light microscopy.

Cytotoxicity Assay

Spleens from experimental and control animals were removed sterilely, cell suspensions prepared, erythrocytes lysed and the remaining cells were washed. The cells were cultured at a concentration of $5x10^6$
cells/well in a 24 well flat bottomed plate (Costar, Cambridge, MA) with 0, 2, or 6 μm of Con A /ml per group of cells and 3 wells per dose in 1 ml of media (RPMI + 10% serum, 1xPen/Strep, sodium pyruvate, nonessential amino acids, and 2-mercaptoethanol). Plates were incubated for 40 hours at 37°C in 5% CO_2. Cells were harvested by

vigorous pipetting. Following centrifugation, the supernatants were collected and stored frozen for future analysis in the IL-2 assay. These cultured effector cells were plated to yield effector-target cell ratios of 50:1, 25:1, 12.5:1, 6.25:1, and 3.125:1 in a 96 well flat bottomed plate (Costar). 50 μl of PHA (100μg/ml) was added to the cultures just prior to adding the ^{51}Cr labelled P815 target cells (5x10^5 cells/well). The plates were gently agitated before being incubated at 37°C in 5% CO_2 for 5 hours. The supernatants were harvested with a Skatron Harvesting Press and counted in an LKB automatic gamma counter.

Con A Stimulation

The media used for the assay was the same as for the cytotoxicity assay. Final doses of Con A were 0, 0.67, 2, 6, 18, and 54 μg/ml. Each dose was plated in triplicate in a 96 well plate. Spleen cells were plated at 1.25x10^6/well. The plates were cultured for three days at 37°C in 5% CO_2. The plates were then pulsed with ^{3}H thymidine (1μCi/well), incubated for 7 hours, and harvested with a Skatron Cell Harvester onto filter paper. The cells were counted in a Beckman LS7500 liquid scintillation counter.

IL-2 Assay

Supernatants from the spleen cell cultures used in the cytotoxicity assay were evaluated for the presence of IL-2

as follows. Several two-fold dilutions of the supernatant and a standard IL-2 solution were aliquoted in triplicate in 96 well U-bottom plates (0.1 ml/well) and 10^4 IL-2 dependent CTLL cells were added per well. The plates were cultured for 20 hours at 37°C in 5% CO_2, then pulsed with ^3H thymidine and returned to the incubator for 6 hours. The cells were harvested onto filter paper with a Skatron Cell Harvester and counted in a Beckman LS7500 liquid scintillation counter.

Radioimmunoassay

Blood from the mice implanted with the DCs containing GH3 cells was obtained by severing the axillary artery after anesthetizing the mouse. Approximately 0.5-1.0 ml of blood was obtained from each mouse, placed in 1.5 ml provials (Costar) and held at -70°C. RIA was conducted with the double antibody technique and the use of kits provided by the NIDDK. Anti-rat GH serum S-4 was used with rat GH RP-2 as the reference preparation.

GH Injections

Mice were given daily intraperitoneal injections of either 5μg of ovine growth hormone (NIDDK) in 200μl of normal saline or a control injection of 200μl of saline for 30 days.

GH Timed Release Pellets

Timed release pellets of ovine growth hormone (NIDDK) were prepared by Innovative Research of America (Toledo, OH). These pellets each contained 75μg of growth hormone which was released at a constant rate over a period of 15 days (i.e. 5μg per day). Pellets were implanted subcutaneously using a trochar (IRA, Toledo, Ohio) every 15 days for a total treatment period of 60 days. Control animals were similarly treated with placebo pellets which were identical in composition except for the growth hormone.

Necropsy

All mice were examined at the time of sacrifice, prior to the removal of the thymus and spleen, for gross pathologies.

RESULTS

GH3 Cells

The mice which received the GH3 cells in diffusion chambers did not exhibit an increase in their thymus mass and morphology or improved cellular immune function. Upon histological examination, the GH3 cells appeared to be healthy and functioning. However, the radioimmunoassay did not detect rat (i.e. GH3 derived) growth hormone in the serum of the mice implanted with GH3 containing diffusion chambers.

GH Injections

The mice which received daily injections of growth hormone for 30 days did not exhibit improvement in their thymus mass and morphology. Histological examination of the thymuses revealed no difference between the growth hormone treated and saline treated mice Figure 2.1. Both groups had involuted thymuses which lacked distinct cortical and medullary areas. A few thymuses contained cysts filled with colloid appearing material and bounded by a plump cuboidal epithelium. In some instances the colloid-like substance had a lamellar appearance. Although such multicystic thymus glands are not an unusual finding in human autopsies (Rosai and Levine, 1976), we have not seen previous reference to this pathology in mice. There was no difference between the experimental and control mice in their cellular immune response. Hepatocellular adenomas were found in 67% of the growth hormone treated mice (described in further detail below) but none of the saline treated mice exhibited abnormal livers.

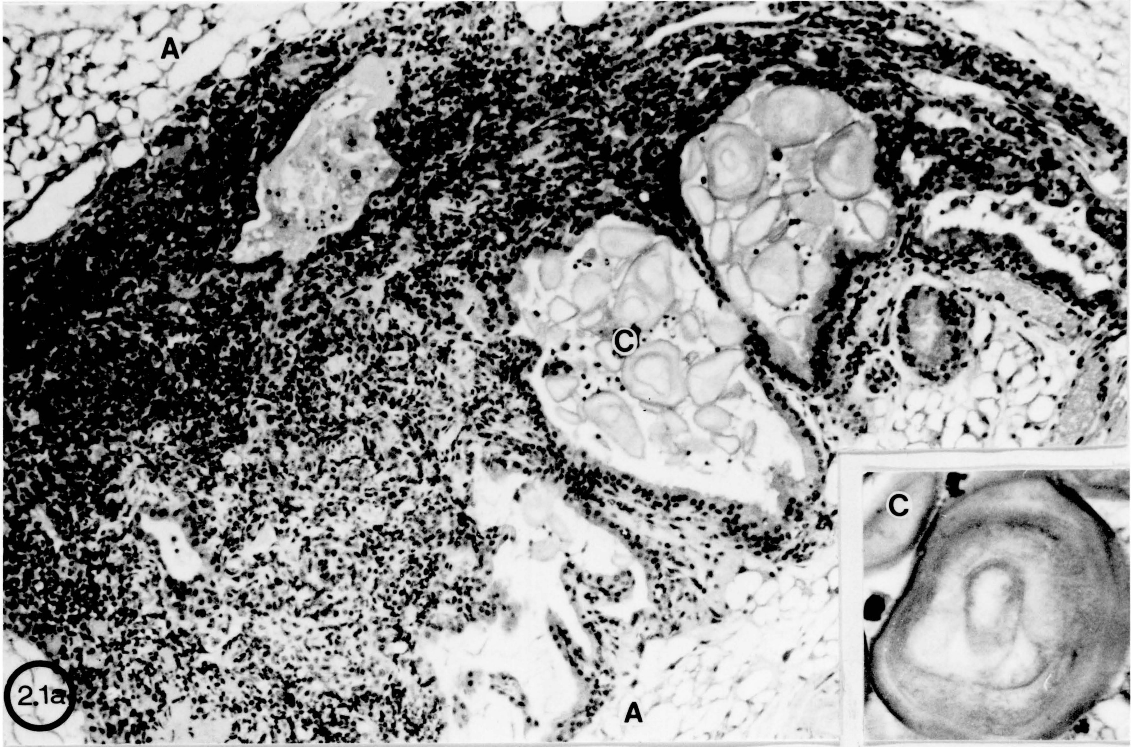
GH Pellets

Thymus Mass

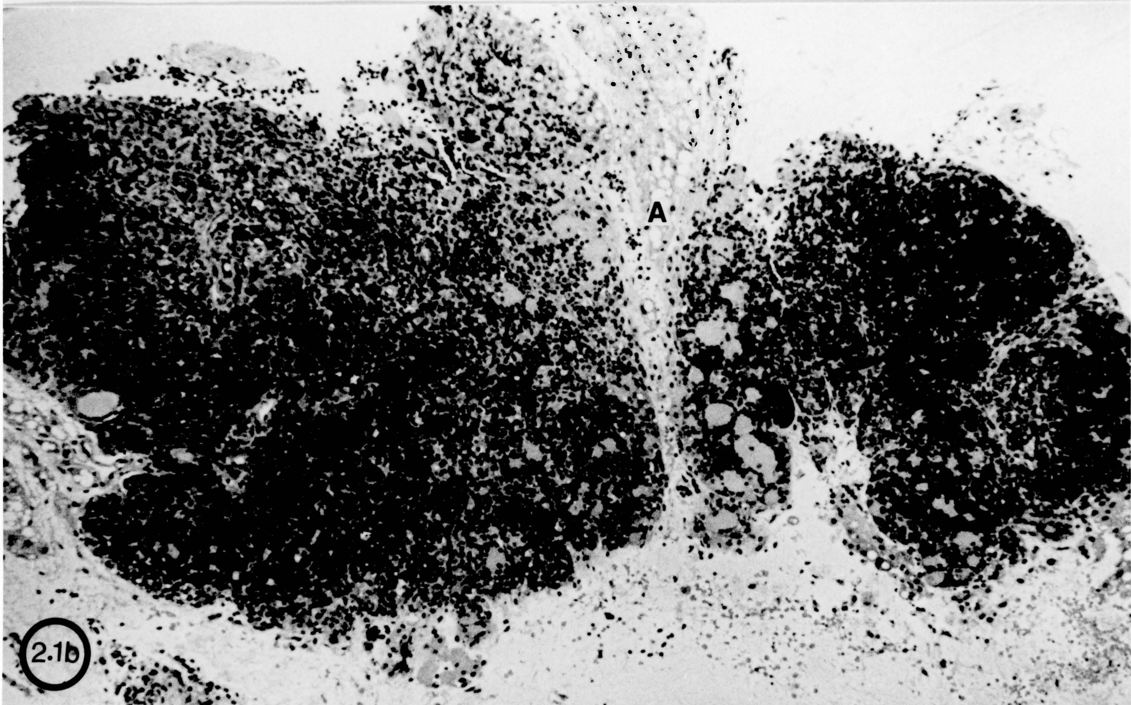
All of the growth hormone treated mice exhibited at least some grossly distinguishable thymus tissue. Two of the placebo treated mice had no grossly distinguishable thymus and the thymuses of the remaining control mice were severely involuted. The mass of the thymus was significantly greater in the growth hormone treated mice

FIGURE 2.1a: Thymus of Twenty-four month old mouse given daily injections of ovine growth hormone for one month. Note the infiltration of fatty connective tissue (A) and lack of distinct cortical and medullary regions. In this particular animal the thymus contained cysts filled with colloid-like material (C) (enlarged in insert). (10X. Insert 40X).

Figure 2.1b: Thymus of a twenty-four month old mouse given daily injections of saline for one month. There is no distinction between the cortical and medullary distribution of lymphocytes (L) and fatty connective tissue is invading the parenchyma (A).(6.3X).



A
C
C
A
2.1a



A
2.1b

compared to the placebo treated mice (p=0.002) (Figure 2.2).

Thymus Histology

Histological examination of the thymuses revealed morphological features similar to young adult, in the growth hormone treated mice. In contrast, the control mice exhibited severely involuted or indistinguishable thymus morphology typical of their advanced age. All but one of the growth hormone treated mice (G2) had distinct cortical and medullary regions. While the thymuses of the growth hormone treated mice possessed good morphology (except G2), they were not uniform. The thickness of the cortex and the degree of fatty connective tissue infiltration of the parenchyma varied even in the thymuses with good morphology. Also variable was the medullary region. Two of the growth hormone treated mice had large vessels in the medulla. However, in all but one animal (G2) normal thymus histology was clearly evident. None of the control mice's thymuses exhibited a distinct cortex and medulla. The placebo treated mice exhibited extreme fatty infiltration of the organ which could be described as a collection of lymphoid nodules interspersed among fatty connective tissue (Figure 2.3).

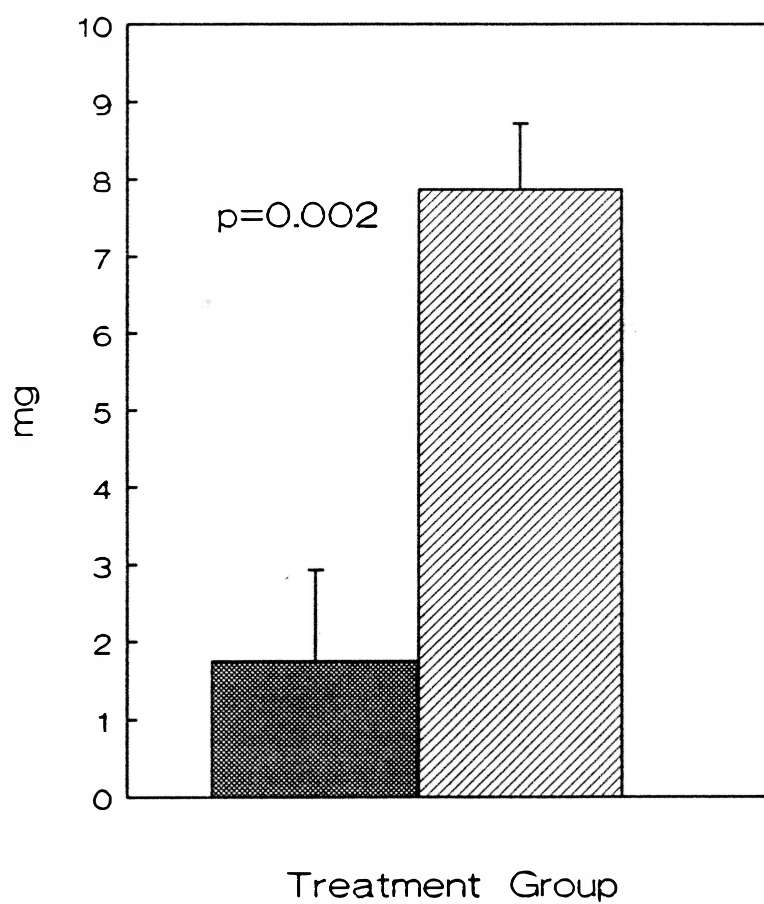


Figure 2.2 Thymus Mass
GH vs. Placebo
Placebo
GH
p=0.002
mg
Treatment Group
0
1
2
3
4
5
6
7
8
9
10

Cellular Immune Function

Con A Proliferation Response

The results of the Con A proliferation are shown in Figure 2.4. As the figure indicates the Con A response was highly variable. Statistical analysis using a repeated measures analysis of variance (SAS, 1985) revealed no significant difference in the Con A response between the growth hormone treated and the placebo treated animals. The unstimulated proliferation level (Con A = 0) and Con A stimulated proliferation response at 6μg/ml were higher in the growth hormone treated animals. These differences were significant when analyzed using a paired T-test but were not statistically significant under the more rigorous conditions of ANOVA.

CTL Activity

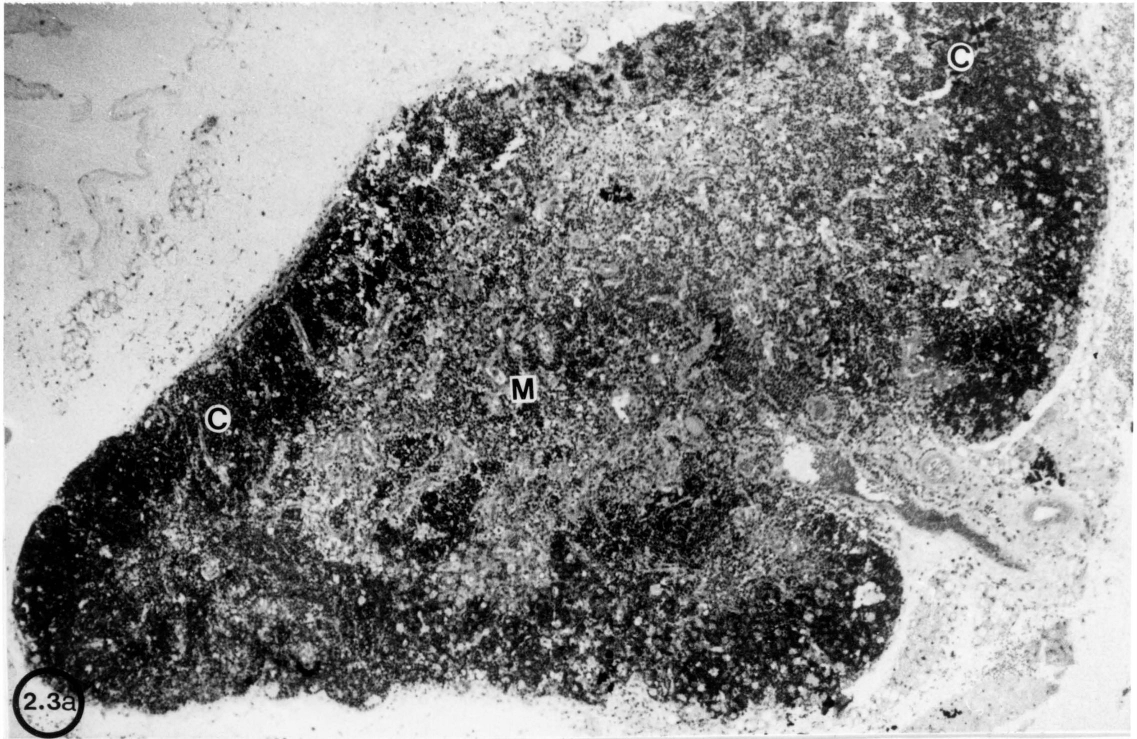
There was no difference between the growth hormone treated mice and the placebo treated mice in their cytotoxic T lymphocyte activity as measured by their ability to lyse ^{51}Cr labelled P815 tumor cells.

IL-2 Production

Production of IL-2 by cultured splenic T cells stimulated with Con A was assayed by the ability of the collected supernatants to promote the growth of the IL-2 dependent T cell line CTLL. There was no difference between the growth

Figure 2.3a: Thymus of a twenty-four month old mice which received timed-release growth hormone pellets for eight weeks. Note the distinct cortical (C) and medullary (M) regions. While not identical to a typical young thymus its morphology is markedly better than the typical 24 month old murine thymus. (4X).

Figure 2.3b: Thymus of a twenty-four month old mouse which received placebo tablets for eight weeks. Note the extensive infiltration of fatty connective tissue (A) and the lack of distinct cortical and medullary regions. (4X).


C
M
C
2.3a

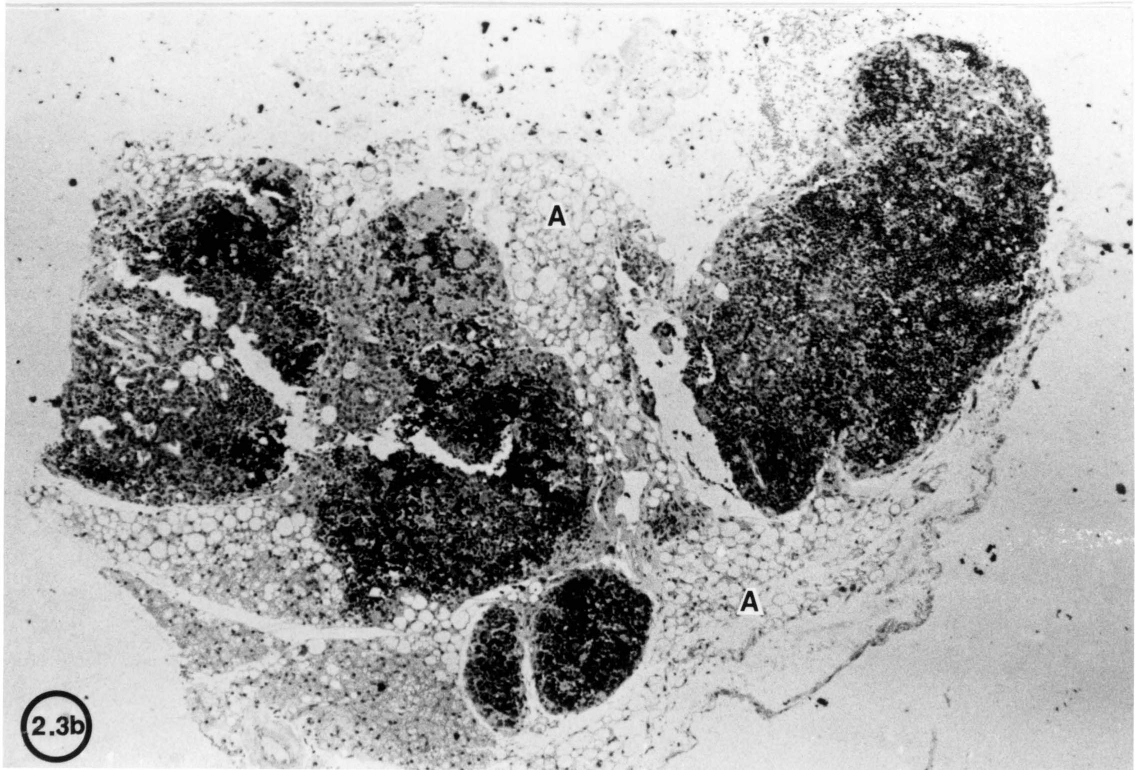
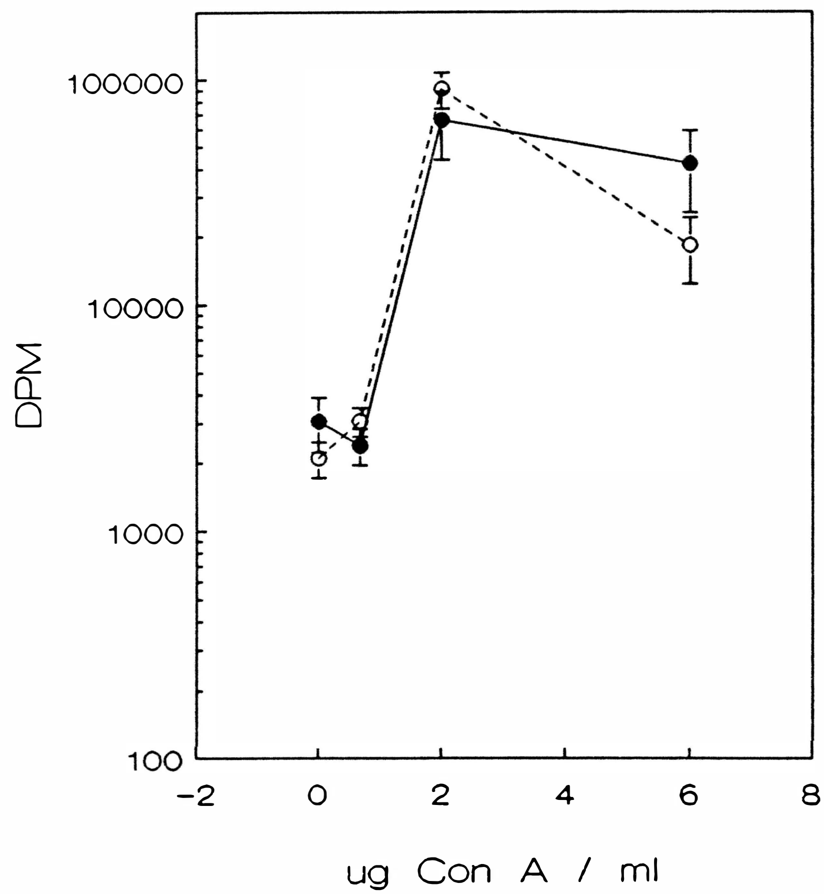

A
A
2.3b

Figure 2.4 Con A Response
GH vs Placebo

—●— Growth Hormone   --○-- Placebo



hormone treated mice and the control mice in their IL-2 production.

Pathological Findings

The incidence of hepatocellular adenomas was extremely high in the GH treated mice when compared to control mice. The incidence of tumors in the mice given GH pellets was 67%. There were no hepatocellular tumors in the control population. The following pathological description applies to the tumors found in both the growth hormone injected mice and the growth hormone pellet treated mice. The adenomas varied in size from small tumors several millimeters in diameter to large tumors encompassing an entire lobe or more. Examination revealed a well defined nonencapsulated mass with superficial vessels and pigmentation slightly darker than normal hepatic tissue. Histological examination showed the tumor to be composed of slightly abnormal hepatocytes. The hepatocyte cytoplasm was more eosinophilic and the nuclei were up to 1.5 times normal hepatocytes with open chromatin and prominent nucleoli. Occasional cells were binucleate. The cellular arrangement was that of irregularly anastomosing cords. There was no evidence of lobular architecture and there were no portal triads or central veins (Figure 2.5).

Two of the mice which had hepatic adenomas also had pulmonary adenocarcinomas (G2 and G6). These tumors exhibited characteristics of spontaneous mouse lung tumors

and were judged to be unrelated to the hepatic tumors Figure 2.6). It should be noted that one of the mice having a lung tumor was the mouse which did not exhibit thymic rejuvenation (G2). The other mouse with a lung tumor (G6) had a thymus with a thin cortex but was otherwise typical of the growth hormone treated mice.

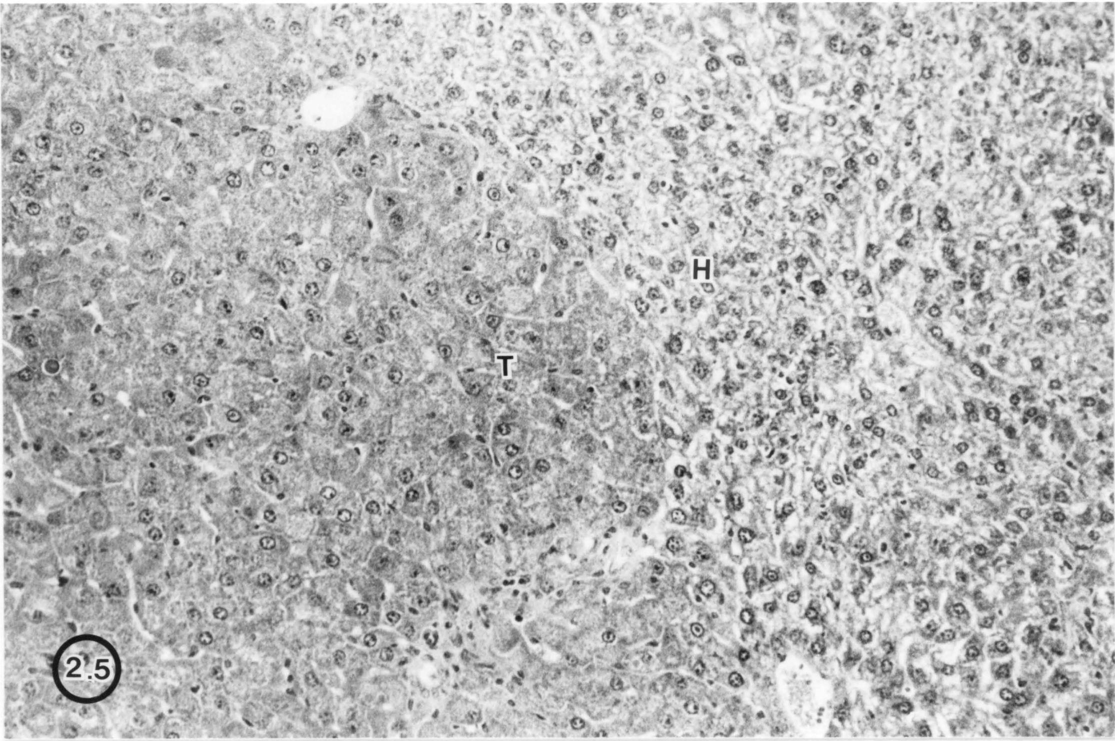
DISCUSSION

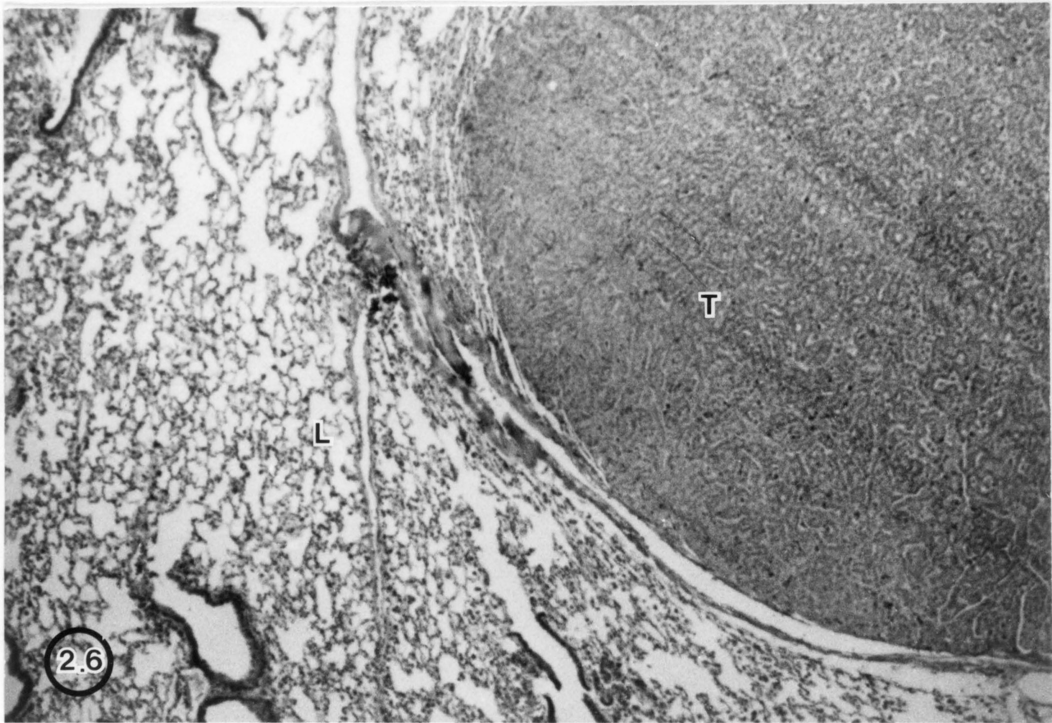
The absence of rat growth hormone in the serum of the mice given diffusion chambers with GH3 cells was quite unexpected. It is unlikely that the rat growth hormone was recognized as foreign and hence eliminated since there is an extremely high degree of conservation exhibited across species. A more likely explanation is that the slow diffusion of GH3 produced growth hormone and prolactin out of the diffusion chambers led to a high local concentration of growth hormone and prolactin which down regulated the production of these proteins. The end result was that only negligible amounts of growth hormone and prolactin were produced.

The results of the experiments in which growth hormone pellets were injected support earlier reports in other species that it is possible to rejuvenate the senescent thymus in vivo (Kelley et al., 1986; Goff et al., 1987; Monroe et al., 1987). The present study indicates that the treatment period required to attain such a rejuvenation in

Figure 2.5: Hepatic adenoma found in 67% of growth hormone treated mice. Area to the right of micrograph (H) is normal hepatic tissue; the tissue to the left is adenoma (T). Note the darker staining and enlarged hepatocytes in the tumor area. (20X).

Figure 2.6: Lung Tumor found in two of the growth treated mice. Note the galndular appearing tumor tissue (T) which has displaced the normal respiratory tissue of the alveolus (L). (20X).


H
T
2.5


T
L
2.6

mice is approximately 8 weeks. This conclusion is based on the lack of rejuvenation of the thymus or cellular immune function in the mice given daily injections of growth hormone for four weeks at the same dose as the timed release pellets. Furthermore, our results would seem to indicate that the rejuvenation of the senescent cellular immune response is not achieved as quickly as the rejuvenation of the thymus gland. The thymus regained much of its normal young morphology after 8 weeks of growth hormone treatment but cellular immune function did not recover as quickly. This can be explained in either of two ways. First, it may be due to the time lag in the seeding of the peripheral lymphoid organs with newly matured and normal functioning thymocytes from the rejuvenated thymus. Alternatively, it may be that the thymus factors responsible for maintaining the vigor of the cellular immune response have not been present for a long enough period of time to restore peripheral lymphocytes to normal functioning levels. The fact that previous studies have demonstrated the benefits of exogenous thymus factors to the senescent immune response (Cowan et al., 1981; Frasca et al., 1982; Frasca et al., 1987; Erschler, 1989) would favor the latter explanation. However, a combination of both newly derived lymphocytes and thymus factors may be the mechanism by which the senescent cellular immune response is restored.

Our analysis of the results found in the Con A proliferation assay indicate that the response has not been significantly improved. The marginally elevated proliferation rate seen in the unstimulated cultures may be due to the recent seeding of the spleen by newly matured T cells exported from the restored thymus. It is possible that the proliferation response at 6μg/ml of Con A is an indication of a nascent improvement that is only evident in marginal proliferation states; the high proliferation rate during more vigorous responses masks this emerging trend. We are currently planning studies that would involve longer treatment periods to determine if this interpretation is correct.

The IL-2 assay did not show a significant increase in the IL-2 production of the growth hormone treated mice. There were, however, indications that perhaps IL-2 production was beginning to improve. The DPM counts were higher for the growth hormone treated mice but the increase was not statistically significant. For example, in the cultures which were stimulated with 6 μg/ml of Con A, the "p" values for the difference between control and treated mice were 0.058 - 0.075. These results lend further support, although far from conclusive evidence, to our hypothesis that the cellular immune response is beginning to improve in the treated mice.

The cytotoxicity assay gave no indication that there was an improvement in this aspect of cellular immunity. The results varied greatly in both the experimental and control populations with no significant differences between them. Unlike the assays of cellular immune function discussed above, there was no indication of a trend toward improvement in the growth hormone treated mice with this assay.

The high incidence of hepatocellular adenoma in the growth hormone treated mice appears to be more than just a chance occurrence related to the age of the mice. Previous reports on the incidence of hepatic tumors in CBA mice vary greatly but none are as high as 67%, although the incidence is higher than in other strains. Phybus and Miller (1942) reported an incidence of 40.7% at an average age 28.6 months in a population of 285 mice. Blankton (1978) reported an incidence of between 11% and 23% depending on the classification of the tumor. Cohen et al. (1984) found hepatic tumors in 62.5% of 24 month old CBA mice. However, they only examined 8 mice. The small size of our study population precludes a definitive finding given that the population under study is known to have a high tumor incidence. However, given the absence of tumors in placebo treated mice, these results do indicate that a correlation between growth hormone and hepatic tumors is likely. We deem it imperative that investigators be aware of possible side effects of any immunotherapy used in order to properly

interpret the results of in vivo studies and have included these observations for that reason.

The mechanism of action for growth hormone on the thymus is undefined and the present study does not provide additional information in this regard. It does seem clear that neuroendocrine factors have a pronounced effect on the thymus and immune function (Fabris and Mochchegiani, 1985; Berczi 1986; Goff et al., 1987). In addition to the anterior pituitary factors discussed above, thyroxine has been shown to have profound effects on the thymus and immune function (Pierpaoli et al., 1970; Schieff et al., 1977; Savino et al., 1984). Thyroxine is also known to affect both growth hormone and prolactin production (Peake et al., 1973; Martial et al., 1977). How the interactions of these various endocrine factors affect immune function in both normal as well as aberrant physiological states we have only begun to understand. The fact that the thymus appears to be functionally intertwined with these endocrine organs may indicate that a more thorough understanding of the thymus entails thinking of it as a member of that system.

CHAPTER THREE

The Effects of PSK on the Senescent Immune System

Involution of the thymus with age and the subsequent suppression of immune function are well documented (Boyd, 1932; Bach et al., 1975a,b; Hirokawa and Makinodan, 1975; Walford, 1980; Kruisbeek, 1981; Cowan et al., 1981; Weksler, 1982, 1983; Steinman et al., 1985; Kraft et al., 1988). However, the suppression of immune function in senescent animals is not absolute and exhibits at least some reversibility (Gerbase-DeLima, 1975; Cowan et al., 1981; Frasca et al., 1982; Bruley-Rosset et al., 1986). Experimental attempts at immunological restoration of immunosenescent animals generally fall into one of three categories: 1. Attempts to replace physiologically active factors that are lost or diminished with age (Weksler et al.,1978; Frasca et al., 1986); 2. Pharmacological treatments (Kwong et al., 1983; Bruley-Rosset et al., 1975); and 3. Nutritional manipulations (Fabris et al., 1986). Limited success has been obtained with each of these forms of intervention. Still, no clinically applicable treatment has been found and the mechanisms of immunosenescence and restoration remain poorly understood.

In all three approaches to rejuvenation of the senescent immune response, substances that act as immunomodulators or biological response modifiers have been employed (Kwong et al., 1983; Goodman and Weigle, 1985; Bruley-Rosset et al., 1986; Cowan et al., 1981; Frasca et al., 1986; Frasca et al., 1987; Ershler et al., 1989). Many investigators studying immunosuppressed states have attempted to restore normal immune function armed with pharmacological agents which are putative immunopotentiators. Examples of this are the work by Wybran (1980) and Friedman et al. (1980) which showed that isoprinosine was an effective immunostimulator in cancer patients. Tsang and Fudenberg (1982) also found this compound to be an effective immunostimulator in an animal model of human osteosarcoma. Based upon these observations, Tsang et al. (1983) examined the effects of isoprinosine on the immunodeficiency of senescence. They found that isoprinosine was able to boost the cellular immune response in aged hamsters.

PSK is a fungal extract commonly used as part of cancer therapies in Japan, where it is widely regarded as an effective immunopotentiator in both laboratory animals and human subjects (review: Tsukagoshi et al., 1984). PSK (Krestin), a protein bound polysaccharide, is an extract from the cultured mycelia of the fungus Coriolus versicolor of the Basidiomycetes family. The physiochemical properties of PSK are the following: an average molecular

weight of 94,000; the protein portion is composed primarily of acidic and neutral amino acids with a small amount of basic amino acids; the major monosaccharide is glucose; the glycoside portion is composed of ß 1-4 glucan linkages branched at the C3 and C6 positions in a ratio of one per several residual groups of 1-4 bonds (Tsukagoshi et al., 1984). PSK is an odorless, tasteless, water-soluble, brown powder. PSK activity in vivo has been achieved with oral, intravenous, and intraperitoneal administration. Its specific mechanism of action remains unclear, but PSK's activity as a biological response modifier has been the object of numerous studies.

PSK has been shown to augment the immune response against allogeneic antigens and increase the survival time of tumor bearing mice (Ehrke et al., 1983). Cell mediated cytotoxicity is also increased by PSK treatment in mice (Taniguchi et al., 1985). Of particular interest is the reported ability of PSK to restore the involuted thymuses of tumor-bearing mice to the normal state (Mizushima et al., 1982; Tsuru et al., 1983; Oguchi et al., 1987). The suppression of immune function in neoplastic disease states is well known (review: Kamo and Friedman, 1977), while the combination of suppressed immune response and thymic involution is reminiscent of the senescent state of the immune system. Even more noteworthy is the reported ability of PSK to restore the immune function of tumor-

bearing aged mice to pre-tumor levels (Matsunaga et al., 1987).

The etiology of the tumor induced immunosuppression and thymic involution is not entirely understood but its similarity to the senescent state is considerable. Given these similarities and the reported success of PSK in reversing these deleterious processes, we were encouraged to investigate the possibility of PSK rejuvenation of the senescent immune system. Previous work has shown that at least one pharmacological agent, isoprinosine, is capable of acting as an immunopotentiator of both tumor suppressed immune systems and senescence induced immunosuppression. The results of previous investigations using PSK indicate that it may have similar therapeutic value.

Materials and Methods

Animals

CBA male mice 18 months of age were obtained from Charles River Laboratories (Kingston, NY) through the National Institute on Aging. Mice were housed 3 per cage. The cages were kept in laminar flow hoods on a 12 hour light/dark cycle. Mice were provided with water and autoclaved Purina mouse chow ad libitum.

PSK

The PSK used in this work was the generous gift of Dr. Jane Ehrke and Kureha Chemical Co. Ltd. (Japan). The brown powder was dissolved in normal saline at a concentration of 175 mg/ml and 1.00 ml was administered three times a week for 4 weeks via intra-peritoneal injections .

Antigen Priming

P815 tumor cells were irradiated with 1000 rads and an inoculum of $1x10^7$ was administered in 0.5 ml of PBS at the end of the PSK treatment period. The mice were sacrificed by cervical dislocation 10 days after P815 priming.

Histology

Thymus glands were removed and fixed in neutral buffered formalin after which they were dehydrated in graded alcohols, infiltrated and embedded in JB-4 embedding plastic. Sections 3-5μm thick were cut and stained with hematoxylin and eosin for light microscopy.

Cytotoxicity Assay

Spleens from experimental and control animals were removed sterilely, erythrocytes were lysed, and cell suspensions prepared and washed. The cells were cultured at a concentration of $5x10^6$ cells/well in a 24 well flat bottomed plate (Costar, Cambridge, MA) with 0, 2, or 6 μm of Con A /ml per group of cells and 3 wells per dose in 1 ml of media (RPMI + 10% serum, 1xPen/Strep, sodium

pyruvate, nonessential amino acids, and 2-mercaptoethanol). Plates were incubated for 40 hours at 37°C in 5% CO_2. Cells were harvested by vigorous pipetting. Following centrifugation, the supernatants were collected and frozen for later use in the IL-2 assay. These cultured effector cells were plated to yield effector-target cell ratios of 50:1, 25:1, 12.5:1, 6.25:1, and 3.125:1 in a 96 well flat bottomed plate (Costar). Fifty μl of PHA (100μg/ml) was added to the cultures just prior to adding the ^{51}Cr labelled P815 target cells ($5x10^5$ cells/well). The plates were centrifuged briefly before being incubated at 37°C in 5% CO_2 for 5 hours. The supernatants were harvested with a Skatron Harvesting Press and counted in an LKB automatic gamma counter.

Con A Stimulation

The media used for the assay was the same as above. Final doses of Con A were 0, 0.67, 2, 6, 18, and 54 μg/ml. Each dose was plated in triplicate in a 96 well plate. Spleen cells were plated at $1.25x10^6$/well. The plates were cultured for three days at 37°C in 5% CO_2, after which they were pulsed with ^{3}H thymidine (1μCi/well), incubated for 7 hours, and harvested with a Skatron Cell Harvester onto filter paper. The cells were counted in a Beckman LS7500 liquid scintillation counter.

IL-2 Assay

Supernatants from the spleen cell cultures used in the cytotoxicity assay were evaluated for the presence of IL-2 as follows. Several two-fold dilutions of the supernatant and a standard IL-2 solution were aliquoted in triplicate in 96 well U-bottom plates (0.1 ml/well) and 10^4 IL-2 dependent CTLL cells were added per well. The plates were cultured for 24 hours at 37°C in 5% CO_2, then plates were pulsed with ^3H thymidine and returned to the incubator for 5 hours. The cells were harvested onto filter paper with a Skatron Cell Harvester and counted in a Beckman LS7500 liquid scintillation counter.

RESULTS

After four weeks of treatment with PSK the mass of the thymus was not increased in the 18 month old mice. The mean values for thymus mass were 7.38 mg for the PSK treated animals and 9.65 mg for the saline treated controls. These values were not significantly different ($p = 0.19$). There was however an unexpected increase in the mass of the spleen in the PSK treated mice compared to both the old saline treated controls and the young controls (Table 3.1). This increase in splenic mass was not due to an increase in white blood cells since cell counts following lysis of erythroid cells did not reveal a significant difference between the PSK and control mice (Table 3.2).

TABLE 3.1 : SPLEEN MASS

Animal	Mass
PSK	178 mg
Saline	108 mg
Young	73 mg

Splenic mass mean values for aged PSK or saline treated and young control mice. PSK treated mice had significantly enlarged spleens when compared to saline treated aged (p=0.003) and untreated young mice (p=0.005).

Table 3.2 : Non-erythroid Spleen Cells

	$\bar{x}$ (x10^3)	σ (x10^3)
PSK	3.33	2.41
SALINE	2.54	1.52
YOUNG	0.75	0.23

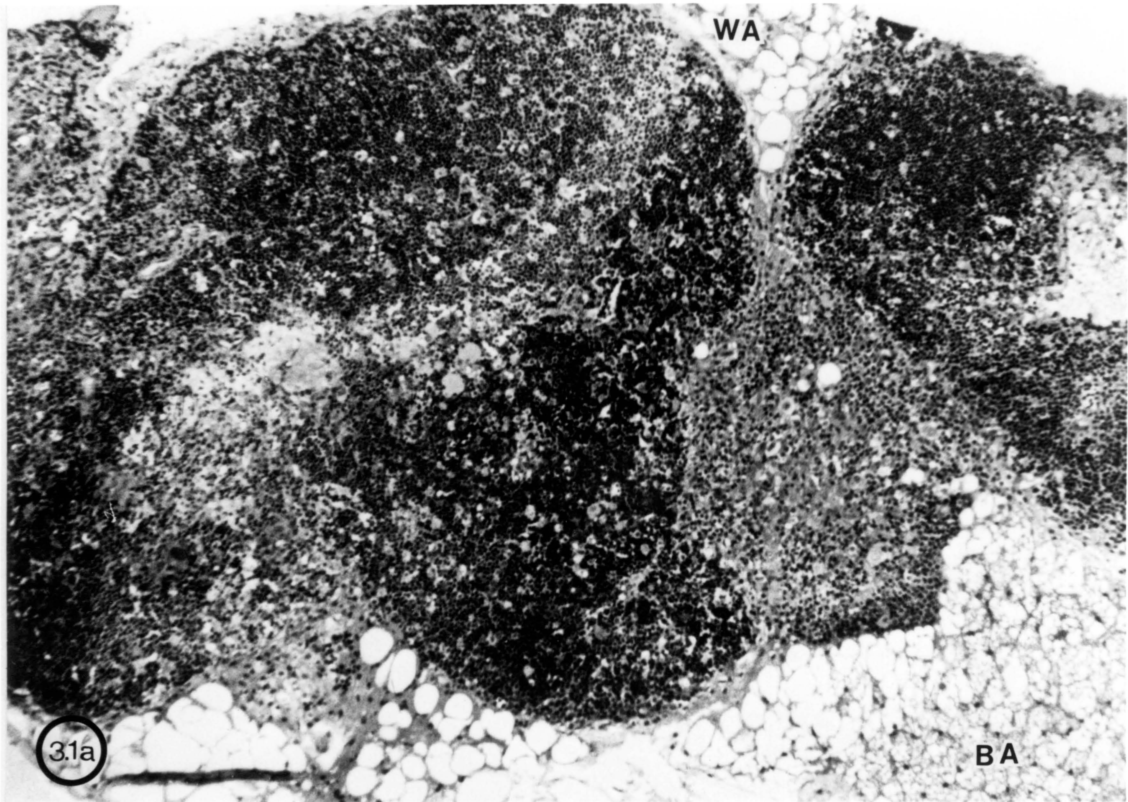
The thymuses of the PSK treated mice were not restored to normal youthful morphology when examined histologically. Both the control and PSK treated mice's thymuses were significantly involuted as seen in Figure 3.1. Only a few of the animals exhibited any distinction between cortical and medullary regions and these were equally distributed between experimental and control groups. The most common appearance of the thymus was an irregularly shaped mass of tissue evenly populated with lymphocytes and containing varying amounts of white and brown fat (Figure 3.1). Many of the epithelial-like cells had a vacuolated appearing cytoplasm. Hassal's corpuscles were not seen. All of the above histological findings are consistent with normal age-related thymus involution.

The Con A response was highly variable as seen in Figure 3.2. The means of the raw data did not indicate any clear pattern of improvement in Con A response in the PSK treated animals. Statistical analysis, using a repeated measures analysis of variance (SAS, 1985), confirmed that there were no significant differences in Con A response between the experimental and control groups at any of the levels examined.

The cytotoxic T lymphocyte activity, as measured by the ability of cultured splenocytes to lyse ^{51}Cr labelled *P815* cells, was not improved by PSK treatment. Preliminary results indicated that the PSK treated mice might have

Figure 3.1a: Thymus of an eighteen month old mouse treated with PSK every other day for one month. Note the lack of a distinct cortex and medulla. In this section both white (WA) and brown (BA) adipose tissue are seen. (6.3X).

Figure 3.1b: Thymus of an eighteen month old mouse given saline injections for one month. Observe that the peripheral area which is dark staining with lymphocytes in a normal young thymus is nearly devoid of lymphocytes at several points (arrows) in this specimen. (6.3X).


WA
BA
3.1a

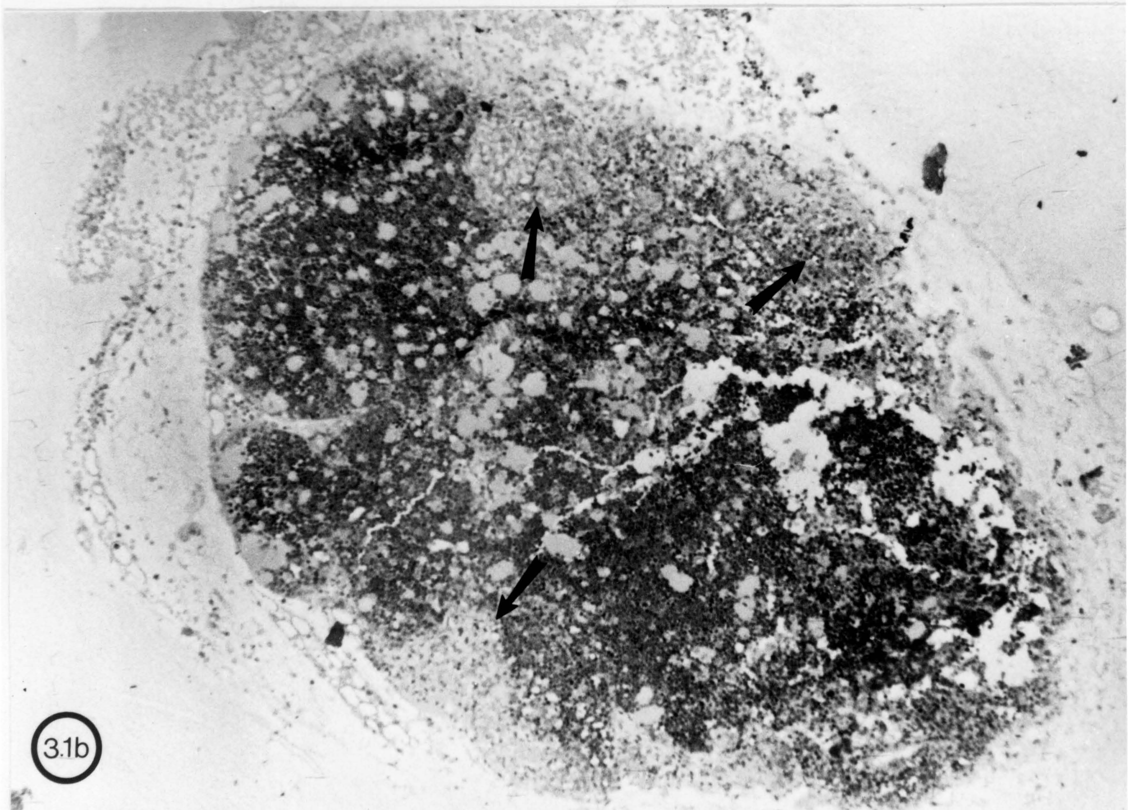
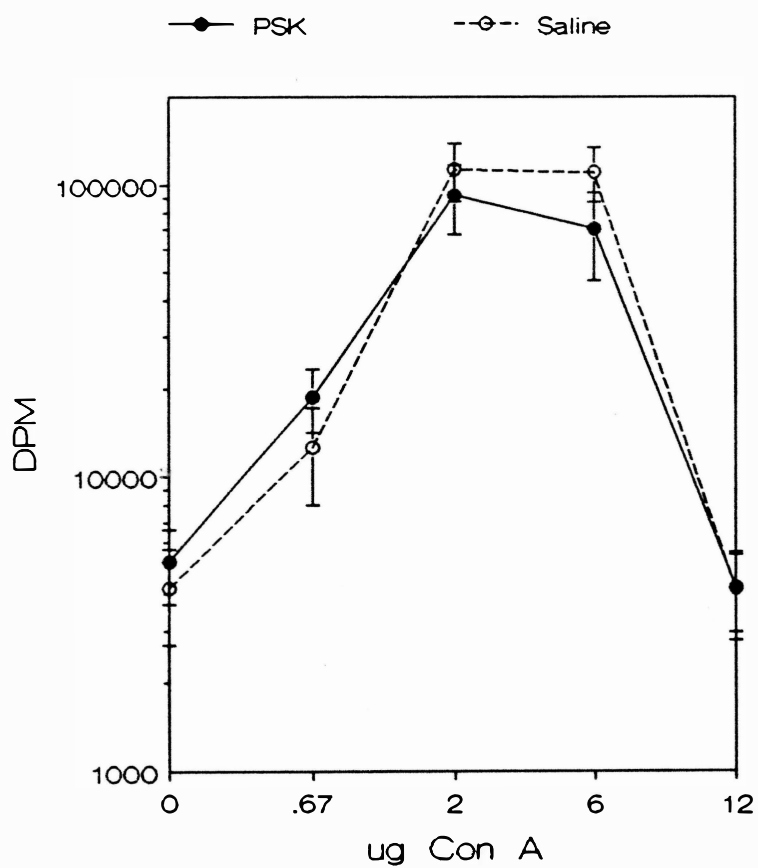

3.1b

Figure 3.2
Con A Response
PSK vs Saline



higher killing than the control treated mice. The results, however, varied between experiments. Pooled data values were higher in PSK treated mice than control mice at high effector:target ratios, equal at middle ratios, and less than controls at low ratios. Due to the high degree of variability, none of these differences was statistically significant.

Production of IL-2 by cultured spleen cells, stimulated with Con A, was assayed by the ability of collected supernatants to promote the growth of the IL-2 dependent cell line CTLL. The production of IL-2 was not increased by the PSK treatment of the aged mice. There was no significant difference between the saline treated and the PSK treated mice.

DISCUSSION

As was discussed in the introduction, the effectiveness of PSK in restoring both thymus structure and cellular immune function in the tumor-suppressed immune response and the similarity of this state to the senescent state indicated a possible role for PSK as an immunopotentiator in aged mice. In this study, treatment of aged mice with PSK did not induce thymic rejuvenation as has been reported in previous studies of PSK treatment of tumor-bearing animals (Tsuru et al., 1983; Oguchi et al., 1987). The thymuses of the PSK treated mice were indistinguishable from the thymuses of the control mice in terms of mass and histological

appearance. Furthermore, cellular immune function in the PSK treated aged mice was not improved over that of the aged controls. The studies of PSK in tumor-bearing animals cited above had also reported a restoration of suppressed immune response. Whatever the mechanism of tumor-induced thymic involution it appears to be different from the senescence-induced thymic involution. Recent work has shown that it is possible to rejuvenate the senescent thymus in experimental animals with growth hormone (Mizushima et al., 1982; Goff et al., 1987; Kelley et al.,1986; Roth et al., 1988; also see Chapter 2), but PSK appears to lack such restorative properties.

There are several plausible explanations of the failure of the PSK therapy to augment the senescent immune response. The dose and length of the treatment period were based on the successful methods of researchers using PSK to bolster the suppressed immune response of tumor-bearing animals and human cancer patients. While these protocols were appropriate for the first phase of this study, it is possible that a longer treatment period may be necessary to counteract the accumulated damages of aging. If this line of investigation is pursued in future studies, a range of doses and treatment periods will be examined. Even if the failure of the current study to show improvement were due to insufficient PSK treatment, one would expect to see at least a trend toward improved immune function or a slight improvement in thymus morphology if that organ were

beginning to rejuvenate. In terms of cellular immune function, it may be a matter of timing and antigen presentation. PSK has been shown to increase or decrease cellular immune function depending on when it is given relative to antigen presentation (Taniguchi et al., 1985). It has been postulated to increase suppressor T cell activity under one set of conditions and to augment cytotoxic T cell activity under other conditions. The protocol used in the current study did not investigate the possibility of such paradoxical effects of PSK on senescent immune function.

One of the most striking results of the PSK therapy was the splenomegaly. The dramatic hypertrophy of the spleen was not expected since it had been previously reported that PSK had no effect on the spleen (Tsuru et al., 1983). This increase in splenocytes suggested to us that perhaps the increased anti-tumor activity reported in previous studies was due to an increase in the number of lymphocytes in the peripheral lymphoid organs. Our examination of the total leukocyte count in the spleens of PSK treated mice revealed that the increase in the number of leukocytes was insignificant (Table 3.2) and therefore this could not be the mechanism by which the previous results in tumor-bearing mice were obtained.

Kikuchi et al. (1988) demonstrated an improvement in IL-2 production in post-chemotherapy cancer patients given daily

doses of PSK. The improvement in this measure of cellular immune function in non-tumor bearing subjects indicates a possible therapeutic role that does not involve the inhibition of tumor derived factors. Several studies, however, have presented strong evidence for a mechanism of action for PSK that involves the inhibition or blocking of tumor-produced immunosuppressive factors to restore the suppressed immune function (Matsunaga et al., 1985; Matsunaga et al., 1986; Oguchi et al., 1987). The results of this study do not indicate any positive therapeutic effect of PSK on the depressed IL-2 production, lectin mediated proliferation, or CTL activity in senescent mice. The absence of positive results in the current study is consistent with the hypothesis that PSK acts by blocking the immunosuppressive effects of tumor produced factors rather than acting directly on lymphocytes.

The only other published study using PSK in older animals (Matsunaga et al., 1987) supports our finding that PSK is incapable of boosting the senescent immune response. They demonstrated that PSK was effective in improving the tumor resistance and the cellular immune function - PHA response and foot pad reaction to sheep erythrocytes - in tumor-bearing aged (60 week old) mice. However, the antitumor effects of PSK were not as great in the older mice as they were in young (30 week old) mice. Further, the improvement in the PSK treated aged tumor-bearing mice was only to the level of non-tumor-bearing aged mice; i.e. the

PSK treatment overcame the tumor induced immunosuppression but did not improve the senescent response. These results reflect a baseline functional level (senescent immune function) which can be further depressed by tumor burden but not augmented by PSK.

It is possible that PSK therapy must start at a younger age in order to restore the senescent immune function i.e. that a window of opportunity exists at a younger age for PSK improvement of senescent immune function; but we think it unlikely. Matsunaga et al. (1987) performed their work on mice which were significantly younger than the mice in the current study. The equivalent age for a CBA mouse, in terms of survivorship, would only be 5 months old. However, the mice were shown to be in an immunosenescent state and therefore were old in terms of biological function even if they did not meet van Zweiten et al.'s (1981) criterion for aged animals (i.e. past the 50% survival age on the strain's survival curve).

It appears that there is no prophylactic role for PSK, only a possible therapeutic role in treating neoplastic diseases. Still, if PSK is effective in this manner for elderly patients it could prove to be an extremely important new ally of the oncologist treating this patient population.

GENERAL DISCUSSION

This work has investigated three possible means of improving the senescent immune system. The first chapter recounted efforts to both improve the status of immune system cells (bone marrow prothymocytes) and add to our understanding of the interactions of the thymus and bone marrow stem cells. The second chapter chronicled our use of a murine model to investigate the reported rejuvenating effects of growth hormone on the senescent thymus and immune system. In the third chapter, an investigation of the possible benefits of the fungal extract PSK on the aged thymus and cellular immune function was reported. These three investigations met with varying degrees of success but both individually and collectively they represent an addition to our knowledge of the immunosenescent state.

The investigation of the effects of thymus supernatant on bone marrow cells from senescent mice revealed that thymus products may be responsible for the maintenance of normal prothymocytes in the bone marrow. As detailed in that chapter, others have speculated that the thymus may act on the bone marrow cells but there has been little investigation of the hypothesis. In the context of

previous work in this laboratory and others, these results elucidate several points. First, bone marrow cells from aged mice have a decreased ability to migrate to the thymus. Second, the similarity of the results found in the in vitro assay of bone marrow chemotaxis and the bone marrow chimeras supports the validity of these assays as measures of the ability of bone marrow cells to repopulate the thymus. Third, the improvement in the thymus directed chemotaxis with thymus supernatant treatment, both in vitro and in vivo, indicates that there is some factor in the supernatant which activates or augments the thymus homing population of bone marrow cells in senescent mice.

The work presented in the first chapter leaves several unresolved questions. It does not reveal what in thymus supernatant is responsible for its ability to improve the thymus homing ability of bone marrow cells. An unexamined possibility is that the responsible factor is not unique to the thymus. We are currently making supernatants from other organs to test this hypothesis. Also unresolved is what role, if any, decreased bone marrow cell function plays in the cellular immune dysfunction of the elderly. Based on our current knowledge of immunosenescence, we do not believe that this is a major factor. It is possible that we under estimate the need for newly derived T cells. If newly derived T cells are needed to maintain normal immune function, this would greatly elevate the importance of a deficit in T cell precursors.

The work presented in the second chapter represents an attempt to replicate the rejuvenation of thymus and cellular immune function previously reported in other species. As such, it does not represent a novel approach to the problem of boosting the senescent immune response. It does represent, however, an attempt to evaluate this putative therapeutic method in the context of a well studied model, the mouse. The value of the results is in the foundation they lay for further studies to elucidate the mechanism of action of anterior pituitary hormones on the immune system in general and senescent immune function in particular. Having demonstrated the possibility of thymic and cellular immune function rejuvenation in the mouse, an animal model that has been extensively studied, the applications and limitations of growth hormone therapy can be evaluated using previously validated tools and techniques. For example, it is possible to evaluate possible deleterious side effects by comparing observed anomalies to previously documented pathologies, as was done here. The mouse is an attractive model for such work because its immune system has been well characterized and can be readily compared to the human immune system.

The third chapter details an attempt to use an unorthodox, but reportedly effective, immunopotentiator of tumor-suppressed immune function to boost the senescent immune response. The attempt failed. The similarities between tumor induced immune suppression and immunosenescence

appear to be analogous end products of very different biological processes. The results of this study do not preclude a possible role for naturally derived biological response modifiers in therapies aimed at mitigating the decline of immune function with age. They do not, however, give any indication that PSK is a promising candidate for such a therapy.

In examining the immunosenescent state in humans one cannot help but be struck by the quandary that modern biomedical advances have created. The failure of the human body to maintain immunocompetence into the later years of life is the catch-22 of the lifespan extension achieved over the past century. We have effectively outlived our immune system's functional lifespan. The result is both a delayed mortality and a greatly increased morbidity. Modern medicine has attempted to contradict evolution and the price which nature has extracted is called immunosenescence.

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