AN ABSTRACT OF THE THESIS OF

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Steven L. Davis

This study was conducted to determine the effects of shipping stress on the immune system in domestic lambs (*Ovis aries*) and to determine the potential of the thymic peptide, thymulin, to reduce those effects of stress on the immune system. Treatments consisted of no shipping (as unstressed controls), shipping (as stressed controls) or shipping plus two doses of thymulin. The shipping procedure was conducted for two consecutive days. The responses were measured in three ways. First, the ability of peripheral blood mononuclear cells (PBMN) to respond to four different doses of the mitogen, Interleukin-2 (IL-2), was measured. Second, antibody response to a standard antigen dose over a three week period following the stress was examined. Third, the plasma cortisol concentrations in stressed versus unstressed and in thymulin treated lambs were compared.

There were no differences between any of the four treatment groups ($p > 0.44$) with respect to the animals' lymphocyte proliferative ability. Although there were no detectable differences, caution should be used in interpreting these results, because of technical difficulties encountered with a key reagent in the assay.

Antibody titers were measured at weekly intervals for each of three consecutive weeks following the stressing procedure. These results also showed no treatment effect between any of the four groups ($p > 0.39$).

A comparison of cortisol levels in the four groups revealed that shipping stress increased plasma cortisol concentrations, and thymulin treatment at either dose and on both days of shipment inhibited ($p < 0.0001$ and $p < 0.047$, for day one and two, respectively) that stress-induced increase in cortisol. Interestingly, these results indicate that treatment with thymulin was effective in negating the stress-associated increase in plasma cortisol levels in the lambs. These *in vivo* data support a possible immunomodulatory function of thymulin.

Interactions Between the Immune System, Stress and Thymulin

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Richard L. Christian, Author — April 7, '97 Date

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TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF FIGURES

LIST OF TABLES

DEDICATION

This thesis is dedicated to the people that have made me the person that I am today:

1) my wife, Wendy Sutton-Christian;

2) my parents, Melvin and Glenda Christian;

3) and, my grandparents, Thelma and Glenn Leverenz.

I would like to especially dedicate this manuscript to the memory of my two brothers, Wayne Edward Christian (1964-1995) and Jerry Lee Christian (1961-1993). I hope this document meets most of the expectations that you had of me.

CHAPTER I

INTRODUCTION

> Any life, no matter how long and complex it may be, is made up of a single moment- the moment in which a man finds out, once and for all, who he is [and what he is made of].
>
> -Jorge Luis Borges (1899-1986)

The effects of stress on the immune system can be profound in all vertebrate species. Recent studies have demonstrated that the mechanism of this interaction lies at least partially in the relationship between the immune and endocrine system. That is, numerous immunosuppressive hormones are released in response to a stressor. The results of numerous studies also show that there exists a thymic-endocrine connection (Spangelo, 1995).

One of the major groups of stress hormones are the glucocorticoids (Minton, 1994; Munck *et al.*, 1984; Fleshner *et al.*, 1995). One of the most prominent glucocorticoids involved in the stress response is cortisol (Kent *et al.*, 1993; Apple *et al.*, 1993; Hashizume *et al.*, 1994;). Cortisol plays an essential role in the regulation of the physiological effects of stress (Dantzer & Mormede, 1983; Fleshner *et al.*, 1995). Although these effects on the physiology of the organism may be slightly different due to acute and chronic stressors (Waern & Fossum, 1993; Makino *et al.*, 1995), it has been reported that even for acute

stressors, there is a dramatic increase in the secretion of biologically active cortisol. This elevated cortisol level can have major and lasting impacts on cortisol-target tissues (Fleshner *et al.*, 1995).

One of the most sensitive target tissues of the glucocorticoids is found in the thymus gland (Cunningham-Rundles *et al.*, 1994). This is especially profound in the context of stress because the thymic micro-environment is responsible for the maturation of all subpopulations of T cells (Marsh, 1993; Marchalonis, 1988; Kuby, 1994).

Recent *in vitro* studies that simulate stress (ie. infusion of corticotropin-releasing hormone into the hypothalamus-pituitary-adrenal axis) have shown a decreased T cell proliferative ability and changes in cytokine expression in rats (Labeur *et al.*, 1995). It is now widely accepted that stress results in a general decrease in immune competence via the actions of glucocorticoids (Scheinman *et al.*, 1995; Munck *et al.*, 1984).

Numerous other conditions have also been linked to a decreased immune response because of interactions in the thymus. Age is perhaps the most well defined "condition" attributed to an immunosuppression (Fabris & Mocchegiani, 1985; Bach, M.-A., 1977; Goya, 1992; Bliznakov *et al.*, 1978). Some of the other conditions that have been shown to cause immunosuppressive effects are metalloion deficiencies,

especially zinc deficiency (Prasad, 1984; Fraker *et al.*, 1977) and malnutrition (Parent *et al.*, 1994).

Interestingly, a peptide originating solely from the thymus gland, thymulin (Savino *et al.*, 1982), has been shown to partially reverse the immunosuppressive effects of malnutrition (Parent *et al.*, 1994), aging (Bliznakov *et al.*, 1978), restraint and isolation stress (Okamoto *et al.*, 1993), and has no apparent species specificity (Bach, 1983). Some, or all, of the above results might be the response to thymulin's ability to induce the expression of a variety of cell-surface markers, and consequently, stimulate and/or regulate the maturation of T cells (Chang & Marsh, 1993; Goldstein, 1984; Villa-Verde *et al.*, 1991).

One of the most troublesome problems related to immune system compromise and stress in the agricultural industry is shipping fever. Shipping fever is the primary cause of infectious diseases in the sheep and cattle industry, and consequently, represents a major financial loss to producers (Kimberling, 1988; McGowan *et al.*, 1957; Pierson, 1970; Wilkie & Shewen, 1988; Confer *et al.*, 1988).

Shipping fever is the direct result of the complex interactions between the host and its environment (Kimberling, 1988; Hansen *et al.*, 1995). The disease is associated with the compromised immune system as the result of external stressors.

The question that has therefore arisen through the coalition of all of this information is whether thymulin treatment could partially, or fully, restore the depressed immune response after restraint (physical) and isolation (psychological) stress. Specific assays were conducted to determine the effects of thymulin treatment by:

1) Measuring and comparing plasma cortisol concentrations in the four treatment groups;

2) Assessing the proliferative ability of PBMN cells *in vitro* in response to mitogen;

3) Measuring the ability of the animals to produce antibodies *in vivo* in response to antigen challenge.

CHAPTER II

REVIEW OF LITERATURE

A mind once stretched by a new idea never regains its original dimensions.

-Oliver Wendell Holmes (1809-1894)

THE IMMUNE SYSTEM AND ITS COMPONENTS

There are numerous components contained within the immune system. This is especially true in the "higher" organisms, like mammals, and more generally, vertebrates. The vertebrate body consists of a variety of tissues, which are comprised of different cell types, each of which is specialized for a particular function. This section of the thesis will describe immunity, the various types of immune cells and how these different cell types interact with one another.

The Two Types of Immunity

It is now recognized that there are two general systems of immunity against pathogens: innate and acquired immunity (Fearon & Locksley, 1996). Innate, or natural immunity, utilizes proteins encoded in the germ line to identify potential pathogens, whereas the acquired, or specific immune system, uses an "almost infinitely adaptable" B and T lymphocyte system to respond to pathogens (Fearon & Locksley, 1996). The latter can be further subdivided into

humoral (B cell-promoted) and cell-mediated (T cell-promoted) immunities (Guyton, 1991).

There are mechanisms by which this high level of adaptability, or diversity, is achieved. However, the discussion of those mechanisms is beyond the scope of this review (for review, see Kuby, 1994; Parham & Ohta, 1996). It is important to note that lymphocytes are found in all vertebrates, including, chondrichthyes, osteichthyes, amphibians, reptiles, birds and mammals (Thompson, 1995). The essence of the difference between the two lymphocyte systems, no matter the species in question, is the way in which they recognize microbes.

B Lymphocytes

The B cell originally derived its name from its site of maturation in the bursa of fabricius in birds. Subsequently, it has been determined that B cells mature in the bone marrow (Kincade, 1981), or the liver of mammals in midfetal life (Guyton, 1991). Antibody differentiation only occurs if antigen is not present (in the presence of antigen B cells undergo clonal selection for that specific antigen). Mature B cells are distinguished from other lymphocytes by their expression of various membrane-bound antibody molecules and complement receptors (Kuby, 1994). Once the B cell undergoes clonal expansion and expresses the antibody, it is termed "antigenically committed". That is, the B cell

only expresses an antibody specific for epitopes on the single, original antigen. Since B cells are able to express major histocompatibility complex (MHC) class II, the B cells are also classified as antigen presenting cells (APC) (Kuby, 1994).

T Lymphocytes

The T lymphocytes were named for their maturation site in the thymus gland (Auerbach, 1961), which will be discussed in a following section. T cells also express membrane-bound receptors for antigen. The key difference between T and B cells is that the T-cell receptor only recognizes antigen as foreign when the antigen is associated with MHC class I or II (Kuby, 1994). Thus the B cell is capable of binding soluble antigen, and the T-cell requires antigen to be bound to self-cells (called antigen presenting cells, or APC).

The first time progenitor T lymphocytes migrate from the bone marrow to the thymus gland occurs during the eighth or ninth week of gestation in humans (Kuby, 1994). This migration is brought about by a chemotactic factor secreted by the thymic epithelial cells in the thymus.[1] That is to say, the thymic epithelial cells secrete a substance that

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[1] For a complete review of this pre-thymic maturation process, see Kuby, 1994, pp.48-62.

attracts the progenitor (immature) T cells to the thymus gland[2].

After the T cells mature in the thymus[3], they are recirculated through secondary lymphoid tissues (lymph nodes, Peyer's patches, tonsils and spleen) (Butcher *et al.*, 1980). The primary regulation of the recirculation of lymphocytes from blood to lymphoid tissues and back to blood (as often as one to two times per day (Kuby, 1994)) lies in the recognition of endothelial cells (Butcher *et al.*, 1980) and the control of diapedesis of the lymphocytes across the vascular walls (Butcher & Picker, 1996). The T cells that have not encountered antigen are termed naïve and are the lymphocytes programmed to recirculate (Butcher *et al.*, 1980).

Essentially both helper and cytotoxic T cell responses can be classified into three phases: 1) activation and expansion; 2) death; and 3) memory (see the following section) (Ahmed & Gray, 1996). Upon presentation of an antigen [bound to an APC, like MHC II/Helper T cells] by the lymphoid tissues, the T cells undergo clonal expansion and differentiation (Butcher & Picker, 1996) into effector and memory cells (Kuby, 1994). The first step in this complex process is the extensive proliferation of helper T cells.

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[2] This maturation process of T cells in the thymus is reviewed in detail in Kuby, 1994, pp. 48-62.

[3] For a complete review of this process, see the following section of this thesis entitled "T-cell Maturation in the Thymus".

The helper T cells secrete a variety of cytokines (see discussion below) or lymphokines[4] (Kuby, 1994), and orchestrate the acquired immune response (Fearon and Locksley, 1996). The lymphokines in turn activate cytotoxic T lymphocytes, which mediate the killing of altered self-cells (e.g. virus-infected cells) (Nowak & Bangham, 1996).

It is interesting to note that the two different types of MHC (class I and II) present antigen to the different subsets of T cells. It is well documented that MHC class I only presents antigen to cytolytic $CD8^+$ cells, while MHC class II presents antigen to helper $CD4^+$ cells (Parham & Ohta, 1996). This is another example of the complex regulation occurring within the immune system.

One final subpopulation of T cells is the suppressor T lymphocyte group. Work in the early 1970s led to the hypothesis that there is a group of T cells responsible for suppressing the immune response (Kuby, 1994). Although numerous experimental systems have since reproduced this suppressive effect (ie. it is a real effect), there is still some doubt as to the existence of a distinct population of suppressor T cells that are responsible for this effect (Kuby, 1994).

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[4] Lymphokines are defined as, "nonantibody proteins, generated by lymphocyte activation, that act as intercellular mediators of immunological response(s)" (Goldstein, 1984).

Natural Killer Cells

As discussed previously, there are two types of immunity: innate and acquired. Innate immunity has been attributed to natural killer cells. Essentially natural killer cells are responsible for a wide range of cytotoxic activity, which is present before an organism is exposed to an antigen (Quan *et al.*, 1982). These cells are large granular lymphocytes, which constitute five to ten per cent of human peripheral-blood lymphocytes (Kuby, 1994).

Immunological "Memory"

Immunity to some pathogens can be extremely long-lived, as per the observations of the Greek historian Thucydides, who noted about the plague of Athens in 430 B.C. that "the same man was never attacked twice" (Finley, 1951). A Danish physician, Ludwig Panum, who investigated a major outbreak of measles in 1846, concluded that 1) "of the many aged people still living on the Faroes who had had measles in 1781, not one was attacked a second time" and 2) "all the old people who had not gone through with measles earlier in life were attacked when they were reexposed to infection" (Panum, 1847). This demonstrates that the immune system can remember an encounter that had occurred years before and that there are inherent mechanisms for maintaining this long-term memory (Ahmed & Gray, 1996). In fact, it has been recently documented that circulating antibody can be

detected in humans for 25 to 50 years after immunization with a non-replicating antigen (Ahmed & Gray, 1996).

It is also quite apparent that memory is responsible for the very rapid and potent antibody response occurring upon re-exposure of the body to the same antigen. This process has been termed the secondary antibody response. The secondary response is also longer-lived than the primary response, because there are many more memory cells present than there were original lymphocytes for the specific antigen (Guyton, 1991), and because the memory cells have a higher affinity for antigen than naive lymphocytes (Kuby, 1994).

There are essentially two opposing hypotheses as to how this memory response may occur in T cells. One view suggests that memory is due to long-lived memory cells, which do not require contact with their specific antigen for survival. The other view contends that memory is the result of continuous stimulation of T lymphocytes by some kind of persistent antigen (Ahmed & Gray, 1996; Kuby, 1994). Although neither group has been able to provide adequate experimental evidence to disprove one or both of the theories, it is clear that memory does occur via some, as yet to be determined, mechanism.

Interactions Between Components

Both cell-mediated and humoral immune responses require interactions among a myriad of cell types to produce a specific and total immune response. Briefly, helper T cells are activated by MHC class II presented on an APC, this in turn stimulates interleukin 2 production. IL-2 then binds to its newly expressed receptor and stimulates helper T cell proliferation (termed clonal expansion). Clonal selection of B cells specifically, is brought about by an interaction between antigen and membrane-bound antibody expressed on the surface of a naïve B cell, coupled with T cell and macrophage interactions (Kuby, 1994). Numerous interleukins are stimulated by the helper T cell at this point, which cause B cell differentiation (for review, see Kuby, 1994). The helper T cell also activates various T effector cells [termed the effector phase (Ahmed & Gray, 1996)], which generate the cell-mediated response and various nonspecific, effector cells.

Collectively, these interactions form the complicated milieu responsible for the immune response. It is still unclear exactly how all of these systems come together and what the role of each of the cell types is, but it is clear that the immune response is a highly controlled and organized network.

THE THYMUS GLAND AND DIFFERENTIATION OF T-CELLS

One of the most critical and responsive parts of the immune system to stressors is the thymus gland. Because of this, researchers have begun looking at the function and regulatory mechanisms of thymic hormones and factors (Cunningham-Rundles *et al.*, 1994). With a better understanding of how these interactions between hormones and the thymus occur, researchers might be able to relieve, or prevent, some of the stress-related problems associated with this thymus responsiveness.

The Thymus Gland

There are numerous thymic hormones that have been described to date, including: thymulin[5], thymopoietin, thymic humoral factor, thymosin α_1, (Hadden, 1992), thymosin α_7, thymosin β_8, thymosin β_9, thymosin β_{10}, thymosin fraction 5 (Goldstein, 1984) thymocyte growth factor (Ernström *et al.*, 1990) and thymosin β_4 (Kuby, 1994; Goldstein, 1984). However, at the time of this review, only the first four (thymulin, thymopoietin, thymic humoral factor and thymosin α_1) have been shown to be functional as thymic hormones (Spangelo, 1995) (ie. they have been identified in the

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[5] Thymulin was historically called "facteur thymique serique", or FTS (Bach *et al.*, 1977).

circulatory system, indicating that they may be produced in the thymus, circulate to and act on other tissues).

Due to the very complex, integrated network associated with the thymus, there are many areas that need further clarification and research. The primary focus of this review will be the aspects that relate to thymulin and its influences on the immune system. However, some of the other areas currently under investigation related to immunodeficiencies include SCID[6], DiGeorge syndrome, ataxia telangiectasia, chronic mucocutaneous candidiasis, disseminated histiocytosis X, Hyper-Immunoglobulin E syndrome, Wiscott Aldrich, and combined immune deficiency (Cunningham-Rundles *et al.*, 1994).

T-cell Differentiation in the Thymus

The thymus gland provides the essential micro environment necessary for thymocyte maturation (Spangelo, 1995; Hadley, 1994). All progenitor T cells, or immature T-cells, must reside in the thymus for a period of one to two weeks in order to mature into a functional T lymphocyte (Marchalonis, 1988). The progenitor T-cells undergo various kinds of differentiation and selection pressures in the thymus throughout the life of the individual. The reticulo-epithelial network, or specifically, the cortical epithelial

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[6] SCID is an abbreviation for severe combined immunodeficiency disease.

cells (Spangelo, 1995), has been implicated in providing the necessary micro-environment for this thymus-dependent T cell development (Marsh, 1993). Most of the differentiation in the thymus relates to the expression of T cell-specific cell-surface markers (like Thy-1[7], GK1.5[8], Ly-1 and Ly-2[9]) at specific times (Marchalonis, 1988) and rearrangements of the germ-line T cell receptor (TCR) genes (Kuby, 1994).

It has further been suggested that the production of thymic hormones (like thymulin) by the thymic epithelial cells may provide the primary control mechanism over the processes that determine what cell surface markers are expressed (or the lineage along which the thymocyte will differentiate) (Kaufman, 1980; Spangelo, 1995). These circulating thymic hormones may also provide a framework for regulatory interactions between the thymus and classic endocrine tissues (i.e., the thymus is a hormone-secreting tissue, suggesting that regulatory pathways between the thymus gland and endocrine system exist) (Figure II.1) (Marsh, 1993; Spangelo, 1995). Chang and Marsh (1993) have recently shown that thymulin modulates the expression of at

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[7] Thy-1 can be defined as a "glycoprotein that is the earliest appearing surface marker of the T cell lineage; also called theta antigen" (Kuby, 1994).

[8] This is another cell-surface marker. It is thought to define distinct functional lineages of T cells ($GK1.5^+$ is expressed on helper T cells, while $GK1.5^-$ is expressed on suppressor and cytotoxic T cells) (Marchalonis, 1988).

[9] These are the markers of all thymus-derived lymphocytes (Kuby, 1994; Marchalonis, 1988).

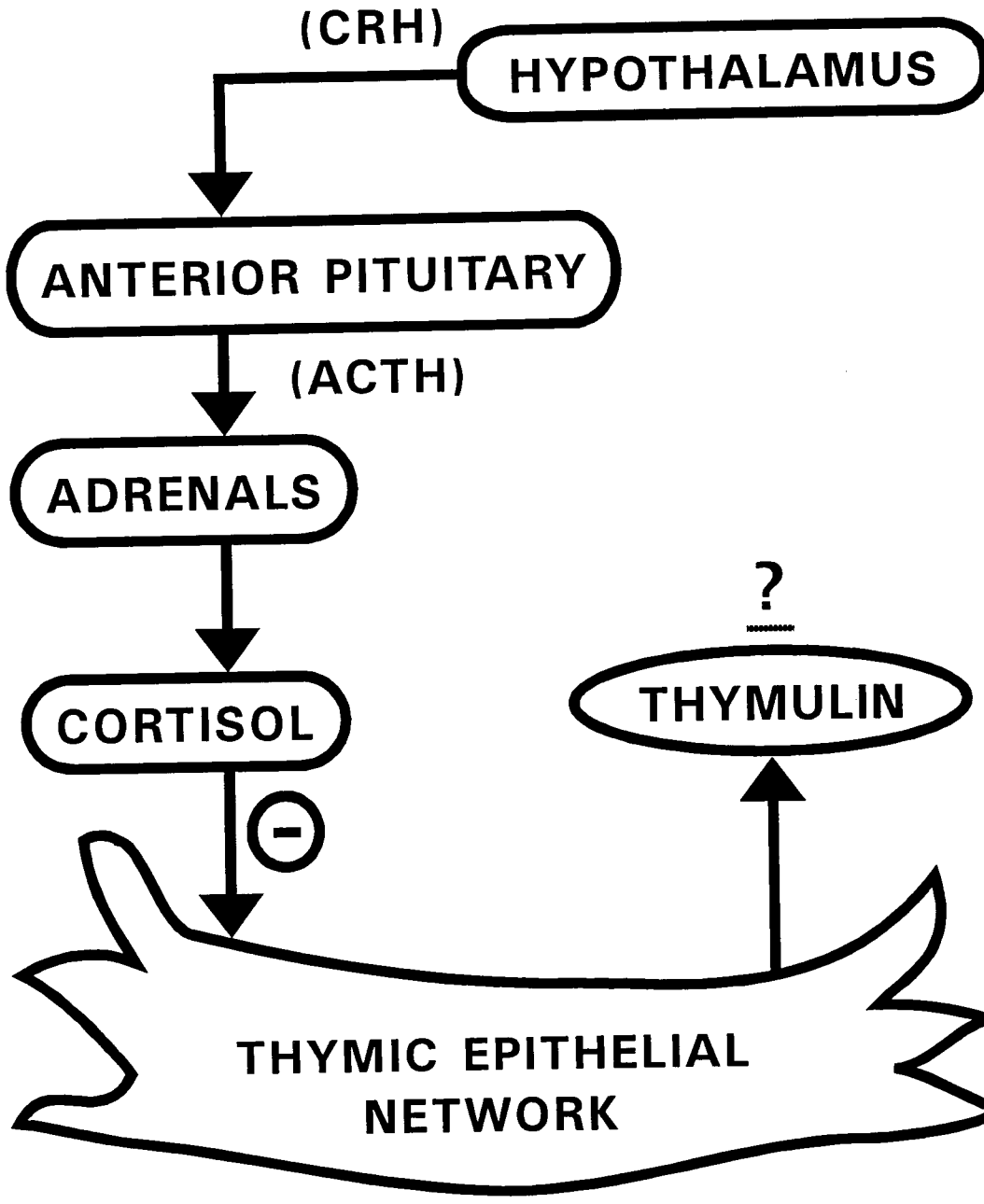

Figure II.1 The diagrammatic representation of the HPA axis and its hormonal interactions with the thymus. The ⊖ represents immunosuppression; the ? represents potential, as yet undefined, interactions.

least some of these cell surface markers on immature avian T cells.

The selection pressures on the T-cell progenitors within the thymus include gene rearrangement to allow for formation of specific T-cell receptors necessary for further selection of the T-cell repertoire. This selection process can be divided into two primary categories, positive and negative selection. Positive selection eliminates thymocytes that cannot recognize self-MHC molecules, whereas negative selection causes thymocytes with high affinity for self-MHC molecules to undergo apoptosis (Spangelo, 1995; Kuby, 1994). This selection process allows for self-nonself discrimination, or the elimination of altered self-cells to avoid problems like autoimmune responses. It is estimated that only about one per cent of all the T-cell progenitors survive these selection pressures (Kuby, 1994).

Within the thymus, the T cells reside in both the cortex and medulla. However, it is still unclear which part is responsible for the final maturation step and exactly where the mature lymphocytes exit the thymus (Marchalonis, 1988). It is clear that cortical cells usually exhibit cell surface markers in predominantly higher amounts than those found in the medulla (Marchalonis, 1988). Consequently, the cortex contains cell populations capable of self-renewal, contribution of cells to the medulla and contribution of progeny to the periphery (Marchalonis, 1988).

Together, these processes result in a wide array of cells that are necessary for a functional T cell arsenal. From the above discussion it is clear that the interactions between all of the cells involved in both the humoral and cell-mediated immune response are very complex, and therefore, difficult to study under controlled, experimental conditions. With advances in technology in the future some of these interactions and the exact mechanisms involved may reveal themselves to researchers.

Intracellular Interactions with the Thymus Gland

In order to gain a better understanding of how thymulin is implicated in regulating immune functions and all of its interactions therein, it is necessary to discuss the effects of the thymus gland on the thymic epithelial cells, including its effects on the immature T-cells. Various kinds of intracellular interactions contribute to the complex network comprising the thymus gland and its many influences. Scientists are really only beginning to gain a better understanding of the complexity of this integrated network. For example, prolactin has been reported to induce the proliferation of thymic epithelial cells[10] and to cause them to begin secreting thymulin (Coto & Hadden, 1991). The suggested mechanism of this action may be to induce the

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[10] The cells that are solely responsible for producing thymulin (Savino *et al.*, 1982).

cytokine[11], interleukin-1 (IL-1), to act on thymic epithelial cells (Hadden, 1992). Recently, it has also become apparent that IL-1 and IL-2 act in synergy with one another to stimulate the proliferation of immature thymocytes and mature T cells (Hadden, 1993). Furthermore, the interpretation of these findings implicate the interleukins in general in the developmental process to be the central, driving components of differentiation and proliferation of T-cells.

A further refinement in elucidating this process has shown that thymulin significantly modulates the release of IL-1 (both α and ß) (Cunningham-Rundles *et al.*, 1994) and IL-6 (Safieh-Garabedian *et al.*, 1993). These cytokines are responsible for the expression of specific T-cell markers, like Thy-1, necessary for further T-cell development (Hadden, 1992). Another study demonstrated that the thymus gland exerts a regulatory influence on circulating thymocytes; the amount of cytokines produced under lethally irradiated stem cells was significantly lower than that obtained from normal, nonirradiated animals. However, if these same irradiated stem cells were allowed to reconstitute their immune systems in the presence of normal

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[11] Cytokines are defined as, "any of numerous secreted, low-molecular-weight proteins that regulate the intensity and duration of the immune response by exerting a variety of effects on lymphocytes and other immune cells" (Kuby, 1994).

thymic epithelial grafts, there was no difference between them and control, nonirradiated animals (Wiedmeier *et al.*, 1991).

Various kinds of neuroendocrine controls are represented in T-cell differentiation and proliferation. The primary mechanism of this control is by the action of the hormones and neuropeptides on thymic epithelial cells. It has been shown that an increase in epithelial growth is a result of prolactin or growth hormone treatment (Dardenne & Savino, 1994). Prolactin and growth hormone have also been shown to stimulate peripheral blood mononuclear cells in a variety of animals (Khosraviani & Davis, 1995; Kelley, 1990; Kooijman *et al.*, 1996; Berczi, 1986). Thus, these hormones exert an influence on the thymic epithelial cells' function, which influences the differentiation of the developing T-cells (Berczi *et al.*, 1981; Kooijman *et al.*, 1996; Berczi, 1986).

In addition to the aforementioned hormones, many other hormone and neuropeptide receptors have been detected in the thymus, including: thyroid-stimulating hormone (TSH), follicle-stimulating hormone (FSH), adrenocorticotropic hormone (ACTH), luteinizing hormone (LH), oxytocin (OT), and vasopressin (VP)(Dardenne & Savino, 1994). Although, their functions or interactions are not yet well understood, it is believed that most if not all of them have an effect on the integrated thymic network, and furthermore, may modulate the production of peptide hormones and neuropeptides (Dardenne &

Savino, 1994). This neuroendocrine control is under the direct influence of complex biological intra-thymic circuitry, which is responsible for the *in situ* production of numerous pituitary hormones and neuropeptides, as well as their respective receptors by thymic cells (Dardenne & Savino, 1994).

IMMUNOREGULATION AND CYTOKINES: CONTROL OF THE IMMUNE SYSTEM

Among the myriad of immunoregulators and stimulants active in the immune system, some of the most prominent are the glucocorticoids and thymic peptides. Cytokines can be viewed as the vehicle that carries a message from the immune system to other tissue types. The thymus produces peptides that provide this messenger function.

Types of Immunoregulators

The primary stimulants can be placed in three categories: 1) microbial derived macrophage activators; 2) thymus-derived T-cell stimulants; and 3) chemically defined stimulants (Hadden, 1993). For obvious reasons, this discussion will focus on the second category. The four aforementioned thymic peptides (thymopentin, thymosin α_1, thymulin, and thymic humoral factor) are all currently under investigation to determine their role in immunoregulation (Hadden, 1993). Thymopentin and thymosin α_1 both promote interleukin production, while thymic humoral factor exhibits

T-cell promoting activity. The purification of thymic humoral factor revealed that it contains eight amino acids, and is currently under further investigation as a potential treatment for HIV-infected, pre-AIDS patients (Hadden, 1993). Interestingly, thymosin α_1 is able to decrease the secretion of a number of pituitary hormones, including: thyroid-stimulating hormone (TSH), adrenocorticotropic hormone (ACTH), and prolactin (PRL) *in vivo* (Dardenne & Savino, 1994). Thymulin is the only one of the four that has been shown to be thymus-specific and to regulate the secretory products of active thymic epithelial cells (Hadden, 1993). Because thymic hormones are able to regulate the release of specific pituitary hormones, there appears to be a regulatory mechanism between thymic peptides and some pituitary hormones.

A more recent calf thymus isolate, called thymocyte growth factor (TGF), has the same amino acid sequence as thymulin, but contains a large N-terminal blocking moiety and is acidic (Ernström *et al.*, 1990). TGF has been shown to initiate DNA synthesis in immature thymocytes, but the active molecule is only stabilized by zinc (Ernström *et al.*, 1990). However, it is unclear at this time whether TGF is a precursor to or a degradation product of thymulin, or if it is simply a completely separate peptide.

Cytokines and the Hypothalamus-Pituitary-Adrenal (HPA) Axis

Cytokines serve as one of the major regulatory mechanisms of the immune system (Labeur *et al.*, 1995). The term cytokine includes those cytokines secreted by lymphocytes, formerly called lymphokines (Kuby, 1994). They are responsible for the activation of B cells, cytotoxic T cells and numerous other players in the immune response (Kuby, 1994). The effect lymphokines have on the HPA axis appears to be primarily mediated by corticotropin-releasing hormone (CRH) (Berkenbosch *et al.*, 1987) (Figure II.1, p. 16). CRH (arginine vasopressin is more important in sheep) acts as a stimulant of the HPA axis to cause an increase of circulating glucocorticoid concentrations, which can cause a suppression of the immune response (Childs *et al.*, 1995; Labeur *et al.*, 1995; McGlone *et al.*, 1993; Munck *et al.*, 1984). The glucocorticoids are potent inhibitors of the synthesis of numerous cytokines [IL-1, IL-6, and TNF-α] (Beutler *et al.*, 1986; Lee *et al.*, 1988).

Glucocorticoids (GC) are also potent inhibitors of the immune system itself (Scheinman *et al.*, 1995; Munck *et al.*, 1984). Although the exact mechanism by which they inhibit immune response remains to be elucidated fully, it is known that GC inhibit the inflammatory response to skin homografts (Medawar & Sparrow, 1956), decrease immune responsiveness to mitogen challenge (Brown-Borg *et al.*, 1993), inhibit

lymphokine synthesis (Goldstein, 1984), inhibit the production of various cell-surface markers and repress immunoregulatory (lymphocytes and other immunoregulatory factors) gene expression (Auphan *et al.*, 1995). Glucocorticoids also increase glycolysis, gluconeogenesis and lypolysis (Niezgoda *et al.*, 1993). By contrast, low levels of GC enhance thymocyte differentiation and antibody formation (Goldstein, 1984) (these low levels would be found under normal, unstressful conditions).

Glucocorticoids also cause the induction of hormone-dependent promoters under various environmental factors, like stress (Nordeen *et al.*, 1995). Some of the earliest studies relating to glucocorticoids revealed that they were responsible for the suppression of lymphocyte proliferation (Gillis *et al.*, 1979a). This suppression may involve a reduction in the clonal expansion of T-cells (Gillis *et al.*, 1979a; Gillis *et al.*, 1979b), via cytokine production (Kuby, 1994). Munck *et al.*, (1984) suggested that this suppression of immune response may help prevent the immune system from overreacting to stimuli like stress.

STRESS AND THE IMMUNE SYSTEM

It is now generally accepted that stress causes a suppression of the immune response, through the interactions of the glucocorticoids with their respective immune target tissues. This depressed response to antigenic challenge has

led to a variety of diseases, both in agricultural and human medicine. The focus of this section will be on the interactions within the body in relation to stress and how these interactions lead to the development of diseases in sheep.

Definition of Stress

What is stress? Everyone has an idea of what stress is, but it is very difficult to provide an adequate definition. There are many different definitions of stress available in the literature, differing mainly in the application for which the definition was intended. Bayne (1975) developed one of the best, most widely applicable, definitions. He defined stress as 'a measurable alteration of a physiological (or behavioral, biochemical or cytological) steady-state which is induced by an environmental change, and which renders the individual (or population, or the community) more vulnerable to further environmental change.' In other words, an environmental change causes a disruption within the organism that renders the animal susceptible to further aggravation or change.

Effects on the Immune System

Numerous stressors have been linked to a depression in immune response, including restraint-stress (Solomon, 1986; Okimuro & Nigo, 1986; Okamoto *et al.*, 1993; Theoharis *et*

al., 1995), restriction of social contact and visual control (Janssens *et al.*, 1994), and heat stress (Minton & Blecha, 1990). Physical, chemical and/or psychosocial stresses disrupt the organism's ability to regulate the normal homeostatic balance of the immune system (Khansari *et al.*, 1990). This balance is disturbed by activating the hypothalamic-pituitary-adrenal (HPA) axis (Chrousos, 1995) and the sympatho-adrenal axis (Minton, 1994). This, in turn, causes a change in the number of circulating immune cells, like mast cells, lymphokines and lymphocytes (Theoharis *et al.*, 1995), a reduction in some aspects of cell-mediated immunity (Coppinger *et al.*, 1991) and an alteration of the equilibrium of many immune system hormones. Ultimately, the activation of the HPA axis results in increased secretion of cortisol (Dantzer & Mormede, 1983), which has an additive effect with repeated application of the stressor (Coppinger *et al.*, 1991). Changes in the sympathetic nervous system are also apparent when glucocorticoid secretion is elevated, hence the immune system is also influenced by the autonomic nervous system (Spencer & Oliver, 1996). This neuroendocrine control of immune response demonstrates the influence of the nervous system on the immune system, and conversely, the immune system's influence on the nervous system (Khansari *et al.*, 1990).

The thymus gland is now known to be very responsive to many types of external stimuli, like stress (Cunningham-Rundles *et al.*, 1994), malnutrition (Parent *et al.*, 1994), and noise (Folch *et al.*, 1991). The immunosuppressive effects of stress have been demonstrated in a variety of animals, including mice (Okamoto *et al.*, 1993), pigs (Janssens *et al.*, 1995; Waern and Fossum, 1993), rats (Siegel *et al.*, 1982), cattle (Suliman *et al.*, 1989; Blecha & Minocha, 1983), horses (Wong *et al.*, 1992), fish (Maule *et al.*, 1989) and sheep (Coppinger *et al.*, 1991; Niwano *et al.*, 1990). It is generally thought that stress induces a decrease in immune response to a challenge in all animals studied to date (Kuby, 1994).

Buckingham *et al* (1992) examined the possibility of an effect of thymulin on the hypothalamic-pituitary-adrenal (HPA) axis. In one study, they failed to find an effect of thymulin on blood cortisol concentrations in unstressed rats. In spite of this observation, they speculated about the possibility of an effect of thymulin o the HPA axis.

The inhibition of the HPA axis by opioids has also been shown to be an effect of stress on various immune regulatory pathways (Odio & Brodish, 1990; Rushen & Ladewig, 1991; McCubbin *et al.*, 1993). This may function as an adaptive mechanism by preventing over-stimulation of the HPA axis in response to short-term stressors (Janssens *et al.*, 1995). This is a logical conclusion, given that it would be very

detrimental to the organism to allow continued HPA axis stimulation during very short duration stressors.

There appears to be no conclusive evidence for the adaptive advantage of increasing cortisol secretion under longer duration stressors. However, there are a couple of plausible explanations for this phenomenon. The first consideration is that there are few natural circumstances that lead to stress which can not be avoided, in contrast to the domestic animal setting. Even if the stress occurs for a short time period, generally, the animal is able to escape the cause of the stress before any negative impacts on other systems can occur. The other possible explanation is that elevated cortisol levels help to re-distribute the energy and resource partitioning occurring within the organism. This would imply that systems not essential to immediate survival are simply shut down, or at least extremely hindered (as is the apparent case with the immune system).

Stress in Relation to Shipping Fever

Stress is known to affect illness in humans (Rabkin & Struening, 1976), as well as a variety of other animals. Numerous studies have demonstrated that whatever the nature of the stress or the organism undergoing the stressor, there is a common pathway. This pathway involves the stimulation of adrenalcortical secretion, a consequential increase in serum glucocorticoids and activation of the sympathetic

nervous system (Khansari *et al.*, 1990). The increased glucocorticoids cause a suppression of the immune system. This compromised immune responsiveness, and subsequent upper respiratory infections, in relation to the stress associated with the shipment of cattle and sheep, results in what is called shipping fever. Irrespective of the management strategy, shipping fever is a primary cause of disease in the world's sheep population (Kimberling, 1988; Salman *et al.*, 1988; McGowan *et al.*, 1957; Pierson, 1970).

OVINE SHIPPING FEVER

Because stress is associated with, or the causative agent in, a wide variety of pathological problems, it is very important to understand the relationship between stress and disease(s). Due to the nature of this thesis, the following discussion will focus on the relationship between stress and ovine shipping fever, as well as describe what ovine shipping fever is and what it does to the organism.

Description of Ovine Pneumonia

In general, domestic animals are very sensitive to stress (Dantzer & Mormede, 1983; Minton, 1994), and sheep are among the most sensitive to stress-induced diseases (Niezgoda *et al.*, 1993). Ovine pneumonia, also called enzootic pneumonia, pasteurellosis, shipping fever and/or hemorrhagic septicemia, is an acute, infectious disease of

sheep (Kimberling, 1988). It is clinically characterized by respiratory distress, elevated temperatures, mucopurulent, oculonasal discharge, depression, anorexia and pyrexia, and pathologically by pneumonitis and pleuritis (Kimberling, 1988).

The occurrence of pneumonia has been demonstrated in all breeds and ages of sheep, although younger animals are more susceptible (Kimberling, 1988). Some of the most important risk factors that can be attributed to higher incidence of pneumonia include stress from: weaning; shipping; a change in feed (Kimberling, 1988; McGowan *et al.*, 1957); drastic changes in weather conditions; and, infectious and/or toxic agents (Hansen *et al.*, 1995; Kimberling, 1988; McGowan *et al.*, 1957). Two of the other prominent risk factors include the anatomy of the respiratory system (Veit and Farrell, 1978) and geographic location of the animals (Hansen *et al.*, 1995; McGowan *et al.*, 1957). The incidence of shipping fever in feedlot lambs occurs from one to three weeks after arrival in the feedlot (McGowan *et al.*, 1957; Kimberling, 1988). The complexity of the incidence of shipping fever is a result of the agent-host interaction with most, if not all, of these factors. However, the "bottom line" is that stress elevates the serum cortisol concentrations with a resulting immunosuppressive effect (Kimberling, 1988), which renders the animals more susceptible to infection.

It is also apparent from the literature that the morbidity and mortality associated with pneumonia in feeder lambs originating from western Oregon, western Washington (Hansen *et al.*, 1995) and the northern coast of California (McGowan *et al.*, 1957) is much greater than lambs coming from other geographical areas. It has been shown that lambs originating from these areas have up to a five times higher death rate, as a result of pneumonia, than their counterparts from other areas (Hansen *et al.*, 1994; Hansen *et al.*, 1995; McGowan *et al.*, 1957). For example, the mortality rate associated with pneumonia was shown to be one per cent for lambs originating in the interior of California, compared to five per cent mortality in the north coastal areas (McGowan *et al.*, 1957).

However, pneumonia appears to be the highest cause of loss to this industry, regardless of the origin of the lambs. For instance, the morbidity of pneumonia can range up to 50 per cent, and mortality as high as ten per cent (Kimberling, 1988; Pierson, 1970). The average national rates of morbidity and mortality to ovine Shipping Fever are approximately 30 to 40 percent and 2 to 5 percent, respectively (Salman *et al.*, 1988; McGowan *et al.*, 1957).

Etiology and Pathogenesis of Ovine Shipping Fever

The disease is the result of a two stage infection: first, one of several viruses invade the upper respiratory

tract, and second, bacteria proliferate and spread to other internal tissues (Kimberling, 1988). *Pasteurella haemolytica* is the primary bacterial organism isolated from infected lung tissues (Kimberling, 1988; Frank & Smith, 1983).

Although *Pasteurella haemolytica* is the primary organism isolated from infected lung tissues, it is only one of several organisms involved in the shipping fever complex (Kimberling, 1988). The first stage of the infection involves the viral damage of the respiratory epithelial lining, which causes inflammation, edema and necrosis and a suppression of the bronchoalveolar macrophages (Kimberling, 1988). This destroys ciliary action and protection provided by mucous, hence allowing colonization of these areas by bacteria and mycoplasma (the second stage of infection) (Kimberling, 1988).

Some of the most common viruses associated with shipping fever include: Parainfluenza Type 3 (Lehmkuhl & Cutlip, 1982), Respiratory Syncytial Virus (Kimberling, 1988; Hansen *et al.*, 1995), Adenoviruses and Reoviruses (Kimberling, 1988). The responsible pathogen(s) may vary with the circumstances surrounding the exact situation. The second stage of the infection results after the virus has damaged the respiratory tract, when bacteria are able to proliferate. The bacteria represent the primary cause of pathological problems associated with shipping fever

(Kimberling, 1988). *Pasteurella* spp are the most prominent organisms, however, *Mycoplasma* spp and *Chlamydia* spp are also present (Kimberling, 1988). There have been at least twelve serotypes and several untypeable isolates of *P. haemolytica* isolated from the lungs of cattle and sheep (Frank, 1979).

Shipping fever probably begins with upper respiratory tract infections and then descends the respiratory tract to its terminus, the lungs. This occurs very early in the infection (Kimberling, 1988). With the aforementioned changes evoked by the viral and bacterial agents, the infection can affect thirty percent or more of the lung parenchyma by day seven, following exposure. The infection then spreads outward to the pleural and pericardial cavities and on into the joints (Kimberling, 1988). Healing can occur by days thirteen to twenty, unless substantial lung damage, intoxication or shock is realized, which results in death (Kimberling, 1988).

THE EFFECTS OF AGING ON THE IMMUNE SYSTEM

Aging is another factor that has been linked to a depressed immune response in a variety of organisms, including humans. What exactly does aging do to the immune system? This question has led to a wide array of research. A select portion of this research is outlined in the following discussion.

Repression of the Immune Response due to Aging

It has been known for a long time that aging has an adverse effect on the immune system. That is to say, the immune response is greatly compromised with aging. One of the early studies related to aging and the immune system demonstrated that aging causes a significant decrease in the cytotoxic activity of spleen cells (Bach, M-A., 1977). This decrease was detectable at 19 weeks of age in mice and remained stable until 46 weeks of age. After 68 weeks, there was another sharp decline in cytotoxic activity; the lymphocyte-mediated cytotoxicity was completely absent after 75 weeks of age (Bach, M-A., 1977). Goya, 1992, established two different categories of this deterioration of immune competency: the first involves the impaired ability of the immune system to deal with non-self or altered self-cells; the second category reflects the loss of tolerance[12] to self-cells. The first category includes a decreased immune response to microorganisms, a weaker primary antibody response, an impaired response to low-dose antigens (Price & Makinodan, 1972) and a loss of antigenic specificity. The second category involves an increase in autoimmunities (Kuby, 1994) and an increased resistance to tolerance, or

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[12] Tolerance can be defined as an "active state of specific immunological nonresponsiveness [to self-antigens] induced by prior exposure to the antigen" (Kuby, 1994).

the ability to be unresponsive to self-antigens (Goya, 1992).

These categories are thought to be a direct result of the loss of the ability of T-cells to undergo clonal expansion (Cheung *et al.*, 1987). The loss in T-cell repertoire has been suggested to include helper T-cell activity (Callard & Basten, 1978), the generation of cytotoxic T-cells (Bach, 1979), and an increase in delayed-type hypersensitivities (Kay *et al.*, 1980). The polymerization of actin in T-cells also declines drastically with age, which could result in a substantial loss in motility, secretion, and internal transporting abilities of T cells (Cheung *et al.*, 1987).

Possible Reasons for the Repressed Immune Response with Age

It is thought that these symptoms are partially a result of thymus involution (Goya, 1992; Fabris & Mocchegiani, 1985). This involution is considered to be one of the primary events in age-associated immunological deterioration (Bach & Beaurain, 1979). It is also believed that this involution is brought about by micro-environmental factors, mediated by the hormones and thymic factors previously described (Fabris & Mocchegiani, 1985).

There are many other things that are affected with aging. For instance, a marked decline in natural killer

cell[13] activity, a marked decline in primary immune response (Bliznakov *et al.*, 1978), a modest decrease in IL-2 stimulation of spleen cells (Muzzioli *et al.*, 1992), and probably most importantly, a marked decline in thymulin levels in the blood (Fabris & Mocchegiani, 1985; Goya *et al.*, 1993; Muzzioli *et al.*, 1992; Fabris *et al.*, 1985; M-A. Bach, 1977; Bach, 1983). This decline in circulating thymulin levels has been demonstrated in a variety of species, including mice (Goya *et al.*, 1992; Bliznakov *et al.*, 1978), dogs (Goff *et al.*, 1987), and humans (Fabris and Mocchegiani, 1985). It is generally accepted that thymulin levels are reduced as a consequence of aging in most animals, but especially in mammals (Chang & Marsh, 1993). It is, as yet, unresolved whether this marked decline in circulating thymulin levels is responsible for some, or all, of the aforementioned depressions in the immune response associated with aging.

THE EFFECTS OF ZINC ON THE IMMUNE SYSTEM

Zinc is a somewhat unique metalloion in that it is held responsible for numerous upregulatory interactions with the immune system. Most of these findings come from the work done with immunodeficient people around the world. More

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[13] They are thought to carry cytotoxic activity towards virus-infected and tumor cells (Muzzioli *et al.*, 1992).

recently, researchers have begun to examine zinc's relationship to the immune system using mice.

Zinc has also been implicated as a major metalloion with regard to the immune system. Experimentally induced zinc deficiencies have been shown to cause a variety of immune system compromises. Some of these include lymphocytopenia, poor wound healing, anergic response to skin sensitization, and recurrent infections (Prasad, 1984). Zinc deficiency has also been demonstrated to cause preferential involution of the cortex of the thymus, depressed responses to T-cell dependent (Fraker *et al.*, 1977) and T-cell independent antigens (Zwickl & Fraker, 1980) and a severely comprised ability to respond to secondary infections (Fraker *et al.*, 1977). Apparently, these depressions in immune responsiveness are also able to be passed on to the second and third filial generations in mice, although not to the same degree as the first generation (Beach *et al.*, 1982).

Another group discovered that zinc deficiency constitutes a stressor which activates the hypothalamus-pituitary-adrenal cortex axis. This leads to a chronic elevation in the glucocorticoid levels (DePasquale-Jardieu & Fraker, 1979). These findings may suggest a mechanism for the negative effects associated with zinc deficiency discussed above. This may also lend credence to the theory that zinc may be acting through thymulin, as thymulin is well conserved among species (See subsequent discussion).

It is also interesting to note that the general patterns of changes in immune status observed in the above studies using mice, appear to correlate well with the observed changes in zinc-deprived humans (Fraker *et al.*, 1985). Regardless of the organism involved however, the question of whether or not zinc is acting through thymulin remains unanswered.

THYMULIN AND ITS INTERACTIONS WITH THE IMMUNE SYSTEM

Because thymulin is produced solely by the thymus (Savino *et al.*, 1982), it has recently gained much attention in the field of immunology. With the isolation, purification and production of numerous analogs of thymulin came many insights into the function(s) of the thymus gland itself in relation to the immune system. The following section answers the question of whether or not there is an interaction between the thymus and immune system.

What is Thymulin and What Does it do?

Many factors have been studied in restoring a depressed response to an immune challenge. For instance, the administration of thymulin showed a restorative effect on depressed thymus-dependent antibody production (Okamoto *et al.*, 1993). Thymulin is a well characterized nonapeptide

produced by the thymic epithelial cells (Bach *et al.*, 1977; Savino *et al.*, 1982; Pleau *et al.*, 1977; Safieh *et al.*, 1990). Thymulin was first isolated and purified from porcine blood (Dardenne *et al.*, 1977), biochemically characterized (Bach *et al.*, 1977) and shown to consist of a nine amino acid peptide with the following sequence (Pleau *et al.*, 1977):

H_2N-Glu-Ala-Lys-Ser-Gln-Gly-Gly-Ser-Asn-OH

Thymulin appears to be the same nonapeptide in all species, as demonstrated by subsequent amino acid analysis in calves and humans (Bach, 1983). In fact, there appears to be no species specificity, as subsequent amino acid analysis revealed the hormone to be identical, or behave in the same manner, in dogs (Morrison *et al.*, 1990), chickens, avian species in general (Chang & Marsh, 1993), sheep (Davis *et al.*, 1994), rats and mice (Okamoto *et al.*, 1993). Pleau *et al.* (1977) determined the molecular weight of thymulin to be about 900 g/mol. Subsequent, more precise, analysis revealed the molecular weight to be 847 g/mol (Dardenne *et al.*, 1977). Thymulin is apparently transported through the blood by albumin and prealbumin, with a small amount found circulating as free thymulin (Burton *et al.*, 1978), although it has a half-life of about ten minutes (Goldstein, 1984; Davis *et al.*, 1994). The short half-life of thymulin can be

extended by the addition of zinc (see discussion on the interaction between zinc and thymulin below), or by substitution of long-lived analogs (Auger *et al.*, 1987; Goldstein, 1984).

Since the original isolation of thymulin, more than forty analogs have been synthesized (Goldstein, 1984). Upon closer examination using these analogs, the hormone's activity was localized on the seven terminal amino acids (Bach, 1983). It was also demonstrated that some of the analogs bind to the thymulin receptor and behave antagonistically, not unlike the actions of an antihormone (Pleau *et al.*, 1980; Auger *et al.*, 1987). Interestingly, five of the analogues examined demonstrated a prolonged activity *in vivo* with respect to natural thymulin (Auger *et al.*, 1987).

The Regulation of Thymulin Production and Release

As previously mentioned, various hormones and neuropeptides act on the thymus; these factors have also been shown to influence thymulin production and release. Specifically, growth hormone and prolactin appear to upregulate thymulin production (Dardenne *et al.*, 1989; Dardenne & Savino, 1994; Hadden, 1993). It has been suggested that the growth hormone upregulation of thymulin is under the direct control of insulin-like growth factor one (IGF-1); consequently, IGF-1 alone can stimulate

thymulin production *in vitro* (Dardenne & Savino, 1994). In another report leu-enkephalin and ß-endorphin[14] were able to directly and specifically modulate the ability of thymic epithelial cells to produce and secrete thymulin (Savino *et al.*, 1990). Therefore, it is apparent that a number of cytokines and hormones are able to stimulate thymulin secretion.

Another report suggested there to be a negative feedback mechanism present, thymulin itself being the inhibitor signal of further thymulin secretion. This is suggested from the observations that the number of thymulin containing cells increases after thymulin depletion *in vivo* and an increase in thymulin-containing cells is slowed with the addition of the hormone (Goldstein, 1984).

Some Actions of Thymulin

Thymulin was shown to restore thymic function in a variety of studies after thymectomy, in aged animals, or after the application of a stressor (Okamoto *et al.*, 1993) . In one such study thymulin was able to restore contact sensitivity after adult thymectomy (Erard *et al.*, 1979). In another study thymulin induced suppressor T-cell activity in NZB mice (Bach & Niaudet, 1976), which may be linked to thymulin's ability to induce the expression of a variety of

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[14] These components of the thymic micro-environment are endogenous opioids (Savino *et al.*, 1990).

T-cell surface markers (Chang & Marsh, 1993). This would indicate that thymulin is essential in restoring the balance between effector and suppressor T-cell subsets and that the effects of aging or thymectomy are reversible (Dardenne *et al.*, 1989; Blitznakov *et al.*, 1978; Gagnerault *et al.*, 1995). Thymulin was also demonstrated to enhance the proliferative response of helper T-cells, but suppressed the response when suppressor T-cells were stimulated with mitogen (Thomson, 1987), which could be the result of the dose used[15]. Safieh-Garabedian *et al.* (1993) linked low doses (1 ng/ml) of thymulin to interleukin-1 (IL-1) stimulation and interleukin-6 (IL-6) inhibition. Therefore, it would appear that the primary biological function of thymulin is the regulation and/or stimulation of T-cell differentiation and maturation within the thymus gland (Dardenne *et al.*, 1978; Chang & Marsh, 1993).

It was further suggested that thymulin may cause specific T-cell subset differentiation by modulating the CD4 and CD8[16] molecules (Villa-Verde *et al.*, 1991), or at least by acting on immature T cell membrane characteristics

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[15] There are two doses proposed for different effects: one dose, used to stimulate helper T cells (1-5 μg/kg); and the other dose (15-20 μg/ml), used to stimulate suppressor T cells (Goldstein, 1984).

[16] CD stands for cluster of differentiation molecules; which are the contact points on the T-cells for antigen presentation. CD4 is associated with MHC class II, helper T-cells...while CD8 is always presented in conjunction with MHC class I, cytotoxic T-cells.

(Gagnerault *et al.*, 1995). At any rate, it is known that thymulin is able to bind to T cell membrane receptors at two different sites with a high affinity (k_d = 10^{-9} and 10^{-7} mol/L, respectively) (Pleau *et al.*, 1980).

Other evidence suggesting thymulin's involvement in the control of immunocompetence comes from the findings that thymulin levels are low and T cell anomalies are observed in human patients with primary immunodeficiencies (Safieh-Garabedian *et al.*, 1992). Collectively these data demonstrate that thymulin is involved in the regulation of the immunomodulation.

THE INTERACTION BETWEEN ZINC AND THYMULIN

How Zinc and Thymulin Interact

It is well documented that thymulin is a metallopeptide that requires zinc, and to a lesser extent, aluminum, copper and manganese salts (Goldstein, 1984), to be biological active (Neve, 1992). Furthermore, thymulin requires zinc to be bound to the N-terminus in equimolar concentrations (Goldstein, 1984; Coto & Hadden, 1991). It has been further documented that numerous metalloproteins or peptides are able to bind to zinc, but few are capable of transporting and exchanging this metal with other body tissues (such as in the case with the IL-1 induction of preferential zinc uptake from various tissues into bone marrow, thymus and

liver (Cousins & Leinhart, 1988)). Some researchers are even proposing that thymulin is a member of a small family of extracellular zinc carriers that are able to present a labile pool of zinc transporters. These transporters are capable of feedback with the tissues that need zinc as the blood levels of zinc and tissue needs for zinc fluctuate (Licastro *et al.*, 1996).

The *in vivo* effects of zinc appear to be linked to other intracellular activities as well. The regulator of corticosteroid synthesis in the pituitary-adrenal axis[17] is dependent on zinc for functional activity (Flynn *et al.*, 1972). In the unbound form thymulin is inactive and may actually competitively inhibit the bound, active form (Fabris *et al.*, 1984; Pleau *et al.*, 1980)[18]. The dissociation constant of thymulin (for zinc) is relatively high ($k_d = 10^{-7}$ mol/L) (Gastinel *et al.*, 1984), and therefore, low plasma concentrations of thymulin may produce an apparent absence of function (Mocchegiani *et al.*, 1993). Zinc has also been implicated in influencing secretion of prolactin from the pituitary gland (Neve, 1992). For instance, low levels of zinc have been implicated in the inhibition of the thyroid-releasing hormone-mediated

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[17] Adrenocorticotropic hormone regulates the corticosteroid synthesis in the adrenal cortex (Neve, 1992).

[18] Here, unbound refers to the free thymulin, whereas the bound form refers to the thymulin-zinc moiety.

secretion of prolactin (Prasad, 1985) and the natural killer T-cell activity in old mice (Muzzioli *et al.*, 1992).

Is Zinc Solely Responsible?

It is clear that the antibody's binding sites for thymulin are dependent on zinc. Zinc determines a specific spatial configuration of thymulin, which yields new antigenic determinants that are specifically recognized by antibodies (Goldstein, 1984). The question is whether or not zinc is also responsible for all of the immunological roles currently attributed to it (Fraker *et al.*, 1985; Licastro *et al.*, 1996).

A plausible alternative explanation that is equally likely is that without zinc present, thymulin is biologically inactive and therefore cannot exert its action(s). Some of the immunological roles now thought to be caused by zinc may in fact be attained through the zinc-thymulin moiety.

CHAPTER III

EFFECT OF THYMULIN ON PERIPHERAL BLOOD MONONUCLEAR CELLS, ANTIBODY PRODUCTION AND PLASMA CORTISOL SECRETION IN STRESSED SHEEP

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ABSTRACT

Four groups of sheep (*Ovis aries*) were used in this study, consisting of 10 animals each of stressed versus unstressed (control), and two doses of thymulin treatment (both of these thymulin treated groups were also subjected to shipping stress). The stressing procedure was conducted for two consecutive days. The effects of thymulin treatment on the ovine immune/endocrine system were examined in three ways. The first method was to compare the proliferative response of PBMN cells challenged with a mitogen in stressed controls and stressed, thymulin treated lambs to assess the cell-mediated immune response. The second method was to measure antibody response to antigen challenge over a three week period following the stress in the four groups to assess the humoral immune response. The final method was to compare plasma cortisol concentrations in the four groups to assess the Hypothalamus-Pituitary-Adrenal (HPA) axis response.

The comparison of the PBMN assay resulted in no statistically significant difference between any of the four treatment groups ($p > 0.44$). Although there were no detectable differences, caution should be used in interpreting these results, because of technical difficulties encountered with a key reagent in the assay.

Antibody titers measured at weekly intervals for three weeks following the stress, also showed no treatment effect between any of the four groups ($p > 0.38$).

A comparison of plasma cortisol concentrations in the four groups, however, showed that shipping stress increased cortisol concentrations as compared to unstressed lambs ($p < 0.0002$), and that both doses of thymulin inhibited that cortisol response to stress on both days of shipment ($p < 0.0001$ and $p < 0.047$, for d 1 and d 2, respectively). These *in vivo* data suggest the involvement of thymulin in regulation of the HPA axis, particularly in stressed animals.

Key Words: PBMN Cells, Cortisol, Immune System, Thymulin, Stress, Ovine Shipping Fever, Sheep

INTRODUCTION

> Surely the gods from the beginning have not revealed all to mortals, but by long seeking mortals can make progress in discovery.
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> -Xenophanes of Colophons

It is common knowledge that stress causes a depressed immune response through the actions of the hypothalamus-pituitary adrenal (HPA) axis (Figure IV.1) in all vertebrates (Theoharis *et al.*, 1995; Solomon, 1986; Dantzer & Mormede, 1983; Brown-Borg *et al.*, 1993). Dantzer and Mormede (1983) have speculated that domestic animals are among the most susceptible animals to stressful stimuli. Domestic animals are subject to a wide variety of stressors, including adverse environmental conditions, management strategies and exposure to pathogens (Hansen *et al.*, 1995; Dantzer & Mormede, 1983; Brown-Borg *et al.*, 1993).

One of the most widely known and least understood pathological problems in the large animal industry (cattle, sheep, horses, etc.) is respiratory disease (Kimberling, 1988; Hansen *et al.*, 1995; Frank & Smith, 1983). The most prominent respiratory disease associated with stress is the condition known as "shipping fever".

It is estimated that shipping fever costs the U.S. cattle industry about six billion dollars per year (Personal Communication, D. Hansen, Oregon State University, 1996;

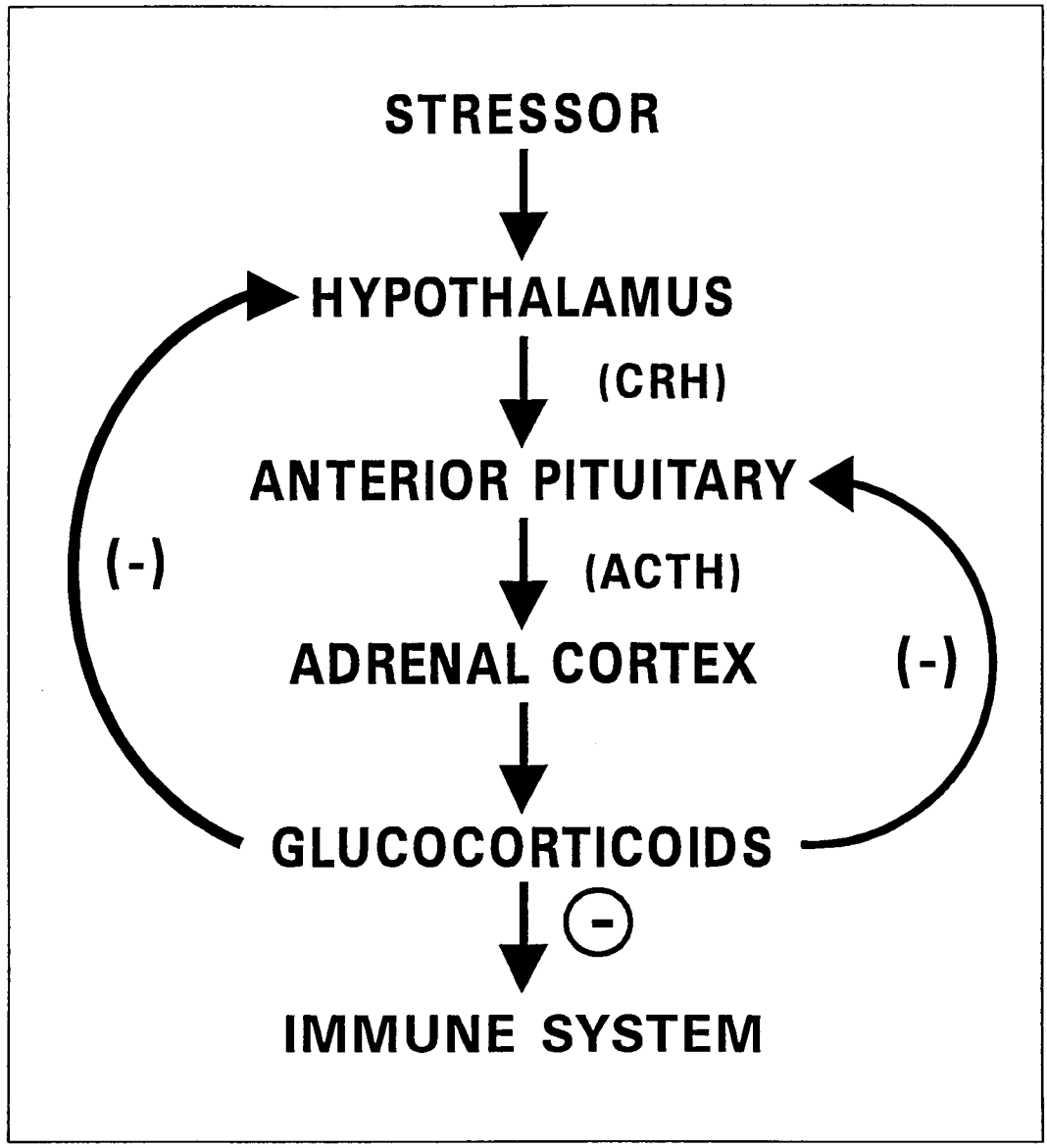

Figure IV.1. Schematic diagram of the effects of stress on the HPA axis. CRH = Corticotropin Releasing Hormone; ACTH = Adrenal Corticotropic Hormone; (-) = negative feedback loop.

Wilkie & Shewen, 1988) and the U.S. sheep industry about twenty-five to one hundred million dollars per year (Salman *et al.*, 1988; McGowan *et al.*, 1957; Kimberling, 1988).

Recent research has unveiled a plausible new approach to the shipping fever problem. Because lymphocytes mature in the thymus gland (Dardenne & Savino, 1994; Kuby, 1994) and the link between the thymus and endocrine system has been established (Spangelo, 1995), scientists have begun to look at thymic hormones as a means to decrease the immunosuppressive effects of stress (Okamoto *et al.*, 1993; Hadden, 1992). The most promising research came from a Japanese group, who concluded that treatment of stressed mice with the thymic peptide, thymulin, restored antibody production to normal levels (Okamoto *et al.*, 1993).

The specific objectives of this experiment were to determine if shipping stress and thymulin treatment had an effect on: 1) cell-mediated immunity, as measured by the peripheral blood mononuclear cell (PBMN) assay; 2) humoral immunity, as measured by the antibody titers; and 3) the HPA axis, as measured by blood plasma concentrations of cortisol in lambs.

MATERIALS AND METHODS

Animals

Six to eight month old crossbred, castrated male sheep (wethers) were used (body weights ranged from thirty-five to forty-four Kg) in the study. All forty animals were contained in one pasture for the duration of the experiment. The animals were allowed access to feed and water *ad libitum*. The feed consisted of a mix of grass forage, supplemented with pelleted barley malt (Barley Malt Screening 60%, Malt 40%, the feed also contained Canola, Milera and Soy Bean Oil) (Personal Communication, B. Bedenbach, Portland, OR, 1996). The feed contained 14% crude protein and 67% total digestible nutrients (Personal Communication, L. Cross, Oregon State University, 1996).

The lambs were randomly assigned to one of four treatment groups (ten animals per group). Thirty animals were removed from the larger group for subsequent injection with various doses of treatment and shipment (Table IV.1). The shipment consisted of trucking the animals for 6 h each d for two consecutive d (Figure IV.2). The treatment consisted of subcutaneously injecting one group with 1ng/Kg body weight thymulin[19] plus vehicle[20] and another with

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[19] Sigma Chemical Company, St. Louis, MO 63178.

[20] The vehicle consisted of a 0.16μg/Kg body weight $ZnCl_2$ + 0.9% sterile NaCl solution.

Table IV.1 General design for the thymulin experiment.

Group	Lamb Numbers	Number of Animals/Group	Treatment
B	11-20	10	Stress Control*
C	21-30	10	Non-Stress Control*
A	1-10	10	1ng Thymulin/Kg BW
D	31-40	10	10ng Thymulin/Kg BW

* These groups received only vehicle (0.16μg/Kg $ZnCl_2$ + 0.9% Sterile NaCl).

BW = Body Weight at the time of the experiment.

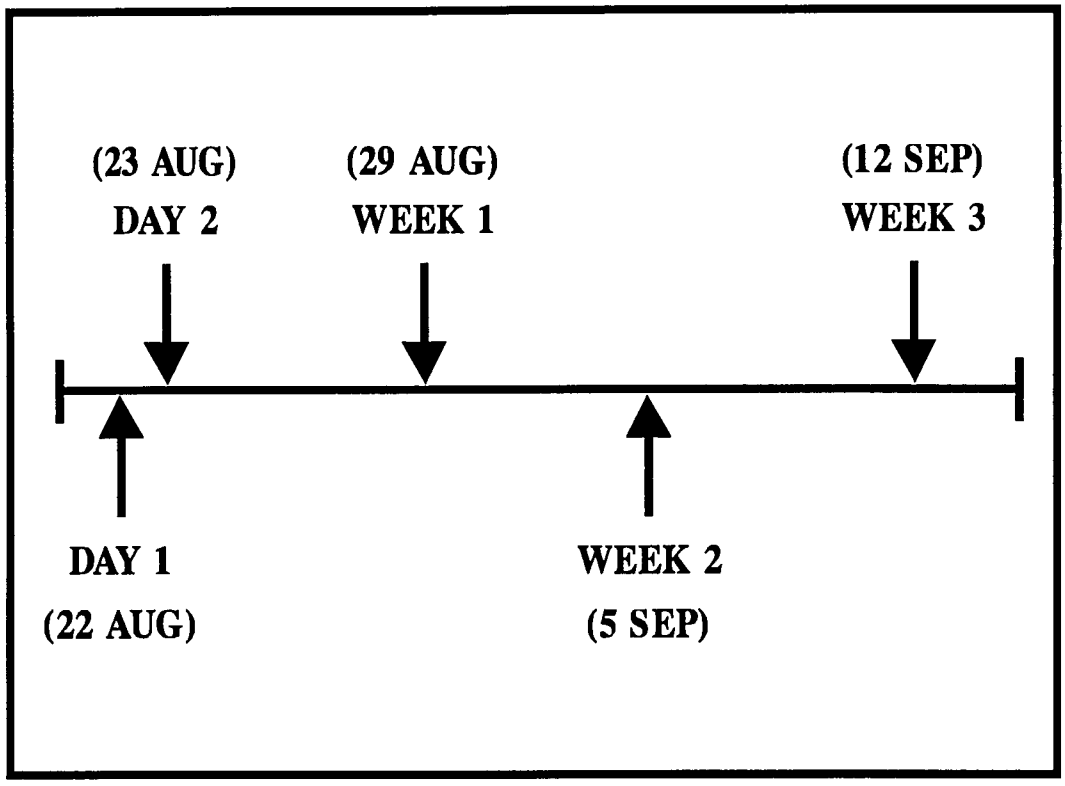

Figure IV.2. Schematic diagram of the timeline for the thymulin experiment. The stressors were applied at day 1 and day 2. Blood collections occurred on all subsequent dates shown.

10ng/Kg body weight thymulin plus vehicle[20], while the stress control group received only vehicle (Table IV.1, p 53). The remaining 10 animals (unstressed controls) also received the zinc chloride in sterile saline injection, however, these animals were left in the pasture at the sheep center for the shipping duration.

On both d of shipping stress, the weather was sunny with ambient temperatures of approximately 15°C. The lambs were placed in a truck with stock racks[21] that allowed free circulation of air around the animals during shipping.

At the end of the shipping period each d, 10 mL blood samples were collected from each animal into heparinized vacutainer tubes[22] (plasma) via jugular venipuncture for all assays[23]. The tubes were placed on ice during collection and transport to the laboratory (the total time elapsed before the samples were processed ranged from 45 to 65 min on all collecting d). Blood was also obtained at weekly intervals for three weeks after shipment[24] (Figure IV.2, p 54).

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[21] The dimensions of the stock truck racks were approximately 3.3 m wide x 5 m long.

[22] Fisher Scientific, Pittsburgh, PA 15219.

[23] All of the blood samples collected - d 1 and one-half of d 2 - were collected for the cortisol assay; those collected on the second half of supplying d 2 were used for the PBMN assay.

[24] The blood samples collected at weekly intervals were used to perform the antibody titer assay.

Following the shipping on d 1, all animals were injected subcutaneously with 2.25 mg of Keyhole Limpet Hemocyanin (KLH)[19] emulsified in Freund's Incomplete Antigen[19]. Because of an error in calculating the amount of KLH solution, there was an insufficient amount to treat all animals. As a result, four of the unstressed control lambs were not injected with the antigen (lamb numbers 12-15).

Experimental Lamb Morbidity

For two weeks following shipment, lambs were monitored for visible signs of pneumonia (labored breathing, nasal discharge, lack of vigor and elevated rectal temperatures (Personal Communication, D. Hansen, Oregon State University, 1995)).

Chemicals

RPMI 1640 medium, Ficoll-Histopaque (with a density of 1.077 μg/ml), Freund's Incomplete Adjuvant, Keyhole Limpet Hemocyanin (KLH), Thymulin, 3,3',5,5'-tetramethylbenzadine and Rabbit α-Sheep IgG were obtained from Sigma[19]. The Streptavidin-Horseraddish Peroxidase (HRPO) and tritiated thymidine ([^{3}H]thymidine) were obtained from Amersham[25]. The

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[25] Amersham, RPN.1004, Arlington Heights, IL.

DMSO and Hydrogen Peroxide solutions were obtained from Oregon State University Chemical Stores[26].

Isolation of PBMN Cells

The PBMN cells were isolated from the whole blood obtained after shipment on d 2, using a previously described density gradient centrifugation method (Boyum, 1968). This method employs the use of a Ficoll Histopaque[19] sucrose gradient to separate red from white blood cells.

Proliferation Assay

The PBMN proliferation assay was conducted by the method previously described (Khosraviani and Davis, 1996). The only modification of this method was that the PBMN cells were cultured in duplicates, which were averaged for statistical analysis.

Antibody Titer Assay

The antibody titer assay [enzyme-linked immunosorbant assay (ELISA)] was performed according to the method described by Minton *et al.* (1991) (Figure IV.3). There were numerous modifications to this protocol, as follows. Flat-bottom, high-binding 96-well ELISA plates were coated with KLH in carbonate buffer (pH 9.2)

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[26] Oregon State University, Corvallis, OR.

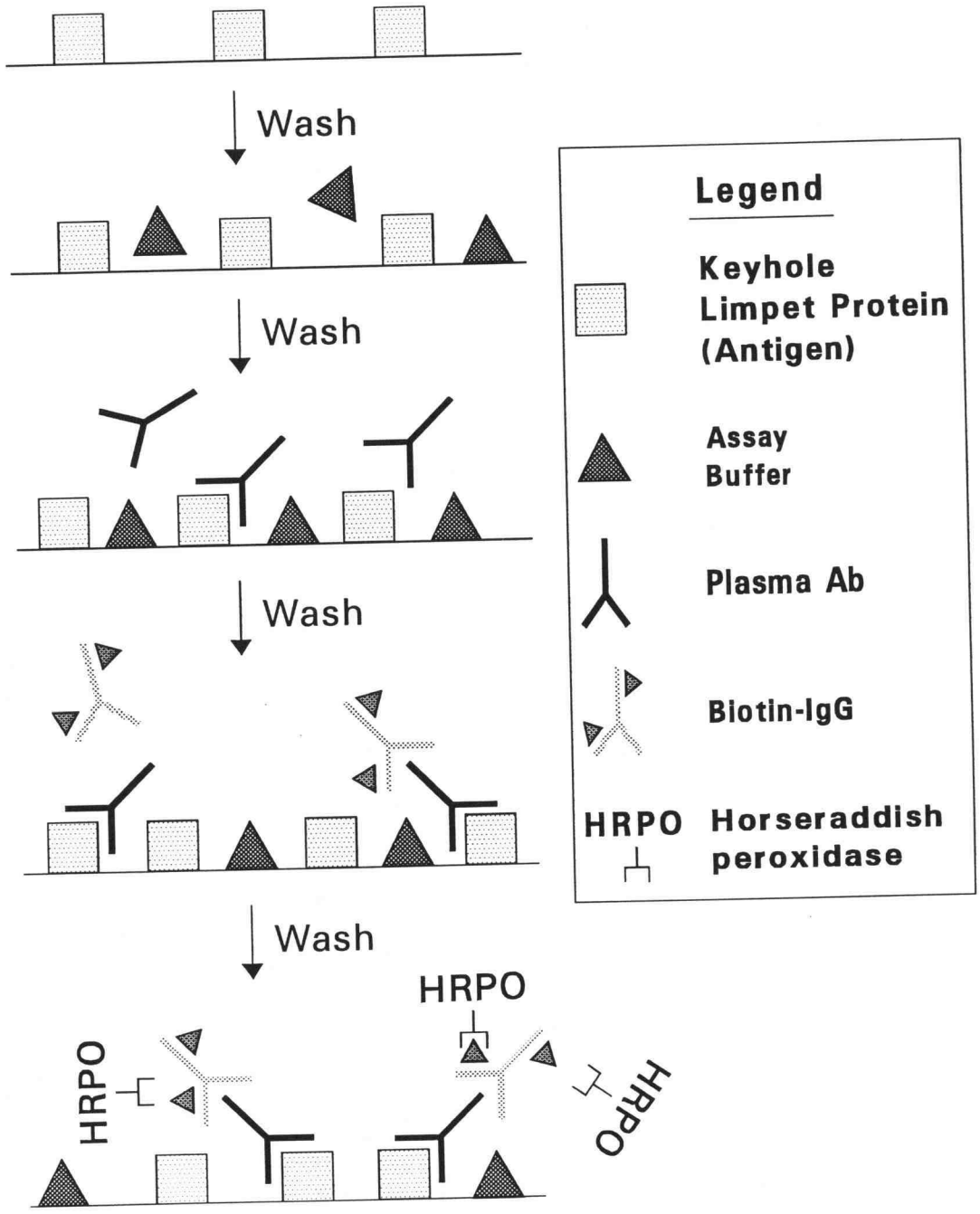

Figure IV.3. Schematic depiction of the antibody titer ELISA.

for 2 h at 37°C. The plates were then blocked with assay buffer, containing 1% bovine serum albumin (BSA), 1 ml Potassium Phosphate, 0.1% Triton X-100 and 0.1% Tween 20. Plasma samples obtained from lambs for weeks 2 and 3 were randomly added in duplicate in the following dilution factors: 10, 50, 100 and 200. Lamb plasma samples from week 1 were examined in the same manner using the following dilution factors: 10, 20, 40 and 100. After a 0.5 h incubation at 37°C, streptavidin horseradish peroxidase (diluted 1:1000)-labeled, biotinylated rabbit-antisheep IgG (diluted 1:2500) was added. Color development occurred with the addition of a chromogen solution (100μl of 3,3',5,5'-tetramethylbenzadine-DMSO stock solution, 1μl of 30% hydrogen peroxide and 10 ml of 0.1 M sodium acetate buffer (pH=5.5)). The color reaction was stopped with 2 M sulfuric acid after 15 minutes, and the plates were read on a Titertek Multiskan® Plus plate reader[27] using a 450 nM filter. Antibody titers were assessed by comparing the optical densities at the 100 fold dilution factor in all samples (ie. the highest dilution factor showing a positive reaction).

The intra-assay coefficient of variation (CV) was calculated for a mid-range (1:50) dilution value and the highest (1:200) dilution value, based on 8 replicates. The total assay variability was estimated by dividing the

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[27] MTX Lab Systems, Inc., McLean, VA 22101.

overall SEM by the overall mean obtained from plasma samples collected during week 2 and 3. There were 3 non-KLH immunized animals included in this assay to obtain an estimate of non-specific binding within the assay.

Cortisol Assay

The blood samples obtained following shipment on each of the two days were centrifuged (1800 x g for 15 minutes), aliquoted and frozen in the -70°C freezer for subsequent analysis. Plasma cortisol was measured by radioimmunoassay (RIA) by Ms. Harriet Lorz in Dr. Carl Schreck's laboratory (which is funded by the United States Department of Agriculture), as described previously (Redding, 1981).

The cortisol assay was tested for parallelism by comparing the plot of the cortisol reference standards to the pooled sheep sera dilutions. This comparison showed the slopes of the two curves to be virtually identical (Personal Communication, Calab Slater, Oregon State University, 1997).

Statistical Analysis

Due to some potential outliers and the variability noted between animals, the ability of the lymphocytes to respond to a mitogen challenge (PBMN assay) and the results of the antibody titer ELISA were analyzed using the nonparametric one-way ANOVA procedure (SAS, 1994). The similarities in cortisol concentrations among groups were

tested using the one-way ANOVA, followed by a Tukey-Kramer multiple range test to determine which group means were different (SAS, 1994). Alpha levels for all assays were set prior to the conduct of the experiment to be 0.05. This study employed a completely randomized, double-blinded experimental design.

RESULTS AND DISCUSSION

Thymulin Concentration in vitro

The concentration of thymulin was not measured in the present study because the ELISA previously published would no longer work, for reasons unknown (Davis et al., 1994). In a previous experiment Davis *et al.* (1994) injected thymulin at a dose of 8 ng/Kg BW intravenously into sheep, resulting in blood serum thymulin concentrations which peaked ten minutes later at 110 pg/ml (compared to normal thymulin concentrations of 15-30 pg/ml in sheep). In the present study, thymulin was injected subcutaneously, which would likely result in a more delayed and gradual peak in blood thymulin concentrations.

Experimental Lamb Morbidity

Of the forty lambs in the study, 8 were observed with symptoms of pneumonia. These animals were distributed over the four treatment groups as follows: 0 from the NSC; 1 from the SC; 3 from the 1 ng/Kg Thymulin; and 3 from the 10 ng/Kg Thymulin group. None of the lambs died of any causes during the 1 mo observation period, following the study.

Effect of IL-2 on Sheep PBMN Cells

The proliferative ability of sheep PBMN cells in response to a mitogen challenge (IL-2) is presented in Table IV.2. There were no statistically significant differences between any of the groups (p-value > 0.44)[28]. Although thymulin treatment had no apparent statistically or biologically significant effects on the cell-mediated immune response under the current experimental regime, it would be beneficial to perform this assay again (see Footnote 28).

Thymulin has been shown to exert its influence on immature and mature T cells (Safieh-Garabedian *et al.*, 1993; Dardenne *et al.*, 1978; Chang & Marsh, 1993). It is also apparent that thymulin acts as an immunomodulator by influencing production of cytokines by PBMN cells (Safieh-Garabedian *et al.*, 1993), which should result in further controlling the differentiation of immature T cells. Specifically, in human PBMN cells, low doses of thymulin (1 ng/ml) resulted in IL-1 stimulation and IL-6 inhibition, while high doses (1000 ng/ml) always resulted in inhibitory actions on these cytokines (Safieh-Garabedian *et al.*, 1993).

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[28] NOTE: One of the key reagents, Ficoll-Histopaque 1077, used in the assay had a severely elevated pH (10), which would cause cell mortality or a decrease in their ability to respond to a subsequent mitogen challenge. There was a recall issued on this product by Sigma[19], indicating that "...during a recent inspection and reassay of this product, we found some bottles which could affect cell integrity." (Appendix A, p 92). We learned of this problem after the assay had been conducted.

Table IV.2 Proliferation of sheep peripheral blood mononuclear (PBMN) cells collected on d 2 *in vitro* in response to interleukin-2 (IL-2) stimulation.

Treatment	Number of Animals	PBMN Response to IL-2 (500 units/ml)	
		CPM/Group** Mean ± SEM@	Per cent Proliferation
Stress Control	10	250.3 ± 15.8	94.8
Non-Stress Control	10	264.1 ± 16.3	100++
1ng Thymulin/Kg BW	10	222.3 ± 9.7	84.2
10ng Thymulin/Kg BW	10	255.5 ± 16.0	96.7

++ The Non-Stress Control group was used as 100% to determine the relative proliferation rate of the other three treatment groups.

** There was no difference between any group means (p-values > 0.44).

@ Standard error of the mean is based on the model assumption of equal variance.

BW = Body Weight of the lambs at the time of shipment.

The results obtained from the PBMN assay (in conjunction with the cortisol results detailed below) are surprising given other evidence that the immunosuppressive effects of cortisol were most likely obtained by controlling T cell proliferative ability (Gillis *et al.*, 1979a; Gillis *et al.*, 1979b). This would indicate that, at least in part, the immunosuppressive effects of cortisol are mediated via T lymphocyte development. Therefore, a differential level of cortisol should result in a difference in the proliferative ability of T cells. That such results were not observed in these experiments could have been due to the problems with the Ficoll reagent.

Effects of Treatment on Antibody Production

The results of the antibody titer ELISA detected no significant effects (p-value > 0.38) on the humoral immune system (Table IV.3). Samples from week three contained the highest optical density values of any of the weeks examined, week two samples were visibly lower (Figure IV.4) and week one titers were essentially zero, after accounting for background titer values; however, p-values from all three weeks were greater than 0.38 (Table IV.3, p.64).

The results from this assay were also somewhat surprising given the current understanding of the relationship between the immune response and stress. For instance, it was recently reported that pigs exposed to mild

Table IV.3 Average antibody response for week 2 and 3 plasma samples.

Treatment	Number of Animals	Antibody Response*	
		Week 2++ Mean ± SEM@	Week 3# Mean ± SEM
Stress Control	6	0.095 ± 0.174	0.456 ± 0.120
Non-Stress Control	10	0.148 ± 0.135	0.281 ± 0.092
1ng Thymulin/Kg BW	10	0.289 ± 0.135	0.457 ± 0.092
10ng Thymulin/Kg BW	10	0.147 ± 0.135	0.263 ± 0.092

* Optical density read at 450nM with an antiserum dilution of 1:100.

@ Standard error of the mean is based on the model assumption of equal variance.

++ $P > 0.45$

\# $P > 0.38$

BW = Body Weight of the lambs at the time of shipment.

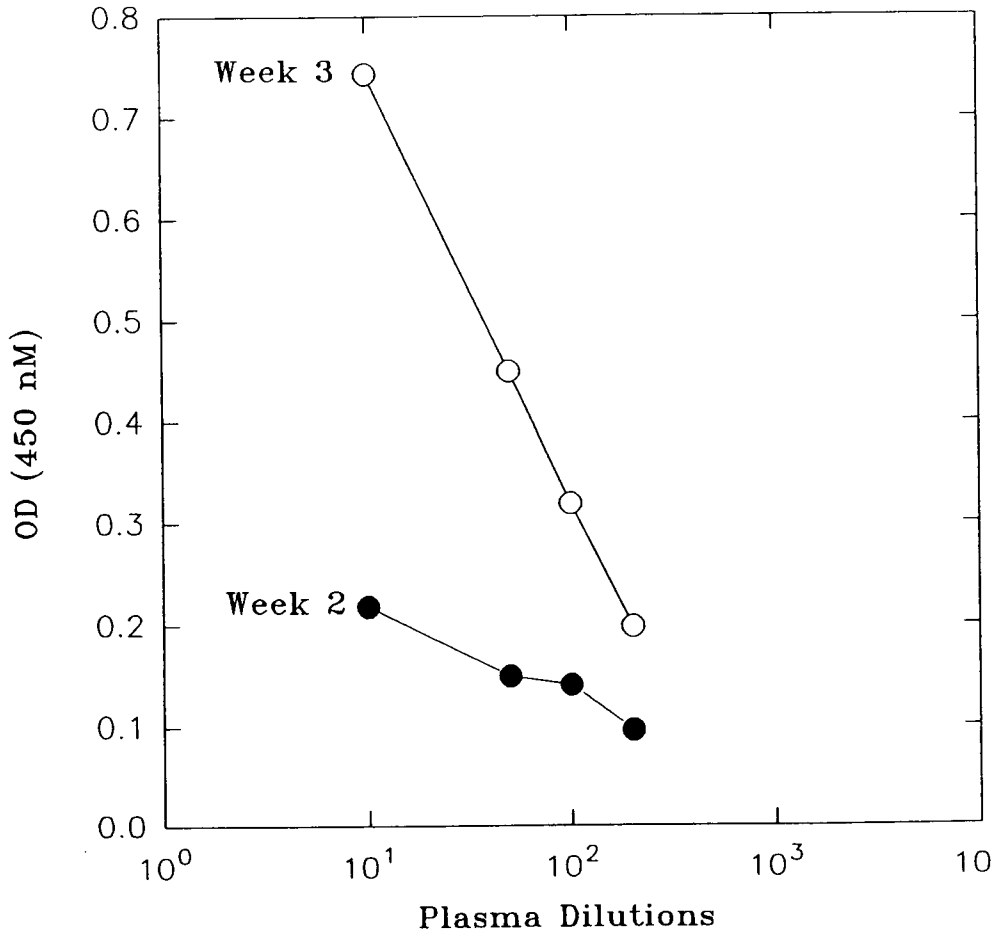

Figure IV.4. Representative antibody dilution curves for week 2 and 3 antibody titer samples.

restraint stress had a decreased antibody proliferative response (Brown-Borg *et al.*, 1993). Another study has shown not only that stress reduced antibody proliferation, but also that thymulin treatment was able to restore antibody response under stressed conditions in mice (Okamoto *et al.*, 1993). However, in view of the large variation in antibody titer (SEM = 46.7-183.2% of mean for week 2; 20.1-35.0% of mean for week 3) 10 animals would be insufficient to detect a treatment effect if one does exist.

The intra-assay CV was determined to be 6.07% at the mid-range dilution value and 6.99% at the highest dilution value. The total assay variability was calculated to be 105.1%, indicating that there is tremendous variability between animals. The non-KLH immunized animals had an average antibody titer value of 0.199, which was 83.8% of the overall mean titer values. This would indicate a relatively high non-specific binding component of the assay.

Effects of Thymulin and Stress on Cortisol Concentrations

Following the first day of the stress treatment, the stress control group had a two-fold higher cortisol concentration than that of the non-stress control group. This effect was apparent on both days of the stress application ($p < 0.0002$). Interestingly, thymulin treatment at both of the experimental doses inhibited the stress-induced increase in cortisol levels to concentrations which

were not significantly different than the non-stress control group (Table IV.4). This also occurred on both d 1 and d 2 of the stress application (p-value < 0.0001 and p-value < 0.047, respectively).

Another interesting comparison is that of the stress control group between the two days of stress. In the stress control group the plasma cortisol levels were lower on d 2, as compared to d 1 (p-value < 0.034).

The cortisol concentrations obtained here are comparable to those in other recent studies conducted with sheep. Apple *et al.* (1993) established that restraint and isolation stress did, in fact, elevate plasma cortisol concentrations, and further, that normal plasma cortisol levels were between 12 and 25 ng/ml, with the higher end of this range being a result of any stress associated with the handling of the sheep. Other papers that incorporated an acclimation period after handling the animals have independently shown slightly different sets of resting mean cortisol levels (these values ranged from 4-8 ng/ml (Cataldi *et al.*, 1994; Hashizume *et al.*, 1994) to 8-12 ng/ml (Thompson *et al.*, 1995; Kent *et al.*, 1993)).

Buckingham *et al* (1992) reported no effect of thymulin treatment on cortisol concentrations, however the rats were not subjected to a stressing procedure. The animals were simply injected with thymulin and the cortisol concentrations were then measured. Therefore, that study failed to address the central question of the current study,

Table IV.4 Mean cortisol concentrations following each of the two days of shipping stress. Cortisol levels were measured to determine if the lambs perceived the shipment as stressful.

Treatment	Number of Animals	Cortisol Concentration (ng/ml)	
		Day 1[++] Mean ± SEM[@]	Day 2[#] Mean ± SEM
Stress Control	10	43.5 ± 3.2	33.8 ± 2.8
Non-Stress Control	10	17.6 ± 2.9	24.4 ± 4.4
1ng Thymulin/Kg BW	10	15.5 ± 2.7	17.9 ± 3.3
10ng Thymulin/Kg BW	10	16.9 ± 2.7	19.5 ± 3.1

[++] $P < 0.0001$
[#] $P < 0.047$

[@] Standard error of the mean is based on the model assumption of equal variance.

BW = Body Weight of the animals at the time of shipment.

whether thymulin modifies an organism's neuroendocrine response to stress.

There are several possibilities as to why the results that were observed here on the interaction between thymulin and plasma cortisol could have been obtained. One of the most plausible explanations is that thymulin, in addition to acting directly on the T cell developmental pathway, acts directly on the HPA axis to decrease cortisol secretion under stressful stimuli (Figure IV.5). It seems logical to assume that some feedback mechanism exists to prevent overstimulation of the HPA axis by stress, given other known mechanisms for preventing the immune system from overshooting in times of acute stressors (like the classic negative feedback of glucocorticoids on the HPA axis) (Munck *et al.*, 1984). This would enable the body to distinguish between acute and chronic stressors, which would ultimately help the organism to respond appropriately to the type of stimuli it is exposed to, without allowing a greater (and potentially harmful) suppression of the immune system.

It also seems logical to postulate that the stress control group may have had lower cortisol levels on d 2 because of their perception of the stressing procedure (i.e. they may not have perceived the second shipping period as as stressful as the first).

The results obtained from the cortisol assay were completely unexpected, based on previous studies reported in the literature. To date, there have been no reports that

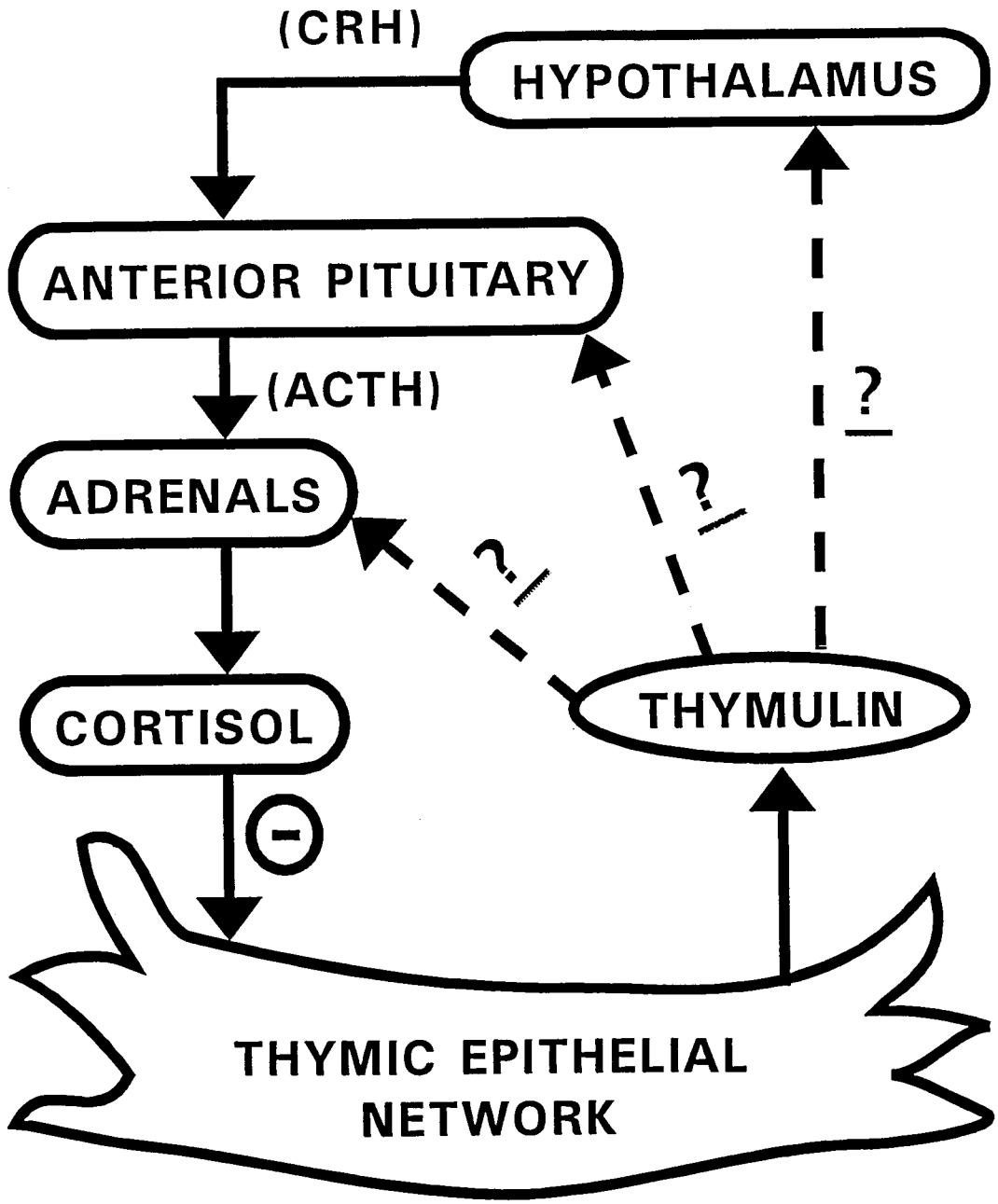

Figure IV.5. The diagrammatic depiction of the HPA axis and its potential interactions with thymulin. The ? represents potential, hypothesized sites of thymulin interaction with the HPA axis; the ⊖ represents an immunosuppressive effect.

the author is aware of on the effects of thymulin treatment on cortisol concentrations under stressful conditions. The current study's results suggest a number of questions: 1) at what point of the HPA axis is thymulin acting (the hypothalamus, pituitary or adrenal); 2) what is the dose range in which thymulin is effective in causing such a response in sheep; 3) does this response in plasma cortisol have an effect on the cell-mediated immune system; 4) can these results be obtained in other species, such as cattle; and, finally 5) can these results be effectively implemented in the sheep and cattle industry to help reduce the incidence of shipping fever?

IMPLICATIONS

This study demonstrated that thymulin treatment in stressed sheep has the ability to reduce stress-induced plasma cortisol to levels not different than unstressed animals. Thus, the negative impacts of elevated cortisol concentrations on the immune system should be virtually eliminated with this treatment. These results also implicate thymulin as a major player in the normal maintenance of the HPA axis and the immune system *in vivo*. Furthermore, thymulin may prove to be an invaluable tool in alleviating the stress-induced immunosuppressive effects now apparent in most domestic animals, fisheries and wildlife species, and perhaps even humans.

CHAPTER IV

SUMMARY

> The future is not a result of choices among alternative paths offered by the present, but a place that is created- created first in the mind and will, created next by activity. The future is not some place we are going to, but one we are creating. The paths are not to be found, but made, and the activity of making them changes both the maker and the destination.
>
> -John Schaar

The interactions that occur in the thymus are very complicated and almost impossible to experimentally isolate, but it is apparent that a few key components must be considered more thoroughly than others in an initial attempt to gain an understanding of this system.

There exists an abundance of information in the literature to lead one to the conclusion that the major control mechanism of T-cell development is found in the thymus, and specifically in the interactions with thymic hormones, like thymulin. There is uncertainty with regard to the exact mechanisms by which this peptide exerts its actions. It is fair, however, to conclude that thymulin is perhaps one of the major ways in which to increase the immune response after the immune system has been compromised, either by age-onset decline or external stimuli (like stress). Numerous other factors need to be taken into account when the final analysis is complete, like the

actions of glucocorticoids, various immune hormones and cytokines on the thymus gland, and whether or not these actions of thymulin are strictly intra-thymic or also endocrine in nature.

This study revealed a hint of one of the possible actions of thymulin under stressful stimuli. It would appear that the peptide is able to decrease cortisol secretion, or more generally, thymulin is perhaps a major player in the normal maintenance of the HPA axis and the immune system *in vivo*. How this regulation/maintenance occurs needs further refinement, but this experiment may lend the critical link needed to gain a more holistic understanding of how the HPA axis is controlled during periods of physical and/or psychological stress.

Based on the review of some of the literature currently available on thymulin and this study, it is the opinion of the author that much more research emphasis needs to be placed on the exact interactions of the peptide *in vivo*. It would appear to have some very major implications as a key player in the normal maintenance of immunocompetence in all mammals from farm animals to humans.

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