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The first experiment was conducted to investigate the modulatory effect of ovine growth hormone (oGH), ovine prolactin (oPRL), and recombinant bovine insulin-like growth factor-I (rbIGF-I) on resting (primary cell culture in absence of mitogens) and recombinant bovine interleukin-2 (rbIL-2)-activated peripheral blood mononuclear (PBMN) cells from non-pregnant female sheep. PBMN cells were treated with oGH, oPRL, and rbIGF-I at various concentrations. Ovine GH and oPRL at 100 ng/mL stimulated PBMN cell proliferation ($p < 0.05$), as measured by their uptake of [^{3}H]-thymidine. However, when oGH was combined with rbIL-2 (500 units/well in tissue culture plate), the proliferative response occurred at the higher dose (2 μg/mL). Ovine PRL did not have a proliferative effect on rbIL-2-activated PBMN cells ($p = 0.07$). Insulin-like growth factor-I had no effect on proliferation of resting or rbIL-2-activated ($p > 0.05$) PBMN cells.

The second experiment was carried out on lamb PBMN cells to assess the development of growth hormone (GH) receptor from birth to 5 months. Growth hormone-binding protein (GHBP) was also measured in plasma from the same animals in which GH receptors were measured.

Specific binding of ^{125}I-hGH to the GH receptor on PBMN cells increased from 0.3% at d 7 to 0.8 $\pm$ 0.1% at d 150 ($p < 0.03$). Plasma GHBP concentrations increased ($p < 0.001$) from d 30 to d 150.

The third experiment was carried out to develop an assay to measure soluble interleukin-2 (sIL-2) receptor in serum from ruminant animals using recombinant bovine interleukin-2 (rbIL-2) and to assess sIL-2 receptor activity in serum from calves treated with selenium (Se), vitamin E and Se + vitamin E. Serum sIL-2 receptor activity also was measured in cows with a positive antibody titer against bovine viral diarrhea (BVD) and infectious bovine rhinotracheitis (IBR) viral diseases. Selenium, vitamin E, and Se + vitamin E had no effect on serum sIL-2 receptor binding activity. However, the occurrence of scours (animal diarrhea) was associated with an increase ($p < 0.01$) in serum sIL-2 receptor activity from 1.8 $\pm$ 0.1 percent specific activity (%SB) in scours-free to 2.5 $\pm$ 0.1 %SB for scours animals. Serum sIL-2 receptor activity was 5.2 $\pm$ 0.8 %SB in infected cows with BVD virus and IBR, which was higher ($p < 0.01$) than that in non-infected cows (3.0 $\pm$ 0.2). Therefore, the serum levels of sIL-2 receptor are elevated in response to several specific infectious diseases, including viral and bacterial infections. These data suggest that serum sIL-2 receptor may serve as a marker for the immune status of an animal to viral and bacterial infections.

According to these studies, lamb PBMN cells carry GH and PRL receptors. Growth hormone and PRL can modulate the proliferation of PBMN cells *in vitro*. Serum sIL-2 receptor might be used to determine the status of the immune system in response to specific diseases.

Interaction Between the Immune and Endocrine Systems in Ruminant Animals

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DEDICATION

This Doctoral Dissertation is dedicated to my wife, Farangis and my daughters, Parisa, Anahitta, and Neelufar.

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CHAPTER I
INTRODUCTION

In order to bring the self-regulated immune system into conformity with other body systems its functioning within the context of an immune-neuroendocrine network is proposed (Besedovsky and Sorkin, 1977). This hypothesis is based on the existence of afferent pathways between immune and neuroendocrine structures. A new interdisciplinary research area has recently emerged that involves the study of interactions between the immune and endocrine systems. Numerous bidirectional interactions between these two systems have been described (Kao *et al.*, 1992). The first point that bears emphasizing is the remarkable near-parallel development of the immune and endocrine systems in various mammalian species during early ontogeny. This can hardly be fortuitous. Species such as mice and rats have, at birth, only marginal levels of immunoglobulins and their immune responses are minimally effective during the first days of extrauterine life. Many endocrine systems are also underdeveloped at this time (Solomon, 1971), notably sexual differentiation, functioning of the hypophysis, thyroid and adrenal glands (Jost *et al.*, 1973).

Among the newly recognized interactions between the immune and endocrine systems is the observation of an increasing number of peptides and proteins that are used in both systems as intercellular messengers (Blalock, 1992). Adrenocorti-cotropic hormone (ACTH) was the first hormone that was found to be synthesized in

the immune system by lymphocyte (Smith and Blalock, 1981). A number of other hormones, including growth hormone (GH) (Baxter *et al.*, 1991a), prolactin (PRL) (Gala and Shevach, 1994) and insulin-like growth factor (IGF-I) (Baxter *et al.*, 1991b) have also been shown to be synthesized by or associated with cells of the immune system. Since these hormones can also function as immunomodulators, it is proposed that cells in the immune system are able to produce GH and PRL-like hormones, which can in turn have autocrine or paracrine effects on the immunocytes themselves. Since 1959 GH has been proposed to be an important immunoregulatory molecule (Shrewsbury and Reinhardt, 1959). Confirmation of this suggestion has occurred through a number of studies both *in vitro* and *in vivo* (Kelley, 1990).

The effects of GH on lymphocyte functional responses are mediated through specific cell surface receptors which have been identified on both thymocytes and lymphocytes (Blalock, 1992). Supposedly, PRL also exerts its immunoregulatory effect through a cell surface receptor on immune cells (Viselli and Mastro, 1993).

Activation of the immune system, usually initiated by a viral or bacterial challenge, brings about a multitude of cellular events leading to the maturation and proliferation of lymphocytes (Reinherz and Schlossman, 1980). Interleukin-2 (IL-2) was first described as a factor in supernatants of activated lymphocyte cultures capable of stimulating the growth and proliferation of human T-cells in culture (Morgan *et al.*, 1976). The receptor for IL-2 has been identified on the surface of T-cells and its increased expression has been shown to occur in response to IL-2

release shortly after antigen binding (Smith, 1988). Rubin *et al.* reported in 1985 that in addition to *de novo* expression of IL-2 receptor on the surface of activated human peripheral T-cells, a released form of soluble IL-2 receptor (sIL-2) could be detected in culture supernatants of activated T-cells.

Therefore, the objective of these studies was to determine if, in the ruminant animal, such interactions also exist. Specifically the studies were conducted to:

1. Measure specific GH binding on PBMN cells in lambs at different ages.
2. Determine binding affinity (K_a) and binding capacity (B max) of these GH binding sites.
3. Characterize specificity of the GH binding.
4. Purify and characterize the GH receptor from PBMN cells membrane.
5. Measure plasma growth hormone binding protein (GHBP) concentrations in growing lambs.
6. Examine the responsiveness of PBMN cells to GH, PRL, IGF-I and glucocorticoid (GC) with and without IL-2.
7. Develop an assay to measure sIL-2 receptor in serum.
8. Measure specific binding of sIL-2 receptor in plasma in growing lambs.
9. Measure serum sIL-2 receptor activity in calves treated with selenium (Se), vitamin E, and Se + vitamin E.
10. Measure serum sIL-2 receptor activity in diseased and non-diseased cows.

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CHAPTER II
REVIEW OF LITERATURE

Cellular Components of the Immune System

The immune system, like the endocrine system, is composed of a number of specialized cell types that have unique functions and particular sets of chemical messengers. The immune system may be regarded as consisting of lymphocytes, macrophages, a series of macrophage-related cells including the dendritic cells of the spleen and the specialized epithelial cells, such as those found in the thymus (Paul, 1984). Lymphocytes differ from one another not only in the specificity of their receptors but also in their functional properties. Two broad classes (or lineages) of lymphocytes are recognized: the T lymphocytes, thymus dependent lymphocytes, and B lymphocytes which are precursors of antibody-secreting cells.

T lymphocytes

T lymphocytes represent one of two major classes of lymphocytes and derive their name from the fact that they and their precursors spend time in the thymus (Nikolic-Zugic, 1991). On all T cells are clusters of differentiation (CD) markers which are cell surface glycoproteins expressed on subsets of thymocytes and mature T lymphocytes (Parnes, 1989). The two main functions of T cells are destruction of host cells with altered (or nonself) surfaces and regulation of immune reactions.

The latter function can occur either by direct interaction with other cells or indirectly through the production and release of soluble mediators called cytokines.

B lymphocytes

The second major class of lymphocytes is termed B-cells, and the name refers to the bursa of Fabricius, which is present in birds and is a lymphoepithelial organ. The primary site for B cell differentiation in mammals is most probably the fetal liver and, following birth, the bone marrow serves in mammals a similar function to the Bursa in birds (Kincade, 1981). The primary marker for B lymphocytes is the cytoplasmic or cell surface expression of immunoglobulins (Ig).

Natural killer cells

A novel cytotoxic activity was described in the lymphoid tissue of rats and mice against a wide spectrum of tumor target cells and the activity was present even before immunization of the animals, it was called natural killer (NK) cells (Quan, *et al.* 1982). Natural killer cells are large granular lymphocytes that lack the major surface markers that are generally associated with T and B lymphocytes. The principal functions of NK cells are the killing of virus-infected and tumor cells in the absence of prior sensitization. The predominant lymphokine that is produced by these cells is interferon alpha (IFN-α).

Endocrinology of Growth Hormone and Prolactin

Growth hormone

The growth-promoting principle of the pituitary gland was isolated in the form of GH in 1944 (Li and Evans, 1944). Growth hormone, also known as somatotropin, is a 191-amino acid single chain protein with a serum half-life of 20 to 25 min. Bovine GH is synthesized as a prehormone with a 26-amino acid hydrophobic signal sequence (Lingappa *et al.*, 1977). Since the signal peptide is cotranslationally cleaved, the prehormone is extremely short-lived. It is secreted by the anterior pituitary gland in all mammals and most vertebrates, and the principal site of degradation of GH is the kidney, where GH is filtered by the glomerulus and subsequently taken up nonspecifically and lysosomally degraded by proximal tubular cells (Johnson and Maack, 1977). Its role in the promotion of growth, coupled with changes in the metabolism of proteins, carbohydrates and lipids, is well documented (Paladini *et al.*, 1983). Although GH secretion is maximal at puberty, it declines after about 30 years of age in humans (Rudman *et al.*, 1990) and low but detectable levels are found throughout the remainder of adulthood. This suggests that this GH may have other functions in addition to promotion of growth.

Growth hormone receptor

In the rabbit liver the GH receptor is a 130-kDa protein. It consists of 620 amino acids divided into a 246-amino acid extracellular domain, a single transmembrane domain of 24 amino acids, and an intracellular domain of about 350 amino acids (Leung *et al.*, 1987). The extracellular domain possesses several potential sites of N-linked and O-linked glycosylation, as in most membrane bound receptors. The difference between the apparent molecular size (130 kDa) and the predicted size by a composition (70 kDa) is thought to be due to a high level of glycosylation and covalent association of the receptor with ubiquitin. A complementary DNA (cDNA) for the bovine GH receptor has been cloned out of a cDNA library prepared from the liver of a Holstein heifer. The cDNA hybridized to a single 4.5 kb mRNA species and shares a high degree of sequence homology with GH receptors cloned from other species (Hauser *et al.*, 1990). The extracellular portion of the hGH receptor is structurally related to the extracellular domain of the prolactin receptor (Boutin *et al.*, 1988) and broadly related to at least eight other members of this receptor superfamily (Cosman *et al.*, 1990; Bazan, 1990).

Specific receptor sites have been shown for human GH in a line of cultured human lymphocytes (4265) which was initiated in 1965 from peripheral blood cells of a patient with chronic myelogenous leukemia (Lesniak *et al.*, 1973). The binding of ^{125}I-hGH to these cells was rapid, saturable, and reversible. Another lymphocyte

cell line (IM-9), initiated in 1967 from bone marrow cells of a patient with chronic myelogenous leukemia demonstrated greater binding per cell (4000 binding sites/cell) and greater sensitivity to low physiological concentrations (1.3×10^{9} M^{-1}) of GH (Lesniak *et al.*, 1974).

Eshet *et al.* (1975) have reported that the ^{125}I-hGH bound by fresh circulating human lymphocytes is displaced only by hGH. Keiss and Butenandt (1985) have reported specific binding of ^{125}I-hGH was rapid, reversible and time and temperature dependent. Scatchard analysis (Scatchard, 1949) of ^{125}I-hGH binding to PBMN revealed a high affinity receptor with a K_a of 1.5×10^{-9} M and a maximal binding capacity (B max) of 7.1×10^{-11} M.

Distribution of growth hormone receptor

Specific GH binding in various tissues have been demonstrated in different species (Davis, 1988; Hughes *et al.*, 1985).

Table II. 1. Distribution of GH receptors

Species	Tissue
Liver	Human (Carr and Friesen, 1976)
	Mouse (Smith and Talamantes, 1987)
	Rabbit (Leung *et al.*, 1987)
	Rat (Baxter, 1985)
	sheep (Chan *et al.*, 1978)
Adipose	Sheep (Chan *et al.*, 1978)
	Rat (Roupas and Herington, 1986)
Thymocytes	Cow (Arrenbrecht, 1974)
	Mouse (Arrenbrecht, 1974)
Lymphocyte	Human (Ashkenazi *et al.*, 1987)
Ovary	Sheep (Chan *et al.*, 1978)
Muscle	Rat (Satyanarayana *et al.*, 1988)
Chondrocyte	Rat (Eden *et al.*, 1983)

Growth hormone receptor regulation

The responsiveness of a target tissue to a hormone is modulated not only by the level of circulating hormone but also by the availability of receptors with which

the ligand can interact. For this reason the regulation of receptor number and affinity has been studied extensively in many hormonal systems. The initial studies of GH receptor regulation examine the end result of regulation (i.e., changes in binding affinity or capacity) rather than the mechanism by which levels of receptors are controlled (Hughes *et al.*, 1985). It is often assumed that an increase in binding activity is caused by an increased rate of synthesis of the receptors while a decrease in binding represents a "turning off" of the machinery involved in *de novo* synthesis. However, regulation of GH receptors may involve additional factors, including the activation/inactivation of pre-existing cryptic receptors that can be switched from a nonbinding to a binding state by an appropriate trigger mechanism (Hughes *et al.*, 1985). Growth hormone receptor exists in a dynamic state of flux and regulation. Because GH receptor appears to be essential to the molecular mechanism of GH action, modulation of these proteins could significantly alter biological responses to GH. Growth hormone receptor appears to be up- or down-regulated by several factors. For example, Kelly *et al.* (1974) have reported that GH binding sites on liver membranes were higher in female rats compared to male controls of all age groups ranging from 10 days to adult. While hepatic GH binding in female rats increased with age, the GH binding in male animals remained almost constant. Posner *et al.* (1980) have demonstrated that hypophysectomy of lambs resulted in a marked reduction, though not a complete disappearance, of their hepatic GH receptors.

Growth hormone-binding protein

The existence of carrier proteins for polypeptide hormones was considered in the early 1960s, shortly after radiolabeled hormones became available for radioimmunoassay (RIA). In 1964, Hadden and Prout reported that GH in plasma was protein bound. Growth hormone binding protein (GHBP) in human plasma is a single-chain glycoprotein with an estimated molecular weight of 50-60 kDa. It binds hGH with high specificity and high affinity (Baumann, 1986, 1990). A serum GHBP has been reported from several other species including sheep, pig, and chicken (Davis *et al.*, 1992), human (Daughaday *et al.*, 1987), rabbit (Ymer and Herington, 1985), mouse (Smith and Talamants, 1988), and rat (Amit *et al.*, 1990). It has been shown that the rabbit and human serum GHBP is related to and possibly derived from the hepatic GH receptor (Leung *et al.*, 1987; Spencer *et al.*, 1988). Characterization of this binding protein shows that it corresponds to the extracellular domain of the GH receptor, and it has been proposed that the GHBP may be produced by proteolytic cleavage of the membrane-bound GH receptor (Spencer *et al.*, 1988), at least in man (Trivedi and Daughaday, 1988). In both rat and mouse, the evidence available suggests that the GH receptor and the GHBP arise independently from the same gene by alternative splicing of the precursor mRNA (Baumbach *et al.*, 1989). Specific mRNA transcripts with estimated sizes of 1.2 kb and 4.0 kb were found for GHBP and GH receptor respectively in mouse and rat (Baumbach *et al.*, 1989). In the human, GHBP is identical to the extracellular domain of the GH receptor but with a hydrophilic tail replacing the hydrophobic

transmembrane domain and the intracellular domain (Baumbach *et al.*, 1989). The physiological significance of the GHBP has been reported to be both prolongation of the half-life of hGH in the circulation (Baumann *et al.*, 1987) and interference *in vitro* with the binding of ^{125}I-hGH to different GH receptor preparations (Waters *et al.*, 1990).

The expression of GHBP increased with age up to puberty suggesting that it is developmentally regulated in a manner similar to GH receptor (Daughaday *et al.*, 1987). The GHBP mRNA was found in all extrahepatic tissues examined that contained GH receptor mRNA. The ratio between the 1.2 kb and 4.0 kb transcripts varied between tissues indicating that GH receptor and GHBP transcripts may be separately regulated.

Signalling mechanism of growth hormone

Although the ability of GH to promote growth and regulate lipid, protein, and carbohydrate metabolism has been known for many years (Davidson, 1987), the molecular mechanism by which GH binding to its receptor elicits its diverse responses has remained an enigma. It has been shown that GH stimulates the expression of c-fos gene (Doglio *et al.*, 1989), an early marker of cell differentiation, and the action of protein kinase C might be involved (Smal and Meyts, 1987). Accumulation of diacylglycerol (DAG) following GH stimulation has been reported in different cell lines (Catalioto *et al.*, 1990). Preliminary data suggest that this DAG may be produced from breakdown of phosphatidyl choline or

other cellular glycerophospholipids, by a mechanism (G-protein) coupling a phospholipase C to the GH receptor (Rogers and Hammermann, 1989).

Additional mechanisms of GH action have also been postulated. For example, GH has been reported to promote the phosphorylation of its receptor on tyrosyl residues in different cell lines (Stred *et al.*, 1990). Although GH receptor lacks homology to receptors with tyrosine kinase activity (Foster *et al.*, 1988), it has been shown that GH added to murine 3T3-F442A fibroblasts promoted the *in vivo* phosphorylation of GH receptors on tyrosyl residues (Stred *et al.*, 1993). Argetsinger *et al.* (1993) have recently identified a protein kinase termed JAK2 "Just Another Kinase", which is a nonreceptor tyrosine kinase with a molecular weight of 130 kDa, as a GH receptor-associated tyrosine kinase. Immunological approaches (immunoprecipitation) have been used to establish GH-dependent complex formation between JAK2 and GH receptor. These data are still fragmentary and do not provide a clear understanding of how the hormonal signal is conveyed inside the cell.

Prolactin

Prolactin is a polypeptide hormone of pituitary origin with close structural relationships to GH (Hwang *et al.*, 1972). Prolactin consists of 199 amino acids in a single peptide chain, and has three intramolecular disulfide bonds .

Prolactin receptor

The PRL receptor in liver is a single transmembrane polypeptide with 210 amino acids on the extracellular domain but only 58 amino acids in the cytoplasm (Boutin *et al.*, 1988). The sequence of the mature PRL receptor, deduced from the cDNA, contains 291 amino acids, with an extracellular region of 210 amino acids, single transmembrane region of 24 amino acids, and a relatively short cytoplasmic domain of 57 amino acids (Boutin *et al.*, 1988).

Complementary DNA clones encoding both long and short forms of the prolactin receptor have been described (Boutin *et al.*, 1988). In all the species studied to date, the prolactin receptor is closely related to the GH receptor, with 25-35% overall homology in their predicted amino acid sequences, especially in the extracellular, hormone-binding domain (Boutin *et al.*, 1988). Comparison of the cDNA sequences of the GH receptors and of the related PRL receptor suggests that they are members of a new family of transmembrane receptors that include the receptors for erythropoietin, IL-2, IL-6 and granulocyte- macrophage colony-stimulating factor (Cosman *et al.*, 1990). The major region of homology between all of these receptors resides in the extracellular domain, whereas the intracytoplasmic domain shows a high degree of heterogeneity, suggesting different signal transduction pathways.

Distribution of prolactin receptor

Specific PRL binding sites in various tissues have been demonstrated in different species and are widely distributed in mammalian tissue (Hughes *et al.*, 1985).

Table II. 2. Distribution of PRL receptors

Species	Tissue
Mammary gland	Mouse (Frantz and Turkington, 1972)
	Rat (Costlow *et al.*, 1974)
	Rabbit (Shiu *et al.*, 1973)
Liver	Mouse (Frantz *et al.*, 1974)
	Rat (Herington and Veith, 1977)
	Pituitary cells Rat (Frantz *et al.*, 1975)
Ovary	Rat, Cow, (Saito and Saxena, 1975)
	Human (Saito and Saxena, 1975)
	Sheep (Posner *et al.*, 1974)
Splenocytes	Rat (Viselli and Mastro, 1993)
Nb2	Rat cell line (Shiu *et al.*, 1983)
Natural killer cells	Human (Matera *et al.*, 1988)
Lymphocytes	Human (Russell *et al.*, 1985)

Prolactin receptor and immune cells

Early reports using ligand-binding methods have indicated that the PRL receptor is present on peripheral blood lymphocytes and spleen and thymus cells (Russell *et al.*, 1984; Bellussi *et al.*, 1987) as well as on large granular lymphocytes (Matera *et al.*, 1988). Recently, using reverse transcription coupled to the polymerase chain reaction, PRL receptor gene expression has been reported for human T-cells, B-cells, and monocytes (Pellegrini *et al.*, 1992) and for normal mouse thymocytes and splenocytes (O'Neal *et al.*, 1991).

Hormone and the Immune System

Growth hormone

Evidence has accumulated indicating that GH not only regulates growth of body tissues but can also control important immune functions. Growth hormone modulates components of the immune system both *in vivo* and *in vitro*. Studies have shown that hypophysectomized rats were unable to exhibit an immune response (Berczi and Nagy, 1981). Treatment of hypophysectomized rats with GH or PRL actually restored immunocompetence, whereas other pituitary hormones had no effect (Berczi and Nagy, 1983). *In vitro* studies have shown that GH increases the proliferation rate of normal lymphoid cells (Davila *et al.*, 1987). These observations are consistent with reports that lymphocytes express specific receptors

for GH (Lesniak *et al.*, 1974; Kiess and Butenandt, 1985). Growth hormone is also responsible for modulating other immunologic activities such as augmenting cytolytic activity of T cells, antibody synthesis, GM-CSF-dependent granulocyte differentiation, TNF-α production, superoxide anion generation from peritoneal macrophages of hypophysectomized mice and NK activity (Kelley, 1990).

Weigent *et al.* (1988) and Hattori *et al.* (1990) have reported that nonstimulated rat lymphocytes produce immunoreactive GH (irGH) which is biologically active and similar in structure to pituitary-derived GH. Lymphocyte-derived irGH is secreted spontaneously in culture while pituitary GH is secreted under the control of growth hormone releasing hormone (GHRH). Additionally, lymphocyte-derived irGH can compete with iodinated pituitary GH for binding to rat lymphocyte GH receptors (Carr *et al.*, 1989). Rat Nb2 lymphoma cells, which are dependent on lactogens for growth, have been used extensively to assay human serum lactogen concentrations (Tanaka et al., 1980) and to study the mechanism of action of these hormones as mitogens.

The role of GH in lymphocyte proliferation has been studied by examining the effect of an antisense oligodeoxynucleotide complementary to GH mRNA in the rat (Weigent *et al.*, 1991). Antisense GH oligodeoxynucleotide treatment inhibits production of irGH by lymphocytes. Antisense GH oligodeoxynucleotide-mediated inhibition of irGH production resulted in a decrease in lymphocyte proliferation. Collectively, these data indicate that lymphocytes synthesize and secrete irGH and

that irGH produced by these cells can stimulate proliferation, suggesting that GH may play an autocrine/paracrine role in lymphocyte replication.

Prolactin

Numerous studies have indicated that PRL is involved in immune functions (see review by Gala, 1991). Hypophysectomized rats have impairments in both humoral and cellular immunity (Nagy and Berczi, 1978), and PRL administration restored these functions (Berczi and Nagy, 1991). Further, the administration of bromocriptine, which specifically blocks PRL secretion, resulted in suppressed immune function similar to that caused by hypophysectomy, and PRL administration corrected the function (Berczi *et al.*, 1981).

Mukherjee *et al.* (1990) have reported that PRL induced the IL-2 receptor on the rat splenic lymphocyte. A more consistent finding *in vitro* has been the blocking of lymphocyte proliferation in response to a number of stimulants and cytokines by anti-PRL antisera (Hartmann *et al.*, 1989: Clevenger *et al.*, 1990). Interleukin-2 stimulation induced the translocation of PRL into the nucleus and PRL receptor to the nuclear periphery. Clevenger *et al.* (1991) suggested that extracellular PRL is requisite for T cell proliferation and that the effects of PRL are exerted in the nucleus. A number of investigators have reported the presence of a PRL-like message and the secretion of PRL-like activity from murine splenocytes involved with immunity (Montgomery *et al.*, 1987). Increased expression of growth-related genes, such as ornithine decarboxylase and c-myc, has been shown to occur in cells

treated with PRL (Berczi and Nagy, 1991). Prolactin has been shown to be secreted from a human B lymphoblastoid cell line variant designated as IM-9-p3 (DiMattia *et al.*, 1988). PRL synthesized by these cells was indistinguishable from human pituitary PRL, however the mRNA species expressed by these cells differed from that of pituitary origin in that it was approximately 150 bases longer than expected. Further confirmation of the identity of this PRL is demonstrated by the fact that the immunoaffinity-purified lymphocyte PRL is of the same molecular weight as human pituitary PRL (DiMattia *et al.*, 1988). A 46-kDa PRL-like molecule has been shown to be secreted by mitogen-driven rodent lymphocytes as compared to the 25-kDa pituitary PRL (Montgomery *et al.*, 1987). These findings would suggest that PRL and/or PRL-like molecules play an important role in positive regulation of lymphocyte functions.

The pre-T cell rat Nb2 lymphoma cell line is almost completely dependent on lactogenic hormones for growth and contains receptors with high affinity for PRL (Shiu *et al.*, 1983). This cell line constitutes a useful system to measure the biological activity of PRL (at the picogram per mL level) and to analyze one of the mechanisms of PRL action, i.e. cell multiplication.

Insulin-like growth factor-I

The insulin-like growth factors comprise a family of peptides which promote proliferation and differentiation of various cell types (Froesh *et al.*, 1985). Insulin-like growth factor-I, a 70-amino acid peptide structurally related to insulin, is

normally considered to be a metabolic hormone which mediates many of the anabolic effects of GH. Insulin-like growth factor-I is predominantly synthesized in the liver, but it can also be produced by a variety of tissues where it can act as an autocrine or paracrine growth factor (D'Ercole *et al.*, 1984). Insulin-like growth factor-I is a single chain polypeptide containing three disulfide bonds with 7.5 kDa and with blood plasma concentration of 200 ng/mL in man (review, Humbel, 1990).

Insulin-like growth factor-I is a mitogenic polypeptide that has been implicated in the regulation of fetal growth (D'Ercole, 1987). The mitogenic effect is mediated by a specific IGF-I receptor which is a receptor kinase and which is homologous to the insulin receptor. *In vitro* effects of IGF-I are commonly classified into short-term insulin-like metabolic effects such as stimulation of glucose uptake and glycogen and lipid synthesis in adipose tissue, and long-term mitogenic effects such as stimulation of protein, RNA, and DNA synthesis and cell proliferation (Humbel, 1990).

A role for IGF-I in the immune system is suggested by the expression of IGF-I in human macrophages and a lymphoblastoid cell line (Merimee *et al.*, 1989). Furthermore IGF-I might be involved in lymphocyte proliferation as suggested by the effects of both growth factors on the in vitro proliferation of lectin-activated lymphocytes (Tapson *et al.*, 1988), and by the increased expression of its receptors on activated T cells.

Glucocorticoid

Steroid hormones are important modulators of eukaryotic gene expression. Generally, these hormones enhance gene expression by stimulating the synthesis of specific mRNA. For example, glucocorticoids (GC) such as dexamethasone stimulate the transcription of growth hormone (Spindler, 1982). Clinically, glucocorticoids are used as immunosuppressive and anti-inflammatory agents. Their immunosuppressive effects may in part be due to their catabolic functions (Arya, 1984). Glucocorticoids may inhibit the transcription of genes responsible for the immune functions of lymphocytes and may interfere with the synthesis or functions of IL-2, eventually resulting in a suppressed immune response (Gillis *et al.*, 1979). Glucocorticoids suppress the proliferation of human T lymphocytes. Gillis *et al.* (1979) have demonstrated that dexamethasone, a synthetic GC, inhibits the production of IL-2 by mitogen-activated lymphocytes.

Interleukin-2

Interleukin-2 is a glycoprotein with an apparent molecular mass of 15.5 kDa (Bazan, 1990). Interleukin-2, also known as T cell growth factor (TCGF), is an immunomodulatory factor produced by T-lymphocytes and has been isolated from a variety of cell cultures and recombinant systems (Smith, 1988). Interleukin-2 has been shown to promote long term growth of activated T cells (Morgan *et al.*, 1976),

activation and proliferation of natural killer (NK) cells, and induction of gamma interferon (gamma INF) and B cell growth factor secretion (Ortaldo *et al.*, 1984).

Interleukin-2 receptor

The receptor for IL-2 plays a crucial role in the regulation of the immune response (Smith, 1988). Binding of IL-2 to its receptor on the surface of T lymphocytes triggers a series of intracellular signaling events that result in the activation and proliferation of resting T cells and, ultimately, in the generation of helper, suppressor and cytotoxic T cells, which mediate immune reactions (Waldman, 1986).

There are three forms of the cell-membrane IL-2 receptor, each with a different affinity for IL-2 (Wang *et al.*, 1987; Sharon *et al.*, 1988). The low affinity receptor (dissociation constant, Kd, approximately 10 nM) is a 55-kDa polypeptide (p55), the intermediate affinity receptor (Kd $\sim$ 1 nM) is a 75-kDa polypeptide (p75), and the high affinity receptor (Kd $\sim$ 10 pM) is a noncovalent complex containing p55 and p75 (Robb *et al.*, 1984) on both T and B cells (Lowenthal *et al.*, 1987). It has been generally believed that the high-affinity receptor is responsible for transducing the IL-2 signal for cellular proliferation (Smith and Cantrell, 1985). In the presence of an antigen, T cells are induced to synthesize and secrete IL-2 as well as to synthesize cell-surface IL-2 receptor. Different affinity forms of IL-2 receptor are synthesized upon stimulation, leading to increased numbers of high affinity receptors on the cell surface. It appears that

binding of IL-2 to high affinity receptors is the trigger necessary to promote cell cycle progression (Smith and Cantrell, 1985). The p55 molecule of the IL-2 receptor does not seem capable of generating such a second signal. The newly described p75 molecule is an attractive candidate for being the signal-generating protein of this receptor complex.

Interleukin-2 receptors are members of the hematopoietic receptor superfamily, which also includes receptors for IL-3, IL-4, IL-6, IL-7, granulocyte macrophage colony stimulating factors (GM-CSF), PRL, GH and erythropoietin (Bazan, 1990). Weinberg *et al.* (1988) have demonstrated that the bovine and human IL-2 receptor coding sequences revealed 71% homology at the nucleotide level.

Serum-soluble form of IL-2 receptor

The generation of anti-IL-2 receptor monoclonal antibodies that recognize different epitopes of IL-2 receptor led to the detection of a truncated form of the receptor, which has an apparent molecular mass of approximately 45 kDa, and which is secreted by activated T cells (Rubin *et al.*, 1986). The secreted form of the IL-2 receptor has been identified as the IL-2 receptor lacking the transmembrane and intracytoplasmic tail (Shimizu *et al.*, 1986). This serum soluble form of IL-2 (sIL-2) receptor binds IL-2 with low affinity (Rubin *et al.*, 1986). The way the high affinity receptor may function is that the p55 molecule rapidly associates with IL-2, bringing it into close proximity to the p75 molecule which might undergo a

conformational change causing IL-2 to associate with faster kinetics (Tsudo *et al.*, 1987). The presence of low levels of sIL-2 receptor has been reported in the serum of mice (Osawa *et al.*, 1986), rats (Mouzaki *et al.*, 1987) and humans (Rubin *et al.*, 1986). The major difference between the soluble and cell-surface forms of low affinity IL-2 receptor is that the former comprises only the ligand-binding domain and lacks both transmembrane and cytoplasmic domains (Robb and Kutny, 1987). Serum soluble IL-2 receptor and p55 contain similar N- and O-linked sugar modifications and both bind IL-2 with the same low affinity (Jacques *et al.*, 1987). The sIL-2 receptor appears to be generated by proteolytic degradation of cell-surface p55, rather than by the posttranscriptional splicing mechanism (Robb and Greene, 1987). Considerable interest has developed in the use of serum sIL-2 receptor as a prognostic or diagnostic indicator of the immune status in an individual. The level of sIL-2 receptor in serum is considered as an estimator of T cell activation and has been reported to increase in a wide spectrum of diseases (Rubin *et al.*, 1990).

Nutritional Effects on the Immune System

The immune system, like all systems in the body, is dependent on the availability and utilization of nutrients for optimal maintenance and function of its components (Chandra, 1985).

The role of vitamin E in immune function

Vitamin E (dl-α-tocopherol) acts as an important antioxidant in cellular membranes; its main function is to protect the unsaturated bonds of cellular membrane phospholipids against free radical attack (Tappel, 1972).

Tengerdy *et al.* (1984) have reported that dietary supplementation with vitamin E leads to enhanced humoral immune responses and increases resistance to bacterial infection in mice and chickens. Tanaka *et al.* (1979) have found that dietary supplementation of vitamin E induces the enhancement of helper T cell activity in mice. Afzal *et al.* (1984) found in rams that dl-α-tocopherol acetate used as an adjuvant to Brucella ovis vaccine decreased overall infectivity against an experimentally induced infection, compared to a Brucella ovis-Freund's incomplete adjuvant preparation. This protective effect of vitamin E was not directly related to an increase in antibody titer but rather appeared to be mediated by effects on cellular and local immunity. It is of interest that injection of vitamin E without the vaccine also decreased infectivity, albeit less than the Brucella ovis-vitamin E adjuvant; a vitamin E injection was more effective than an oral supplement (Tengerdy *et al.*, 1983).

Vitamin E enhances antibody biosynthesis and cell mediated immunity *in vivo* (Meydani *et al.*, 1990). Gogu and Blumber (1993) have reported that vitamin E increases cellular growth and glycosylation in the murine cytotoxic T-cell line (CTLL), perhaps via increasing the expression of the IL-2 receptor. Vitamin E has been demonstrated to affect the immune response by enhancing antibody biosynthesis

and cell mediated immunity *in vivo* (Gogu, 1993). The effect of vitamin E on T cell differentiation in the thymus of F344 rats has been examined (Moriguchi *et al.*, 1993). The ratio of $CD4^+$ $CD8^-$ /$CD4^-$ $CD8^-$ T cells increased in the high vitamin E group and significantly decreased in the vitamin E-free group compared to that of the regular group. Production of IL-2 by thymocytes following stimulation with concanavalin A (ConA) for 48 h increased about threefold in the vitamin E group compared to the regular group (Moriguchi *et al.*, 1993). Conversely, thymocytes from rats fed the vitamin E-free diet showed a significant decrease in IL-2 production compared to the regular group.

Selenium

A number of minerals appear to influence the immune response. It is, however, difficult to dissociate the effect of one mineral from the other. Roy *et al.* (1993) have shown that dietary ($1x10^{-7}$ M) supplementation with selenium (Se,) results in a significant upregulation of the expression of both p55 and p75 subunits of the IL-2 receptor on the surface of ConA-stimulated lymphocytes from mice. Selenium deficiency is associated with a depression of humoral-immune responsiveness, with lower eventual titer of antibody. Mice fed selenium-deficient diets during gestation and lactation had reduced growth and lower IgM and IgG responses to antigens in plaque assays than did mice fed the same diets only after weaning (Mulhern *et al.*, 1985). The deficiency of Se is corrected with vitamin E,

suggesting close interactions between these two nutrients. T-cell activation is dependent on adequate amount of Se (Fischbach *et al.*, 1984).

Viral Diseases

Bovine viral diarrhea virus

Bovine viral diarrhea (BVD) virus has been associated with diarrhea and enteric problems (Radostits and Littlejohns, 1988), abortion (Kahrs, 1968), and immunosuppression (Markham and Ramnaraine, 1985). Signs of acute disease include diarrhea, ulceration of the mucosal surface of the mouth, esophagus, stomach and intestines, fever, depression, and leukopenia (Kahrs, 1971). When pregnant cows become infected with BVD virus up to day 125 of gestation, the calf may be aborted or become chronically infected (Radostits and Littlejohns, 1988).

Occasionally researchers have reported isolation of BVD virus from lungs of cattle with respiratory disease. Reggiardo (1979) stated that BVD virus was isolated from 21% of bovine lungs with pneumonia in cases of shipping fever.

Infectious bovine rhinotracheitis

The respiratory form of infectious bovine rhinotracheitis (IBR) is demonstrated by fever, anorexia, rapid breathing and clear to mucopurulent discharge (Rahrs, 1977). An alpha herpesvirus of the family Herpesviridae was

isolated which is now called bovine herpesvirus type 1 (BHV-1) or IBR. Incubation is 2-6 days, morbidity ranges from 10-100%, but mortality is low (0-10%). In field conditions, about 25% of BHV-1-infected cows abort 8 to 100 days (Rahers, 1977).

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CHAPTER III

EFFECT OF GROWTH HORMONE, PROLACTIN, INSULIN-LIKE GROWTH FACTOR-I AND GLUCOCORTICOID ON RESTING AND INTERLEUKIN-2-ACTIVATED PERIPHERAL BLOOD MONONUCLEAR CELLS IN SHEEP

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Abstract

This study examined the effect of growth hormone (GH), prolactin (PRL), insulin-like growth factor-I (IGF-I), and glucocorticoid (GC) on modulating the proliferative responsiveness of peripheral blood mononuclear (PBMN) cells in either the presence or the absence of recombinant bovine interleukin-2 (rbIL-2) stimulation. Blood samples were drawn from crossbred 2-year old sheep. Peripheral blood mononuclear cells were separated by Ficoll gradient centrifugation. The proliferation of PBMN cells were measured by counting the [^{3}H]-thymidine incorporation into PBMN cells cultured *in vitro*. Tritiated thymidine uptake by cells was both time and dose-dependent as it was measured by mitogen stimulation with concanavalin A (ConA), phytohemagglutinin-M (PHA), and rbIL-2. Ovine growth hormone (oGH) and ovine prolactin (oPRL) at 100 ng/mL had a stimulatory effect ($p < 0.05$) on PBMN cell proliferation in an *in vitro* assay. However, when oGH was combined with rbIL-2 (500 units/well), the proliferative response occurred at the higher dose of 1 or 2 μg/mL. Ovine PRL did no have a proliferative effect on rbIL-2-activated PBMN cells ($p= 0.07$). Treatment with rbIGF-I had no effect on PBMN cell proliferation, either of resting or rbIL-2-activated PBMN cells ($p > 0.3$ and 0.7 respectively). Dexamethasone (a synthetic glucocorticoid) had a suppressive effect with rbIL-2-activated cells at a dose as low as 63 ng/mL. These data indicate that functionally significant receptors for GH, PRL, and GC are present on PBMN cells in sheep.

Furthermore, the results suggest that cells which were stimulated by rbIL-2 had reduced numbers of activity of GH or PRL binding sites.

Key words: Growth hormone, prolactin, interleukin-2, and PBMN cells

Introduction

Numerous interactions between the endocrine and immune systems have been described in recent years. Both systems share a set of hormones and their respective target cell receptors, which can be used for intra and intersystem communication (Blalock, 1989). Growth hormone and PRL reportedly modulate immune function both *in vivo* and *in vitro*. The injection of GH into hypophysectomized animals enhanced the antibody response to antigen and accelerated skin graft rejection (Nagy *et al.*, 1983). *In vitro* studies have shown that GH increases the proliferation rate of normal lymphoid cells in rodents (Davila *et al.*, 1987). Weigent *et al.* (1988) have reported that nonstimulated rat lymphocytes produce immunoreactive GH (irGH) which is biologically active and similar in structure to pituitary-derived GH. Growth hormone produced by lymphocytes is similar to pituitary GH in terms of bioactivity, antigenicity, and molecular weight (Hattori *et al.*, 1990). Weigent *et al.* (1991) have reported that an antisense GH oligodeoxynucleotide treatment inhibits production of irGH by lymphocytes in rat. Antisense GH oligodeoxynucleotide-mediated inhibition of irGH production and it resulted in a decrease in lymphocyte proliferation.

Prolactin is involved with a variety of physiological effects on growth and reproduction in male and female vertebrates of several species (Nicoll, 1974) and also plays a role in the regulation of the humoral and cellular immune system (Berczi and Nagy, 1986). Prolactin can induce interleukin-2 (IL-2) receptor

expression in splenocytes from ovariectomized (OVX) female rats (Viselli *et al.*, 1991). Prolactin potentiates proliferative responses of T-cells to suboptimal doses of the T-cell mitogen ConA (Spangelo *et al.*, 1985). Prolactin receptors have been detected on human B- and T-lymphocytes (Russell *et al.*, 1985) and on cells from sheep lymphoid tissues.

A role for IGF-I in the immune system is suggested by the expression of IGF-I receptors in human macrophages and a lymphoblastoid cell line (Merimee *et al.*, 1989). Furthermore IGF-I might be involved in lymphocyte proliferation as suggested by its effect on the *in vitro* proliferation of lectin-activated lymphocytes (Tapson *et al.*, 1988) and also by its inhibitory effect on lymphocyte proliferation as reported by Hunt and Eardley, 1986.

Corticosteroids are thought to be "lympholytic" *in vivo* because their administration is followed by lymphopenia and decrease in size of lymphoid organs, particularly lymph nodes and thymus (Claman *et al.*, 1971). Corticosteroids are also immunosuppressive, and this property is felt to be correlated with their lympholytic activity. The immunosuppressive effects of GCs are thought to be mediated by intracellular GC receptors (Wiegers, 1993).

The objective of this experiment was to:

1. Assess the mitogenic effect of ConA, PHA, and IL-2 on sheep PBMN cells *in vitro*.
2. Determine the effects of oGH, oPRL, and IGF-I, on resting primary PBMN cells in culture.

3. Determine the effects of oGH, oPRL, IGF-I, and GC on sheep PBMN cells activated *in vitro* by IL-2.

Materials and Methods

Chemicals

Ovine prolactin (oPRL), ovine growth hormone (oGH), and human growth hormone (hGH) were gifts from the National Hormone and Pituitary Program[1]. Fetal bovine serum (FBS) and RPMI medium 1640 were obtained from Gibco Laboratories[2]. Tritiated thymidine (^{3}H-thymidine) was purchased from Amersham[3]. Ficoll-Histopaque, with a density of 1.077 μg/mL, and dexamethasone, were obtained from Sigma[4]. Recombinant bovine IGF-I (rbIGF-I) was obtained from the Monsanto Company[5].

Isolation of PBMN cells

Heparinized blood samples were drawn from 2 year old sheep via jugular venipuncture in vacutainer tubes and then the tubes were placed on ice while being

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[1] National Hormone and Pituitary Program, NIDDK, USDA, Rockville, MD 20850. OGH, NIDDK-oGH-15, oPRL, NIDDK-oPRL-20, and human GH, NIDDK-hGH-I-3.

[2] Gibco, Grand Island, New York.

[3] Amersham, PRN .1004, Arlington Heights, IL.

[4] Sigma Chemical, St. Louis, MO 63178

[5] Monsanto Company, St. Louis, MO 63038

transported to the laboratory. The PBMN cells from whole blood were isolated using a density gradient centrifugation method (Boyum, 1968) using Ficoll Histopaque at 1000 x g for 30 minutes at room temperature (RT) and then resuspended in complete RPMI 1640 with L-glutamine containing 25 mM HEPES buffer, 25 mM sodium bicarbonate, and gentamicin[4] (50 μg/mL). Cultures initiated on the same day as that blood samples were collected. Freshly isolated sheep PBMN cells from six animals were examined for their capacity to respond to oGH, oPRL, rbIGF-I, and dexamethasone. The PBMN cells were counted using a hemocytometer chamber. Cell viability was determined by exclusion of 1% trypan blue, and it exceeded 95% in all preparations.

Proliferation assay

To assess immune function, mitogen-induced proliferation of the lymphocytes was determined. The PBMN cells (0.5 x10^6) were cultured in triplicate in 200 μL volumes in culture medium containing RPMI 1640 supplemented with 5% heat inactivated (45 min at 56^o C) fetal bovine serum[2] (FBS) in 96-well tissue culture plates[6]. Various concentrations of different mitogens were incubated with isolated PBMN cells. Then the tissue culture plates were incubated in a 37^o C, 5.0% CO_2 environment for 72 h. To measure DNA synthesis as an indicator of cell replication, 1 μCi [^{3}H]-thymidine was added to each well after 72 h, and

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[6] Becton Dickinson

incubated overnight (16 h), and then cells were harvested onto 102 x 256 mm glass fiber[7] filters with a 12 multiwell cell harvester[7] and the filter papers were air dried. Incorporated radioactivity was measured using a liquid scintillation cocktail (Cytoscint[8]) and counted in a Beckman gamma counter[9] and triplicate determinations were averaged.

Experiments:

Effects of mitogens on the PBMN cells

Various concentrations of several mitogens were incubated with isolated PBMN cells to determine the optimal concentrations of mitogens for optimum responses and also to determine the incubation period. Ten μl of mitogen (either PHA[10] or ConA[10]) at concentrations starting at 0.125, 0.25, 0.50, 1.0, and increasing to 2 μg/mL, and recombinant bovine IL-2[11] at concentrations from 10, 100, 500, 1000, and 2000 units/mL, were added to each well. One unit of rbIL-2 contained 0.01 ng/mL of IL-2. Controls contained PBMN cells and media only.

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[7] Skatron Inc. Sterling, VA

[8] ICN Biomedicals Ins. Irvine, CA 92713

[9] Beckman Instruments Inc., Fullerton, CA

[10] Calbiochem corporation, La Jolla, CA

[11] Dr. C. Morrison, Ciba Geigy. Saint. Aubin, Switzerland.

Effects of oGH and oPRL on the PBMN cells

In this experiment, resting or rbIL-2 activated cells from 8 sheep were treat ed with oGH and oPRL at concentrations of 0.01, 0.1, 0.5, 1 and 2 μg/mL. Activated PBMN cells were treated with 500 units of rbIL-2 per well. The tissue culture plates were incubated in a 37^{o} C, and 5.0% CO_2 environment for 72 h. Controls for resting PBMN cells contained cells and media only; controls for activated cells contained rbIL-2 (500 units/well), cells and media. This experiment was repeated with PBMN cells of 8 sheep. Proliferation responses are expressed as [^{3}H]-thymidine uptake at percent of control.

Effects of IGF-I and dexamethasone on the PBMN cells

In this experiment, resting or rbIL-2-activated PBMN cells were treated with rbIGF-I at concentrations of 0.001, 0.01, 1.0, 100, 500, and 1000 ng/mL. Dexamethasone at concentrations at 63.0, 125, 250, 500, and 1000 ng/mL was added to the activated cells. Activated PBMN cells were treated with 500 units/well in the tissue culture plate. Controls for resting cells contained cells and media only; controls for activated cells contained rbIL-2 (500 units/well), cells and media. The plates were incubated in a 37^{o} C and 5.0% CO_2 for 72 h.

Statistical analysis

Because absolute proliferation levels of PBMN cells displayed large heterogeneity between animals, results are expressed as a percentage of control (mean $\pm$ SE). Effects of oGH, oPRL, and rbIGF-I on resting and rbIL-2-activated PBMN cells were investigated using a factorial treatment structure in a randomized complete block experimental design. The statistical model used for analysis included the main effects of resting or activated cells, hormone treatment at different concentrations (0, 10, 100, 500, 1000, or 2000 ng/mL), and the interactive effects of cells x hormone treatment. The blocks correspond to individual animals. That is, PBMN cells from the same sheep were randomly assigned to all treatment combinations formed from the two factors. The general linear models (GLM) procedure in SAS, 1985, was used for the analysis of variance and comparison of treatment means. Since proliferation values were not normally distributed among the subjects, the natural logarithm was used for statistical analysis.

Results and Discussion

Effects of mitogens on sheep PBMN cells

Proliferative responses for nonstimulated and mitogen-stimulated PBMN cells are presented in Table 1. Intraassay coefficients of variance, based on triplicate measurements on the same samples, were 8 to 10%. Both ConA and PHA significantly increased proliferation ($p < 0.0001$). The effect of ConA (Fig. 1) was present at a dose of 0.125 μg/mL while the PHA effect was observed at doses of 0.25 μg/mL and above. peripheral blood mononuclear cells proliferated in vitro in response to concentrations of rbIL-2 by as low as 100 units per mL at ($p < 0.0001$), as shown in Fig. 2.

Proliferation of PBMN cells in response to oGH and oPRL

Whether GH and/or PRL regulation of PBMN cells proliferation is facilitative or inhibitory appears to depend upon hormonal concentration. There were a statistically significant interaction between resting or activated cells and treatment effect of oGH ($p < 0.01$) or oPRL ($p < 0.01$) on cell proliferation. The effect of the oGH or oPRL was analyzed separately for resting and activated PBMN cells. Growth hormone had a significant dose-related effect on cell proliferation, no matter whether PBMN cells were activated with rbIL-2 or not (Figs. 3 and 5). Ovine GH had a proliferative effect on resting cells at 100 ng/mL ($p < 0.01$) and on rbIL-2-activated cells ($p < 0.002$) at 2 μg/mL.

These results with sheep PBMN cells are in agreement with those of Mercola *et al.* (1981) who showed that hGH had proliferative activity at 100 ng/mL on healthy human PBMN cells. Ovine PRL had a proliferative effect on resting cells at 100 ng/mL ($p < 0.01$) (Fig. 5). However, when oPRL was used in combination with rbIL-2-activated cells, the proliferative effect of PRL on PBMN cells was not significant ($p = 0.07$) (Fig. 6). Schimpff and Repellin (1989) have shown that hGH had no proliferation effect on PHA-activated human lymphocytes which is in agreement with our results from rbIL-2-activated cells. Resting PBMN cells treated with GH and/or PRL shows two different response curves (Figs. 3 and 4), one from 0 to 0.1 μg/mL and another from 0.5 to 2 μg/mL. This result may suggest that there are two populations of receptors, one with high affinity and one with low affinity. The low affinity response is still present in IL-2 activated cells (Fig. 5 and 6), suggesting that a disappearance of the high affinity receptors. Elvinger *et al.* (1991) have reported that bovine somatotropin (BST) treated cows had not proliferative response up to 1000 ng/mL on the resting or mitogen activated (ConA, PHA) lymphocytes. In contrast, Burton (1990) indicated that lymphocytes isolated from BST-treated cows had enhanced responsiveness to stimulation with ConA. Clevenger *et al.* (1990) reported that PRL is needed for cell proliferation of the murine T-helper lymphocyte L2 cell line (an IL-2-driven T-cell) because anti-PRL antibody in the cell culture medium prevented cell proliferation in comparison to a control culture which contained 10 μg of exogenous PRL per mL of medium and exhibited normal cell proliferation. The results show that the response of PBMN

cells to rbIL-2 is influenced by the presence of oGH and/or oPRL at low doses. Croze *et al.* (1988) have reported that after five passages of Nb2 (a lymphoma cell line) cells in serum-free medium supplemented with hGH, the maximal proliferation of cells in response to IL-2 gradually declined to that of the control cells which just treated with IL-2.

Effects of other hormones on proliferation of sheep PBMN cells

Treatment of resting PBMN cells with rbIGF-I is shown in Fig. 7. Insulin-like growth factor-I did not modulate cell proliferation ($p < 0.3$) in tissue culture. In bone marrow transplant studies, rhIGF-I increased the rate of T and B cell repopulation of the periphery after lethal irradiation, suggesting that rhIGF-I acts at the level of the bone marrow rather than inducing proliferation of peripheral lymphocytes (Clark *et al.*, 1993). Indeed, a recent article by Landreth *et al.* (1992) indicates that IGF-I is produced locally by bone marrow stromal cells and may play a role in regulating primary B cell lymphopoiesis. Tapson *et al.* (1988) have reported that IGF-I stimulated uptake of [^{3}H]-thymidine into resting human T lymphocytes to a significantly greater extent than occurred in control cells, or cells incubated with IL-2 alone. The effect of IGF-I on rbIL-2-activated PBMN cells is shown in fig. 8. Insulin-like growth factor-I did not have proliferative effect on rbIL-2-activated PBMN cells ($p > 0.7$). It seems in figure 8 that rbIGF-I had an inhibitory effect on cells activated by rbIL-2 at the dose of 1 μg/mL. In various biological systems, IGF-binding proteins (IGFBPs) have been shown to inhibit IGF-I

function (review by Hintz, 1990). In tissue culture, IGFBPs can be produced by monocytes (Kooijman, 1992). In our study, our PBMN cell preparation contained about 4 to 6% monocytes. If these cells produced enough IGFBP, this could interfere with the effect of IGF-I on cell proliferation in our studies. Another possibility is that the fetal calf serum contained high concentrations of endogenous IGF. Therefore, additional IGF-I would produce no additional effects. Hunt and Eardley (1986) have also reported that IGF-I at a concentration of 1 μg/mL had a suppressive effect on IL-2-activated splenocytes in tissue culture. Although the biologic significance of these observations still needs clarification, the possibility that IGF-I links the endocrine and immune systems deserves further consideration. It is interesting that the concentration of IGF-I reaches its greatest height at a time when at least some mechanisms of neonatal immune tolerance are hypothesized to be occurring (Strober, 1984).

Effect of dexamethasone

The effect of Dexamethasone on the number of rbIL-2 activated PBMN cells is shown in Figure 9. Dexamethasone inhibited ($p < 0.0001$) the proliferation of the rbIL-2-activated PBMN cells. Wiegers *et al.* (1993) have demonstrated that a low circulating level of corticoid maintained the immune response while a rather high dose of corticoid completely abolished the proliferative response in adrenalectomized rats in an *in vitro* assay using PBMN cells activated with ConA. Arya *et al.* (1984) have reported that the dexamethasone suppresses the proliferation

of human T lymphocytes by inhibiting the synthesis of IL-2 mRNA and also inhibits the accumulation of gamma-interferon mRNA in these cells.

Implications

Growth hormone and PRL have regulatory effects on PBMN cells *in vitro*. Thus, GH and PRL may play a role in the control of proliferation of PBMN cells in domestic animals and may have usefulness as immune-response modifiers. In addition, These studies show that sheep PBMN cells have receptors for several hormones and suggested that these hormones may be normally involved in maintaining the immune system.

Table III. 1. Proliferation effect of ConA and PHA on PBMN cells in sheep.

Mitogens	0	0.125	0.25	0.5	1.0	2.0 μg/mL
ConA	809* ±127	10477 ±625	24450 ±769	26435 ±626	29391 ±690	27054 ±2335
PHA	809 ±127	1027 ±58	4737 ±1062	19386 ±265	25810 ±678	27718 ±584

* Numbers represent counts per minute of [^{3}H]-thymidine uptake by cells.

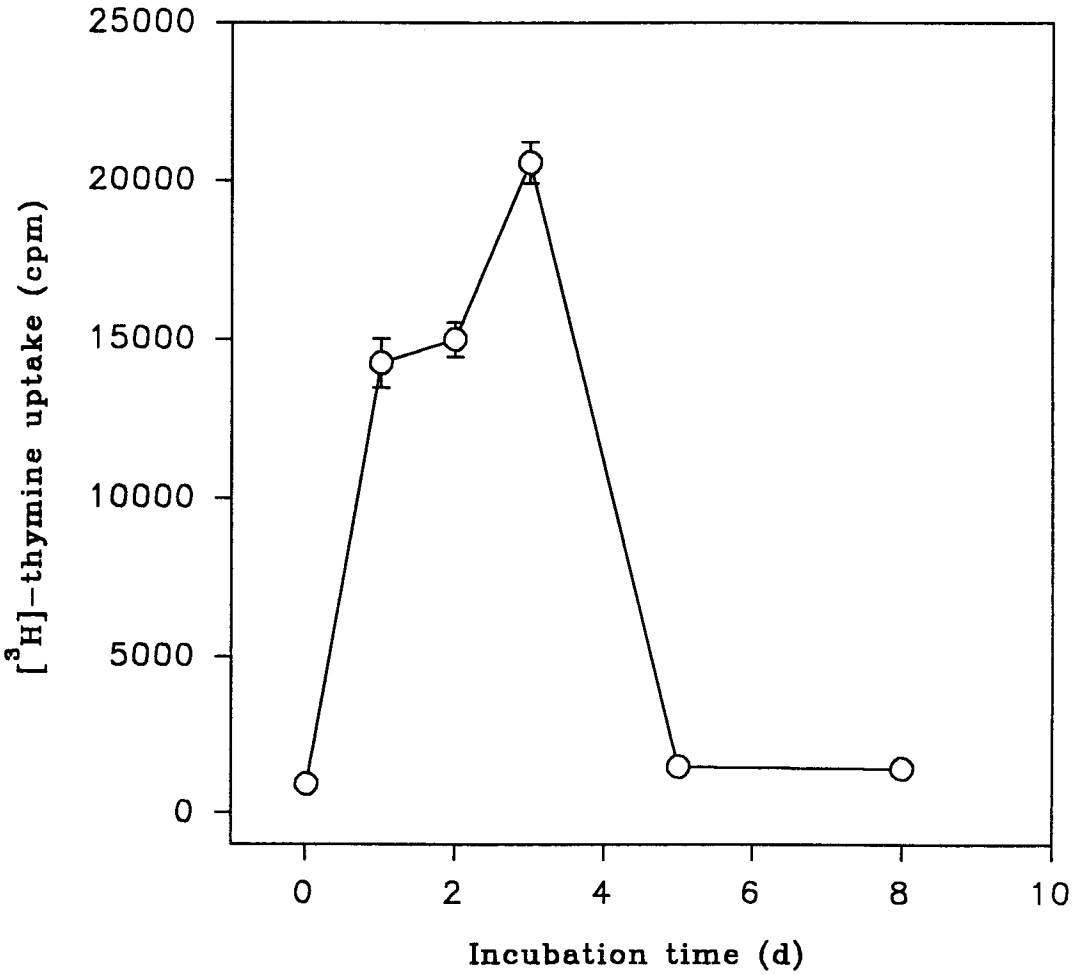

Figure III. 1. Incubation time vs proliferation of PBMN cells in the presence of 2.0 μg/mL ConA.

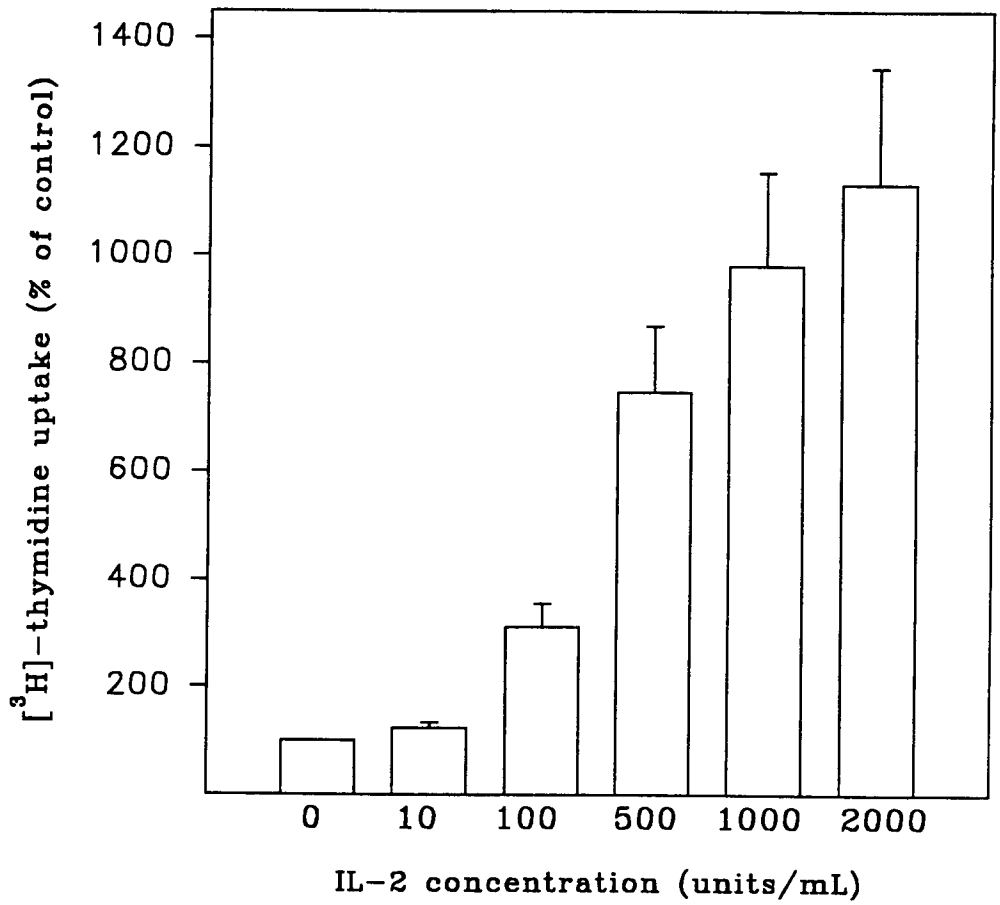

Figure III. 2. Effect of increasing concentration of rbIL-2 on lamb PBMN cells in tissue culture.

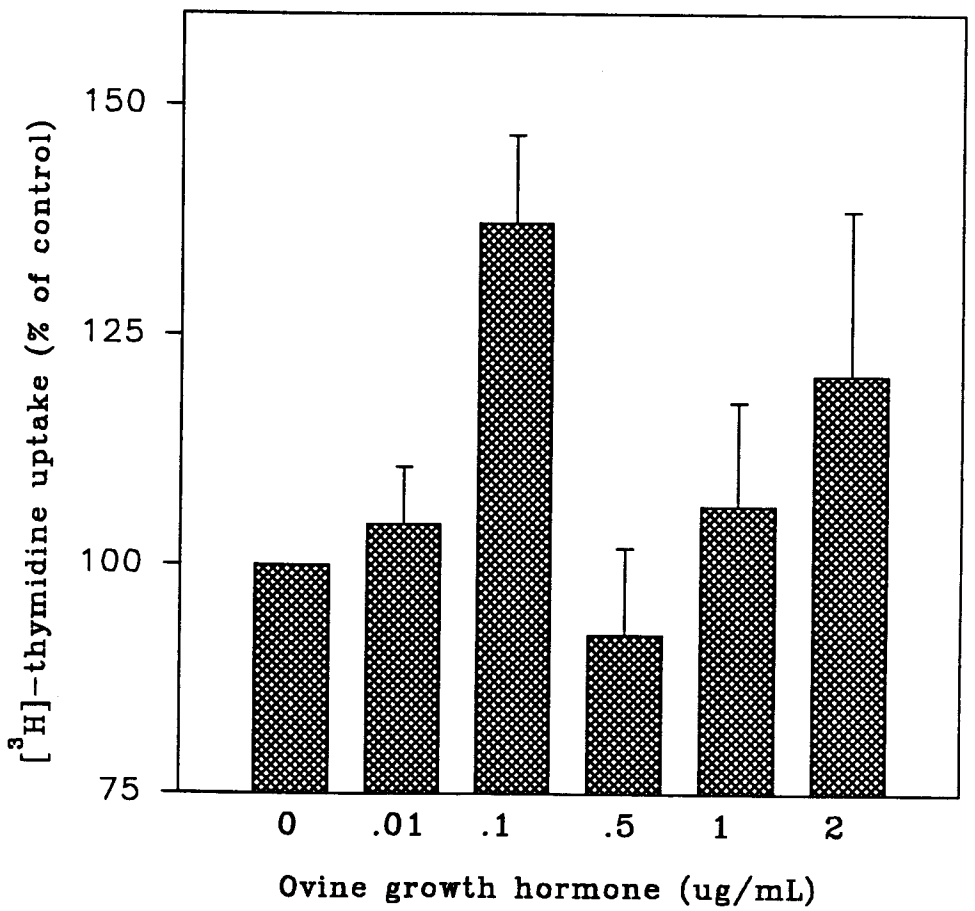

Figure III. 3. Effect of oGH on proliferation of resting PBMN cells.

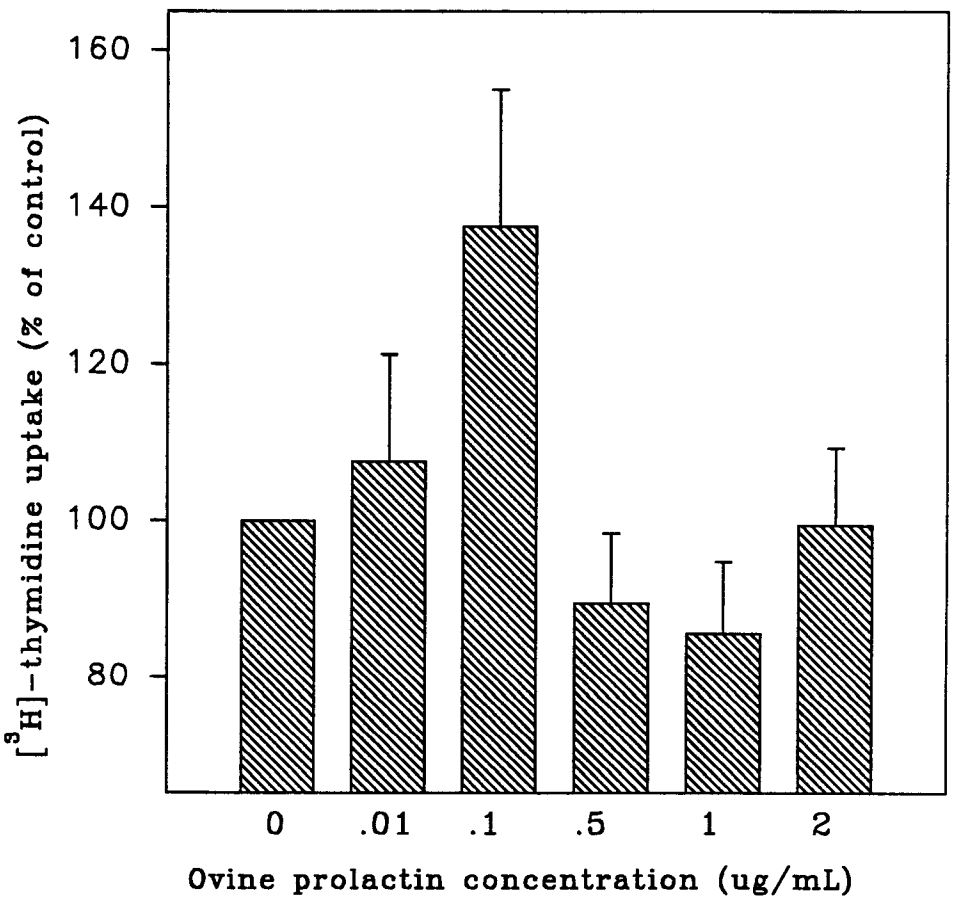

Figure III. 4. Effect of prolactin on proliferation of resting lamb PBMN cells.

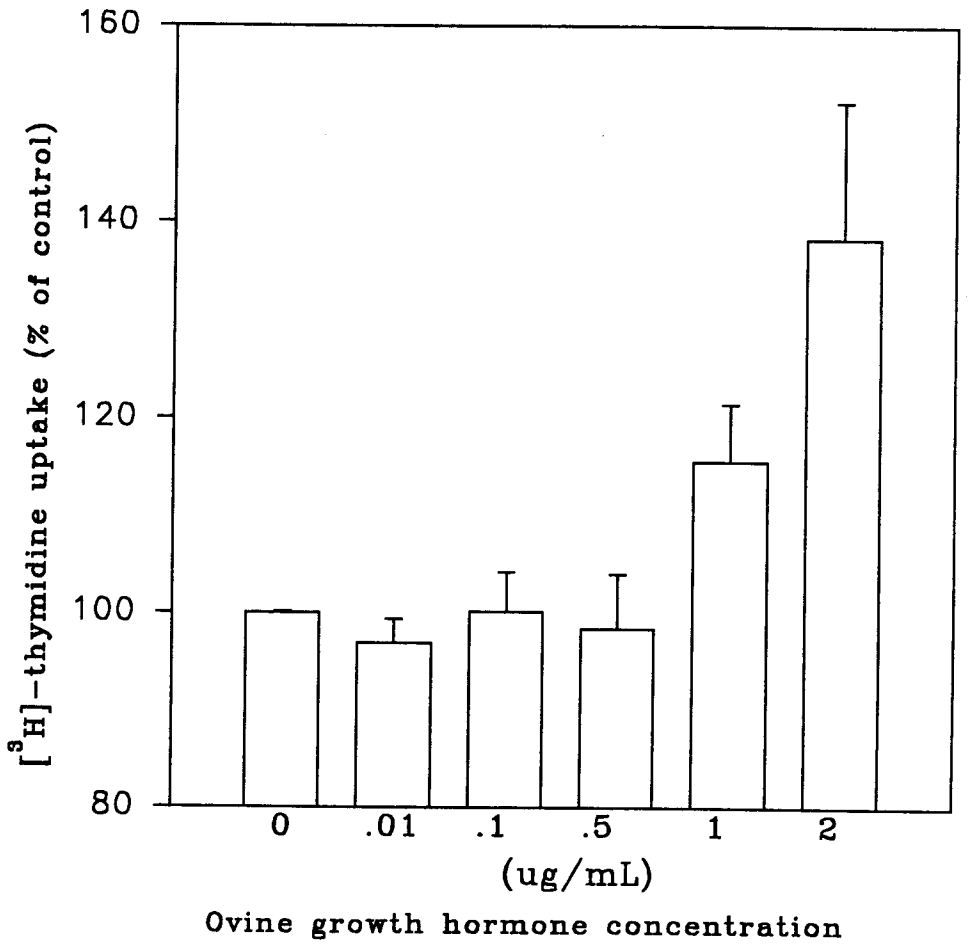

Figure III. 5. Effect of growth hormone on rbIL-2-activated PBMN cells *in vitro* with rbIL-2 (500 units/well).

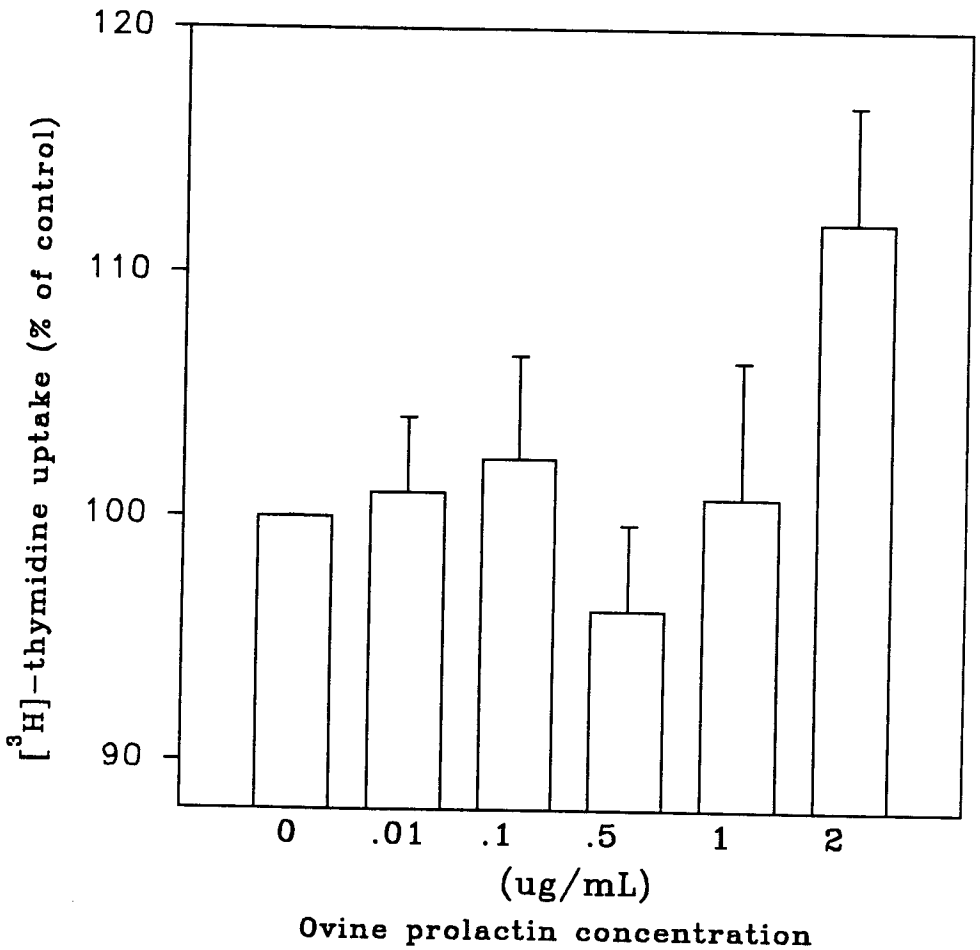

Figure III. 6. Effect of prolactin on rbIL-2-activated PBMN cells *in vitro* with rbIL-2 (500 units/well)

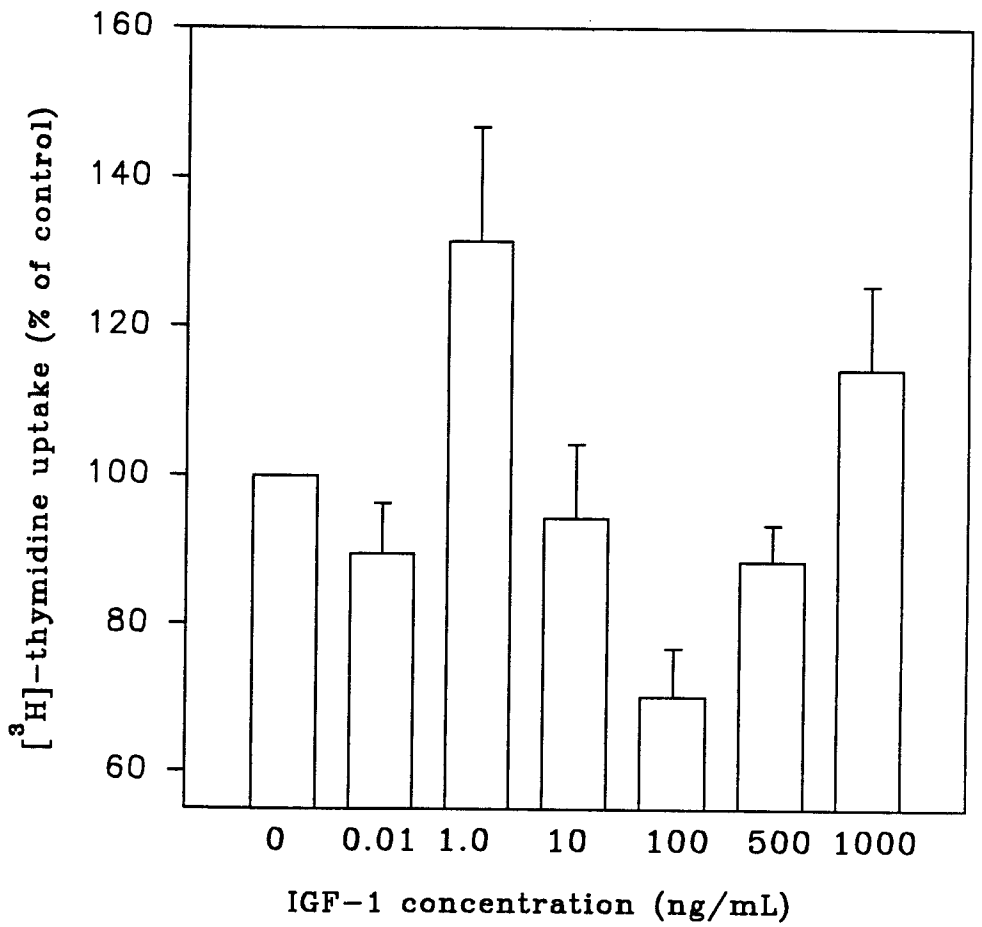

Figure III. 7. Effect of IGF-I on resting PBMN cells.

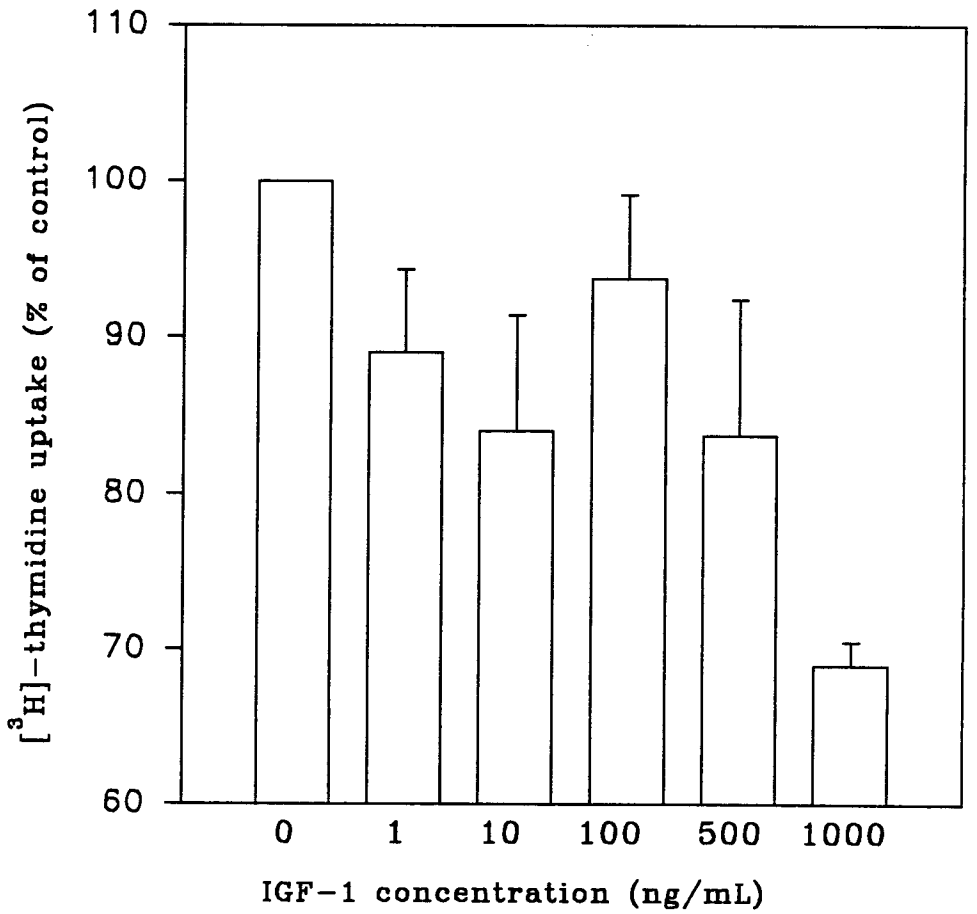

Figure III. 8. Effect of rbIGF-I on rbIL-2-activated PBMN cells *in vitro* with rbIL-2 (500 units/well).

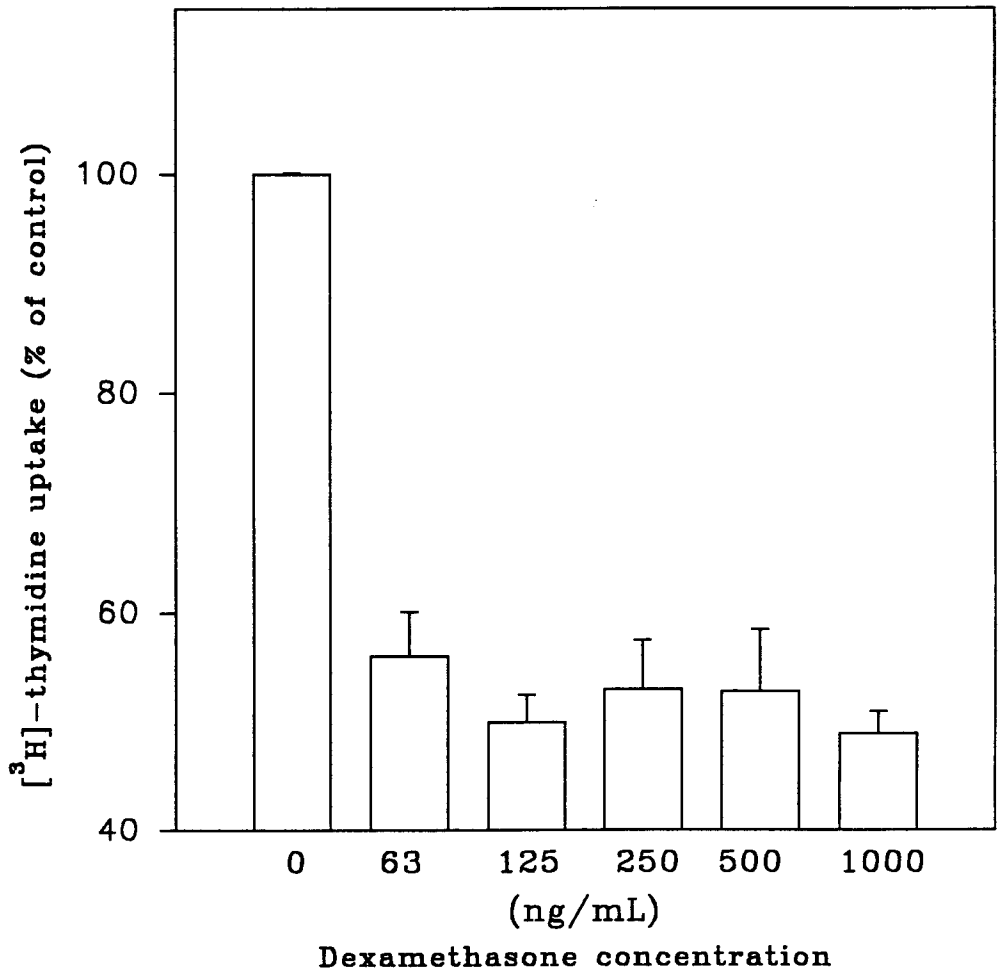

Figure III. 9. Effect of dexamethasone on rbIL-2-activated PBMN cells *in vitro* with rbIL-2 (500 units/well).

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CHAPTER IV

ONTOGENY OF GROWTH HORMONE RECEPTOR ON PERIPHERAL BLOOD MONONUCLEAR CELLS AND ON PLASMA GROWTH HORMONE-BINDING PROTEIN IN LAMBS

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Abstract

This experiment was conducted to determine if growth hormone (GH) receptors are present on immune cells of the lamb and if they change with age. Simultaneously, we examined age-related changes in plasma growth hormone binding protein (GHBP). Blood samples were drawn from crossbred lambs at ages of 7, 14, 30, 90, and 150 d after birth. Peripheral blood mononuclear (PBMN) cells were separated from whole blood by Ficoll density gradient centrifugation method and plasma was stored at -20° C.

Specific binding of ^{125}I-hGH to PBMN cells increased from 0.3% at d 7 to 0.8 $\pm$ 0.1% at d 150 ($p < 0.03$). Likewise, plasma concentrations of GHBP were lowest during the first several weeks of age but increased ($p < 0.0001$) between ages of 30 and 150 d. Specific binding of GH receptors and GHBP concentrations in plasma were positively correlated ($r = 0.89$; $p = 0.049$) over age, suggesting similar age dependent regulation. Results indicated that PBMN cells have very low GH receptor activity in the lamb. Observation of increased GHBP concentration with increased age is in agreement with previous reports showing an age related increase in serum GHBP concentration in the pig.

Key words: Growth hormone receptor, Growth hormone-binding protein, Peripheral blood mononuclear cells, lamb.

Introduction

A new interdisciplinary research area has emerged that involves the study of interactions between the immune and endocrine systems (Kao *et al.*, 1992). One emerging area of research on the endocrine-immune system interrelationship concerns investigation of the presence of hormone binding sites (receptors) on immune cells and their roles at the molecular and cellular level. Binding of GH to its receptor is required for regulation of the actions of GH on all tissues (Nicoll *et al.*, 1986).

Binding sites for human GH (hGH) have been previously demonstrated on cultured human cell lines such as IM-9 cells (Lesniak *et al.*, 1974 and Asakawa *et al.*, 1986) and on human PBMN cells (Kiess and Butenandt, 1985). Keiss and Butenandt (1985) have reported that the specific binding of ^{125}I-hGH by human PBMN cells was rapid, reversible, and time and temperature dependent. Scatchard analysis (Scatchard, 1949) of ^{125}I-hGH binding to human PBMN cells revealed a receptor with a mean affinity constant of 1.5×10^{-9} M and a maximal binding capacity of 7.1×10^{-11} M. Human GH binds to GH binding sites in ruminant animals with a higher affinity than does their own GH (Nicoll *et al.*, 1986b).

In some studies, GH receptor assays have been carried out on solubilized receptors from human lymphocytes (McGuffin *et al.*, 1976). These researchers observed more binding to solubilized GH receptors than to intact membrane receptors on the circulating immune cells. The development of methods for

solubilization and partial purification of GH receptor from rabbit liver (McIntosh *et al.*, 1976) and from lymphocytes (Stewart *et al.*, 1983) has been reported.

The extracellular domain of the human growth hormone receptor (residues 1 to 246) occurs naturally in serum in the form of a GHBP, which binds hGH with approximately the same affinity as the intact receptor (Fuh *et al.*, 1990). Growth hormone binding protein has been identified in human sera (Baumann *et al.*, 1986), as well as in rabbit (Ymer and Herington, 1985), mouse (Smith and Talamantes, 1988), and rat (Amit *et al.*, 1990). It has been shown that the rabbit and human serum GHBP is related to and possibly derived from the hepatic GH receptor (Leung *et al.*, 1987; Spencer *et al.*, 1988). Growth hormone binding protein in human plasma is a single-chain glycoprotein with an estimated molecular weight of 50-60 kDa. A serum GHBP has also been reported from several species including sheep, pig, and chicken (Davis *et al.*, 1992). In most species, it is thought that the serum GHBP is derived mainly from the enzymatic lysis of the extracellular portion of the GH receptor (Baumann, 1990).

Although specific GH receptors have been demonstrated in various tissues of a number of species, the presence of GH receptors on domestic animal PBMN cells have not been investigated. This study was conducted to

1. Characterize GH binding sites in PBMN cells of the lamb and age related changes in binding activity.
2. Determine concentration of the plasma GHBP from 7 d to 150 d of age.
3. Purify and characterize GH receptors from sheep PBMN cells.

Materials and Methods

Animals and blood collection

Fifteen crossbred lambs, ages from 7 d up to 150 d were used. Twenty mL blood samples were collected via jugular venipuncture in heparinized vacutainer[1] tubes. Blood samples were placed on ice during collection and transfer to the laboratory.

Chemicals

Human growth hormone (hGH) was a gift from the National Hormone and Pituitary Program[2]. Carrier-free sodium ^{125}Iodide was purchased from Amersham[3]. RPMI medium 1640 was obtained from Gibco Laboratories[4].

PBMN cell separation

The separation of the PBMN cells from whole blood was performed by the

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[1] Becton Dikinson, Rutherford, N. J. 07070

[2] National Hormone and Pituitary Program, NIDDK, USDA, Rockville, MD 20850. Human GH, NIDDK-hGH-I-3.

[3] Amersham, PRN .1004, Arlington Heights, IL.

[4] Gibco, Grand Island, New York.

Ficoll-Histopaque density gradient centrifugation method of Boyum (1968) with modification as follows: The heparinized blood was taken from normal sheep in sterile vacutainer tubes and centrifuged at 1800 rpm at 8° C for 15 min. The buffy coat was diluted 1:3 with RPMI-1640 supplemented with gentamicin[5] (50 μg/mL). The buffy coat/RPMI mixture was layered onto 8 mL Ficoll Histopaque[5] (density 1.077 μg/mL) in 50-mL sterilized centrifuge tubes. The tubes were then centrifuged at 450 x g for 30 min. The PBMN cells were found in the top band of the tube. They were harvested, resuspended with RPMI 1640 and washed twice at 1200 rpm for 10 min and maintained in the same medium. Before use, the cells were counted in a hemocytometer chamber to determine cell number. The viability of the separated cells was tested by the trypan blue exclusion method (Moore *et al.*, 1966). The preparations contained about 85 to 90% lymphocytes and 4-6% monocytes as determined by Wright stain. Contamination with granulocytes was less than 5%.

Iodination of growth hormone

Labeled hGH was prepared by a modification of the method of Greenwood and Hunter (1963) which was adapted in our laboratory. A typical iodination mixture contained the following reagents which were added in sequence: 25 μL of 0.5 M sodium phosphate (pH 7.4), 2.5 μL of hGH (1mg/mL), 0.5 mCi sodium 125-Iodide (carrier-free) , and 30 μL chloramine-T (0.5 mg/mL, Sigma).

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[5] Sigma, St. Louis, MO 63178

The reaction mixture was immediately diluted with 1.0 mL of 0.05 M sodium phosphate buffer, pH 7.4. The specific activity of iodinated hGH was determined by precipitation of ^{125}I-hGH with 10% trichloroacetic acid. The ^{125}I-hGH was purified by passage through a 1.5 x 6 cm anion exchange column (AG 1-X8[6]) and eluted with 0.05 M sodium phosphate buffer. Free iodide was removed with a 1 x 55 cm Bio Gel P-100[6] column (mesh 100-200) using phosphate buffered saline (PBS) (0.14 M NaCl, 0.01 M $NaPO_4$, pH 7.4) for elution. Fractions of 2.0 ml were collected in tubes containing 1 mL PBS plus 1% bovine serum albumin[5] (BSA). The ^{125}I-hGH prepared in this way was used immediately for radioreceptor assay (RRA) or stored at 4^{o} C for no longer than 2 weeks.

Radioreceptor assay

The radioreceptor assays were performed in triplicate at room temperature (RT) on the intact freshly separated PBMN cells from whole blood. The PBMN cells (5 x 10^6 cells/tube) were added to 12 x 75 mm borosilicate tubes containing 100 μL of ^{125}I-hGH (30,000 cpm) in the absence or presence of an excess (1 μg) of unlabeled hGH in a total incubation volume of 0.3 mL in 0.01 M PBS, containing 10 mM $MgCl_2$ and 1% (wt/vol) BSA. Incubation was carried out for 2 h at 32^{o} C in a constant temperature water bath with vigorous shaking.

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[6] Bio Rad Laboratories, Hercules, CA 94547

[7] Beckman Instruments, Inc. Palo Alto, CA 94303

At the end of the incubation period, 0.5 mL ice-cold PBS-1%BSA buffer was added and the tubes centrifuged at 1000 x g at 4° C for 10 min. Tubes were decanted, air dried and the resulting pellet was counted in an automatic gamma counter[7] (gamma 5500). Non-specific binding (NSB) was defined as cpm ^{125}I bound in the presence of 1 μg unlabeled hGH; this was determined for every sample. Specific binding was expressed as the difference between total binding and nonspecific binding. A preparation of liver microsome was used as a positive control to demonstrate that the ^{125}I-hGH preparation was indeed able to bind to its receptor. PBMN cells also were treated with 4 M $MgCl_2$ to remove the endogenously bound growth hormone as described by Baginga *et al.*, 1990.

Solubilized receptors

The PBMN cells were separated from whole blood and then PBMN cell receptors were solubilized immediately in Tris-HCl buffer, pH 7.5, and Triton X-100 (1 mg/1 mg protein) as follows: After stirring the solubilized receptors in a shaker for 1 h at room temperature (RT), the mixture was centrifuged at 100,000 x g for 1 h. The supernatants were stored in aliquots at -20° C for later binding studies. Protein was determined by a method which has been adapted to measure protein in samples containing Triton X-100 (Chandrarajan and Klein, 1975). Solubilized receptors (200 μg) were incubated with 30 x 10^3 cpm of ^{125}I-hGH in the absence or presence of 1.0 μg of unlabeled GH in a final volume of 0.5 ml Tris-HCl

buffer, pH 7.5, containing 1% BSA, 10 mM $MgCl_2$ and 10 mM $CaCl_2$. The samples were incubated at RT overnight on a reciprocal shaker.

RRA of solubilized GH receptor

The Triton X-100 extract containing 50 mg protein was incubated 3 hours with ^{125}I-GH (30,000 CPM) in 0.5 ml Tris-HCl pH 7.5, containing 0.1% BSA, 10 mM $MgCl_2$, and 10 mM $CaCl_2$. Then, polyethylene glycol (Stewart *et al.*, 1983) was used to precipitate the hormone-receptor complex. The incubation was stopped by placing the tubes in ice, adding 0.2 ml of cold 12.5% polyethylene glycol in 25 mM Tris-HCl buffer, and then mixing vigorously. After waiting 10 min to allow complete precipitation, the tubes were centrifuged at 2500 x g for 20 min, then the tubes were decanted. The tubes were kept inverted on filter paper for 2 h at RT and then the radioactivity was counted in a gamma counter.

GHBP concentration

Plasma concentrations of GHBP were measured using a competitive enzyme linked immunosorbent assay (ELISA) using recombinant bovine GHBP (rbGHBP)[8] and a polyclonal rabbit anti rbGHBP[8] (Davis *et al.*, 1994). Briefly, rbGHBP (25 ng/well in 100 μL of carbonate buffer, 0.1 M, pH 9.6) is added to Costar

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[8] Monsanto Company, ST. Louis, MO 63038

high-binding EIA plates and incubated for 48 h at 4° C to coat the well with GHBP. On the same day, equal volumes (60 μL) of assay buffer (0.1 M PBS, 1 % BSA, 0.1 Triton X-100, 0.1 % Tween-20), serum or reference standard in assay buffer and antiserum (diluted to 1:50,000 in assay buffer) are added on d 1 to wells in a low-binding Costar microtiter plate and incubated for 48 h at 4° C. On d 3, the GHBP coated plates were washed 3 times with nanopure water containing 0.1 % Tween 20. The GHBP coated plates are then blocked by adding 100 μL of assay buffer and incubating at room temperature (RT) for 30 min. The plates were again washed 3 times and then 100 μL of incubated mixtures from low-binding plates were added to the coated plates and incubated for 24 h at 4° C. On d 4, biotin conjugated anti-IgG[3], streptavidin conjugated to horseradish peroxidase[3] and chromogen (3,3,5,5-tetramethylbenzadine were used to for color development. Plates were read on a Titertek plate reader at 450 nM. The computer is programmed to the reference standard curve and to calculate the relative concentration of GHBP in samples by extrapolation from the standard curve.

Liver microsomal isolation

A liver from a 1 year old male lamb was collected after slaughter, and liver microsomes were prepared as described by Orian *et al.* (1991). Approximately 1 g of liver was diced and then homogenized in 5 volumes of a solution containing 0.3 M sucrose at 4° C. The homogenate was centrifuged at 1,500 x g for 20 min at 4° C. The supernatant was collected and centrifuged at 15,000 x g for a further 20

min at 4° C. The 15,000 x g supernatant was then centrifuged at 100,000 x g for 90 min at 4° C. The pellet from the 100,000 x g centrifugation was resuspended in 1 volume of a solution containing 25 mM HEPES, pH 7.5, 10 mM $MgCl_2$, and the protein concentration was determined by the method of Lowry *et al.* (1951). Membrane preparations thus prepared were stored at -20° C until used for binding studies.

Statistical analysis

The general linear model (GLM) procedure in SAS, 1985, was used for the analysis of variance and comparison of treatment means. The correlation coefficient was defined by Pearson's correlation matrix test.

Results and Discussion

Ontogeny of growth hormone receptor

Developmental changes in specific binding of ^{125}I-hGH to intact PBMN cells are shown in Fig. 1. Percentage of specific binding per 5 x 10^6 cells increased ($p < 0.03$) from 0.3% at d 7 to 0.8 $\pm$ 0.1% at 150 d. In the presence of a large excess (1 to 2 μg/tube) of unlabeled hGH only a small percentage of the ^{125}I-hGH was bound to PBMN cells. This is referred to as "nonspecific" binding. Nonspecific binding tubes were used for each sample to calculate total specific binding. Radioreceptor assay was done at room temperature (RT) and at 2 h according to the procedure by Lesniak *et al.* (1974). Cell viability was always more than 95% of the PBMN cells as determined by trypan blue exclusion. Specific binding of ^{125}I-hGH was expressed as percent bound/total radioactivity added per tube. Receptors were considered GH-specific because ovine prolactin (oPRL) failed to displace ^{125}I-hGH from PBMN cells at doses up to 5 μg. In our experiment, we found that the average binding of ^{125}I-hGH was less than 1.0% of total binding. However, cells from some individual lambs and some older ewes exhibited up to 2 %SB (with 5 x 10^6 cells per tube). This is consistent with the observation by Eshet *et al.* (1975) who reported specific binding of ^{125}I-hGH to human circulating lymphocytes of 3 and 4% when 10 x 10^6 lymphocytes were used in their assay.

In our study, solubilized receptors from cell membranes did not increase the specific binding of ^{125}I-hGH to GH binding sites from PBMN cells. The specific

binding stayed the same as that on the freshly isolated PBMN cells and it was under 1%. Divalent cations and chelating agents such as Triton X-100 have been shown to have important effects on some receptor hormone interactions (Gorden and Weintraub, 1985). Bonifacino *et al.* (1981) have reported that solubilization of the membrane receptor increased the specific binding (%) of ^{125}I-hGH to GH receptors in rat liver. To investigate whether high concentrations of endogenous sheep GH obscured the detection of specific GH receptors on PBMN cells, specific binding of ^{125}I-hGH was examined after pretreatment of PBMN cells with a high concentration (4 M) of $MgCl_2$. Pretreatment with $MgCl_2$ to remove endogenously bound hormone did not have any effect on the %SB.

Scatchard plots could not be done because of low binding, so binding affinity was not estimated. In addition, because of the low yield of GH binding sites from solubilized PBMN cells, it was not possible to purify and further characterize the binding.

Growth hormone-binding protein

Plasma concentrations of GHBP were low during the first several weeks of age (20 to 27 ng/mL) but increased to 43 ng/mL ($p < 0.001$) from 30 to 150 d of age. These results are agree with those of Mullins and Davis (1992) who have reported that GHBP activity increased with age in both sexes of pigs (boar and gilt). There is now good evidence that the principal form of GHBP is identical to the extracellular domain of the GH receptor (Leung *et al.*, 1987) and that GHBP may be

produced by proteolytic cleavage of the full-length GH receptor (Trivedi and Daughaday, 1988; Spencer *et al.*, 1988) or derived from a separate mRNA (Baumbach *et al.*, 1989). Studies of the ontogeny of the GHBP activity in man (Daughaday *et al.*, 1987) and the changes in the concentration of the GHBP and hepatic GH receptor in pregnant mice (Smith and Talamantes, 1988) suggest that the serum GHBP levels could be a peripheral indicator of GH receptor activity. This idea is supported by the finding that patients with Laron-type dwarfism, a syndrome caused by the lack of functional hGH receptors, also lack GHBP activity in serum (Baumann *et al.*, 1987).

Relationship between GH receptor and GHBP

Although GH receptor levels were very low on PBMN cells at all ages examined, there was a high positive correlation ($r = 0.89$, $p = 0.049$) between GH receptor and plasma GHBP levels. This observation suggest that GH receptor on PBMN cells and circulating GHBP are developmentally regulated in a similar fashion.

Implications

In growing lambs, percent specific binding of hGH to PBMN and serum concentrations of GHBP were lowest at birth but increased up to 5 months of age. These results suggest that concentration of specific GH receptors on PBMN cells increases with age and is associated with an increase in GHBP in serum. The presence of GH receptors on PBMN cells suggests that GH may be involved in modulating the cellular component of the immune system in lambs.

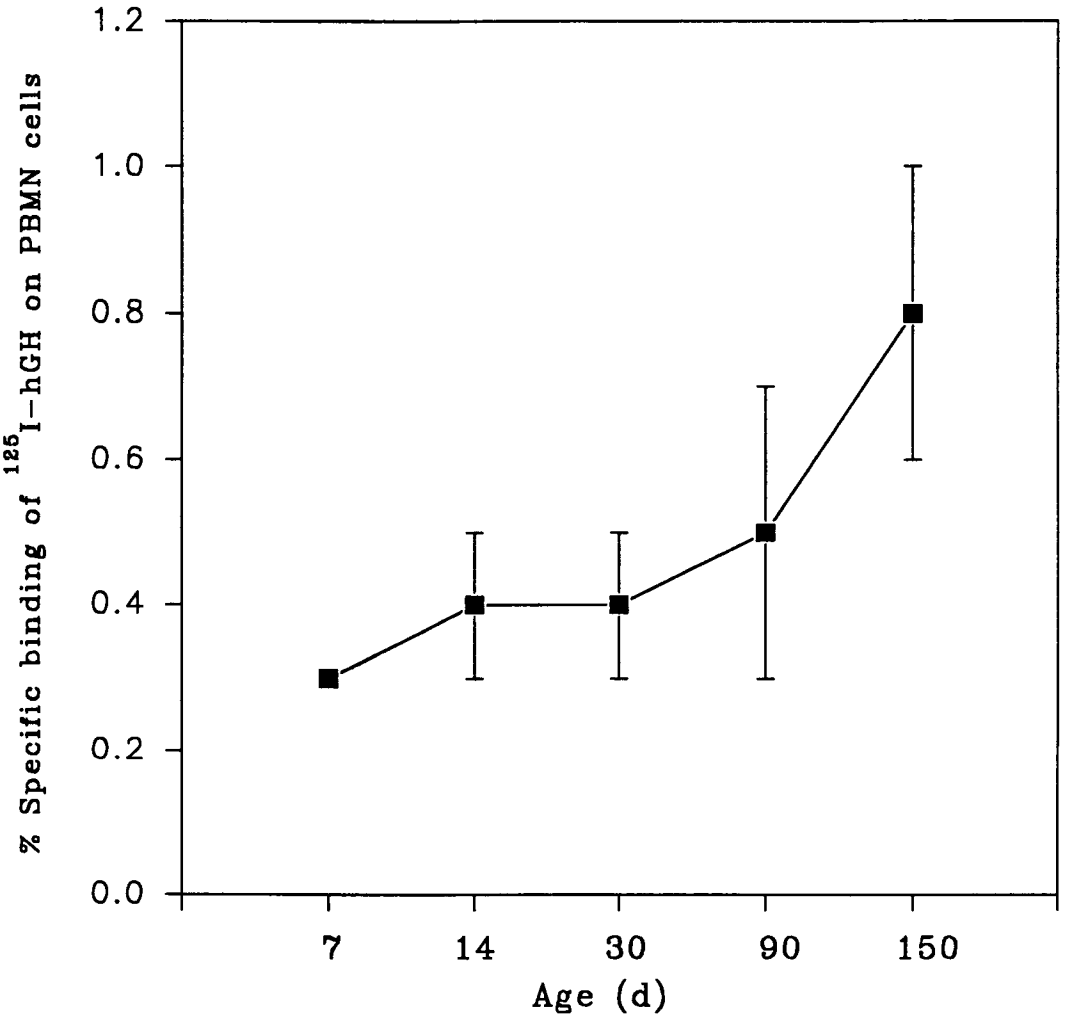

Figure IV. 1. Ontogeny of the GH receptor on PBMN cells.

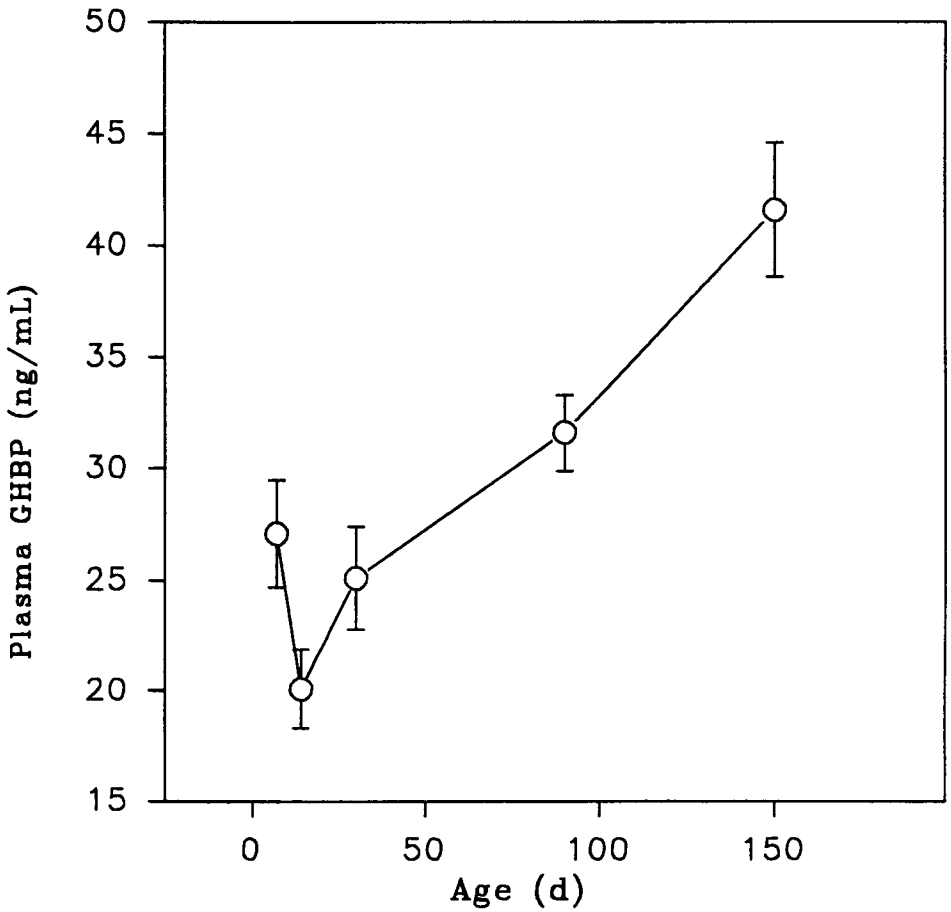

Figure IV. 2. Age related changes in the plasma GHBP concentrations in lamb.

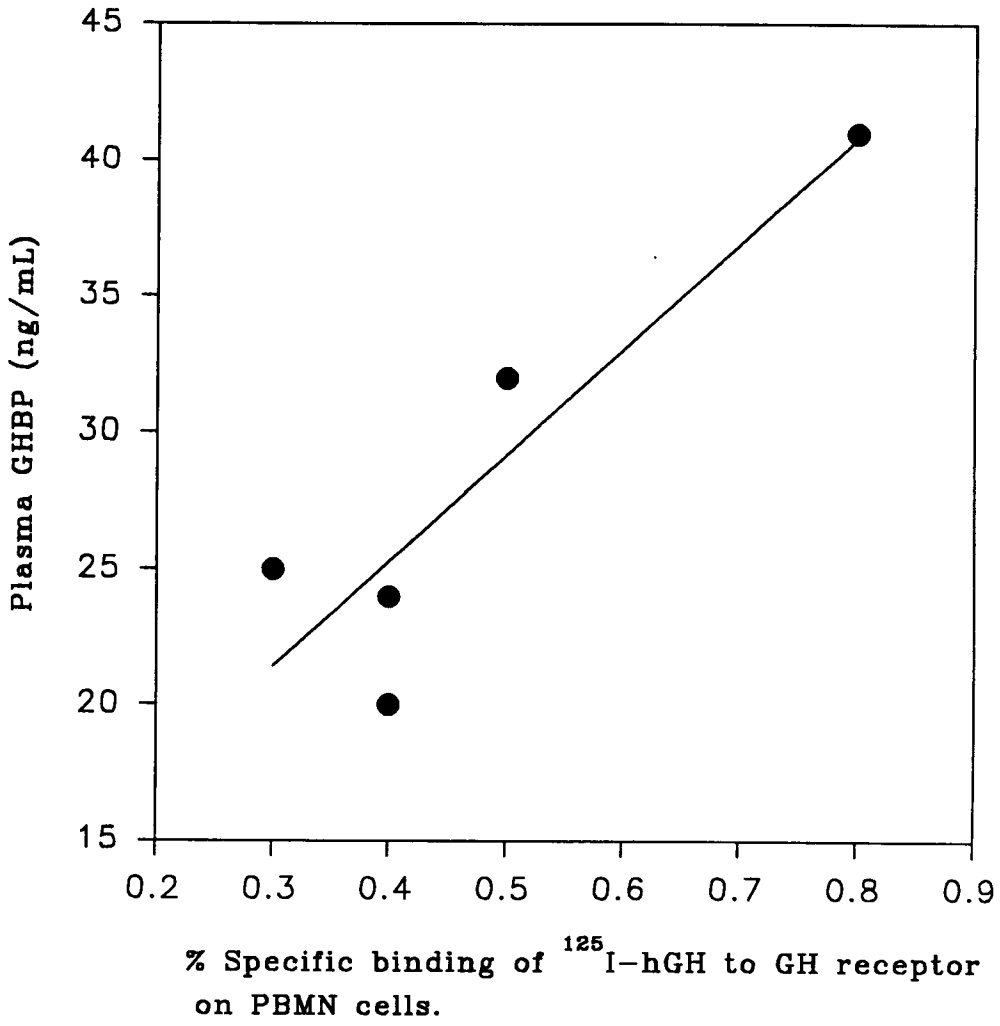

Figure IV. 3. Relationship between GH receptor on PBMN cells and plasma GHBP.

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CHAPTER V

SOLUBLE INTERLEUKIN-2 RECEPTOR IN BOVINE SERUM: EFFECTS OF DISEASE AND TREATMENT WITH VITAMIN E, SELENIUM, AND VITAMIN E + SELENIUM

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Abstract

This study was conducted to determine if soluble interleukin-2 (sIL-2) receptor activity in serum may be used as an indicator of the status of the immune system in ruminants. An assay was developed to measure sIL-2 receptor in serum in ruminant animals by using recombinant bovine interleukin-2 (rbIL-2). Blood samples were drawn from cows and serum was collected and stored at -20° C until assayed. Specific binding (SB) of ^{125}I-rbIL-2 to the sIL-2 receptor in bovine serum was time dependent (plateau at 6 h) and dose dependent (highest binding at 200 μl of serum). Scatchard analysis of the binding revealed a single order binding with low binding affinity (ka= 1.5 $\pm$ 0.7 x 10^{-7} M^{-1}) and moderate capacity (Bmax= 5.6 $\pm$ 1.2 fmol/mg).

Serum sIL-2 receptor activity was higher ($p < 0.01$) in cows infected with Bovine Viral Diarrhea (BVD) and/or Infectious Bovine Rhinotracheitis (IBR) than in healthy cows [percent SB (%SB) of 5.2 $\pm$ 0.8 and 3.0 $\pm$ 0.2, respectively]. In addition, serum sIL-2 receptor activity was measured in serum from calves treated with selenium (Se), vitamin E, and Se + vitamin E. Neither Se nor vitamin E treatment had an effect ($p > 0.05$) on serum sIL-2 receptor binding activity in calves. The occurrence of Escherichia coli (E. coli) scours, however, was associated with an increase ($p < 0.01$) in serum sIL-2 receptor activity (%SB of 1.8 $\pm$ 0.1 and 2.5 $\pm$ 0.1 for control (without scours) and scours calves, respectively). These results confirm that a soluble form of IL-2 receptor in serum is present in the

bovine species and that the relative amount of this serum receptor is increased in diseased calves and cows. Serum IL-2 receptor, therefore, may be of value as an indicator of immune response to disease.

Key words: Soluble interleukin-2 receptor, bovine, vitamin E, selenium, viral disease

Introduction

Interleukin-2 (IL-2), is also known as T cell growth factor, is an immunomodulatory factor produced by certain subsets of T-lymphocytes. It has been isolated from a variety of cell cultures and recombinant systems (Smith, 1988). Bovine IL-2 is a glycoprotein with an apparent molecular mass of 15.5 kDa and it has an amino acid homology of 65% with human IL-2 and 50% with murine IL-2 (Cerretti *et al.*, 1986). Interleukin-2 has been shown to promote long term growth of T cells, activation and proliferation of natural killer (NK) cells, and B cell growth factor secretion (Morgan *et al.*, 1976; Ortaldo *et al.*, 1984; Inaba *et al.*, 1983).

The receptor for IL-2 plays a crucial role in the regulation of the immune response (Smith, 1988). Binding of IL-2 to its receptor on the surface of T lymphocytes triggers a series of intracellular signaling events that result in the activation and proliferation of resting T cells and ultimately, in the generation of helper, suppressor and cytotoxic T cells; all three mediate immune response Waldman, 1986). There are three forms of cell-surface IL-2 receptors, each with a different affinity for IL-2 (Leonard *et al.*, 1983; Wang and smith, 1987). The low affinity receptor (Kd $\sim$ 10 nm) is a 55-kDa polypeptide (p55), the intermediate affinity receptor (Kd $\sim$ 1 nm) is a 75-kDa polypeptide (p75), and the high affinity receptor (Kd $\sim$ 10 pm) is a noncovalent complex containing p55 and p75 (Sharon *et al.*, 1988; Burton et al., 1990). The p55 protein provides a helper binding function with no signalling capacity of its own, while the p75 protein can stimulate cell division without the assistance of the p55 chain (Robb and Kutny, 1987). In the

absence of antigen stimulation, the concentration of cell-surface IL-2 receptor on the majority of T cells is not high enough to induce proliferation (Cantrell and Smith, 1984). In the presence of antigen, T cells are induced to synthesize and secrete IL-2 as well as to synthesize cell-surface IL-2 receptor (Meuer *et al.*, 1984). Different affinity forms of IL-2 receptor are synthesized upon stimulation by antigen, leading to increased numbers of high affinity receptors on the cell surface. It appears that binding of IL-2 to high affinity receptors is the trigger necessary to promote cell cycle progression (Smith and Cantrell, 1985). Antigen- or lectin-activated T cells and certain T- and B-cell lines release a soluble truncated form of p55 IL-2 receptor that has an apparent molecular mass of approximately 45 kDa (Rubin *et al.*, 1985). Reportedly, there are low levels of sIL-2 receptor in the serum of mice (Osawa *et al.*, 1986), rats (Mouzaki *et al.*, 1987) and humans (Rubin *et al.*, 1986). The major difference between the soluble and cell-surface forms of low affinity IL-2 receptor is that the former comprises only the ligand-binding domain and lacks both transmembrane and cytoplasmic domains (Robb and Kutny, 1987).

Considerable interest has been developed in the use of serum sIL-2 receptor as a diagnostic indicator of the immune status in an individual. The level of sIL-2 receptor in serum is considered as an estimator of T cell activation and has been reported in a wide spectrum of human diseases (Rubin and Nelson, 1990).

Vitamin E enhances antibody biosynthesis and cell mediated immunity *in vivo* (Meydani *et al.*, 1990). Gogu and Blumberg (1993) reported that vitamin E increases cellular growth and glycosylation in a murine cytotoxic T-cell line

(CTLL), perhaps via increasing the expression of the IL-2 receptor. Vitamin E has been demonstrated to affect the immune response by enhancing antibody biosynthesis and cell mediated immunity *in vivo* (Gogu and Blumberg, 1993).

Roy *et al.* (1993) have shown that dietary supplementation with selenium (Se) results in a significant up-regulation of the expression of the IL-2 receptor on the surface of ConA-stimulated lymphocytes from mice. Dietary supplementation of Se, along with vitamin E, inhibits tumorigenesis and retard tumor growth in animals (Stites and Terr, 1991).

Bovine viral diarrhea (BVD) virus has been associated with diarrhea and enteric problems (Radostits and Littlejohns, 1988), abortion (Kahrs, 1968), and immunosuppression (Markham and Ramnaraine, 1985). Signs of acute disease include diarrhea, ulceration of the mucosal surface of the mouth, esophagus, stomach and intestines, fever, depression, and leukopenia (Kahrs, 1971). When pregnant cows are infected with BVD virus up to day 125 of gestation, the calf may be aborted or become persistently infected (Radostits and Littlejohns, 1988). Occasionally researchers have reported isolation of BVD virus from lungs of cattle with respiratory disease. Reggiardo (1979) stated BVD virus was isolated from 21% of lungs with pneumonia in cases of shipping fever.

The respiratory form of infectious bovine rhinotracheitis (IBR) is demonstrated by fever, anorexia, rapid breathing and clear to mucopurulent discharge (Rahrs, 1977). An alphaherpesvirus of the family Herpesviridae was isolated which is now called bovine herpesvirus type 1 (BHV-1). The virus can

infect goats, deer, and other hoofed ruminant mammals (Yates, 1982). In field conditions, about 25% of BHV-1-infected cows abort within 8 to 100 days.

The objectives of this study were to:

1. Develop an assay to measure the sIL-2 receptor in bovine serum.
2. Characterize sIL-2 receptor activity in bovine serum.
3. Determine if disease status in the bovine is associated with increased or reduced serum IL-2 activity.
4. Determine if either vitamin E or selenium treatment influences serum IL-2 receptor activity.

Materials and Methods

Animals and blood samples

Experiments to determine the binding capacity and binding affinity of sIL-2 receptor were carried out using serum from three 3-year old Holstein dairy cows. Thirty mL blood was drawn from each animal via jugular venipuncture into plain Vacutainer[1] tubes. Blood samples were centrifuged at 4° C and 1200 x g for 15 min. The serum was separated and then frozen at -20° C. Sera from diseased and healthy adult cows were provided by Dr. Donald E. Mattson[2]. Sera from calves (Hereford) treated with Se and vitamin E were provided by Dr. Harley A. Turner[3].

Iodination

Iodinated rbIL-2 was prepared by a modification of the chloramine-T method described by Khosraviani and Davis (1994). A typical iodination mixture contained the following reagents added sequentially into a 2-mL glass vial: 2.68 μL of rbIL-2[4],

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[1] Becton-Dickinson, Rutherford, N. J. 07070

[2] College of Veterinary Medicine, Oregon State University, OR 97331

[3] Eastern Oregon Agricultural Experiment Station, Oregon State University, Burns, OR 97331

[4] Dr. C. Morrison, Ciba-Geigy, Saint. Aubin Switzerland, CH 1520.

25 μL of 0.5 M sodium phosphate buffer at pH 7.4, 0.5 mCi carrier-free sodium 125-Iodide[5], and 30 μL chloramine-T (0.5 mg/ml of 0.05 M sodium phosphate buffer). The mixture was swirled for 50 sec and then 50 μL of sodium metabisulfate (125 μg/50 μL of 0.05 M sodium phosphate buffer) was added. The reaction mixture was immediately diluted with 1.0 mL of 0.05 M sodium phosphate buffer at pH 7.4. The contents of the iodinated vial were transferred to a disposable anion exchange (AG-108)[6] column to separate the iodinated rbIL-2 from free Na-iodide. The Na-^{125}Iodide uptake by rbIL-2 was determined by precipitation of an aliquot of ^{125}I-rbIL-2 with 10% trichloroacetic acid. The remaining ^{125}I-rbIL-2 was further purified by passage through a 1 x 55 cm P-100[6] (mesh 100-200) column with 0.01 M phosphate buffered saline (PBS) at pH 7.4 for elution. The iodinated rbIL-2 preparation was used immediately for radioreceptor assay or stored at 4^{o} C for a maximum of 2 weeks.

Assay conditions

After the initial studies were performed to establish optimal binding conditions, subsequent binding studies were carried out as follows. The assay contained two sets of tubes, total binding tubes (TB) and non-specific binding (NSB) tubes for each serum sample.

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[5] Amersham Life Science, Arlington Heights, Illinois 60005

[6] Bio-Rad Laboratories, Hercules, CA 94547

One hundred μL of serum was added, in duplicate, to 12 x 75 mm borosilicate tubes[7] containing 100 μL of ^{125}I-rbIL-2 (30,000 cpm) in the absence (for TB) or presence (for NSB) of 2.5 μg of unlabeled rbIL-2 in a total incubation volume of 0.5 ml PBS buffer containing 0.5% (wt/vol) bovine serum albumin (BSA). Incubation was carried out for 6 h at 32° C in a constant temperature water bath with vigorous shaking. After incubation, 0.5 ml ice-cold dextran coated charcoal (2 g activated charcoal, 100-400 mesh and 0.2 g of dextran in 0.01 M PBS) was added to each tube. The tubes were vortexed, then centrifuged at 2500 x g at 4° C for 15 min. The supernatants, containing bound IL-2, were collected and counted in a gamma counter[8].

Effects of time and serum volume

Binding of labeled rbIL-2 to the soluble form of the IL-2 receptor in serum was measured at incubation times varying from 0.5, 1, 2, 4, 6, and 8 h. In a separate assay, serum volumes from 50 to 200 μL were tested. Percent specific binding (SB) was expressed as the difference between TB and NSB. This method is similar to that described by Davis *et al.* (1992) for measuring serum growth hormone-binding protein activity.

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[7] Fisher Scientific, Pittsburgh, PA 15219

[8] Beckman 5500, Beckman Instruments, Inc. Palo Alto, CA 94303

Binding affinity and capacity

Scatchard analysis was carried out on data obtained from a competition experiment for various concentrations of unlabeled rbIL-2. The assay procedure was described in assay condition. Then, binding capacity and affinity were estimated using the LIGAND program (Munson and Rodbard, 1980) which was adapted for the microcomputer by McPherson (1985).

Effect of viral diseases on sIL-2 receptor in the cow

Serum-virus neutralization (SN) tests were performed to measure BVD and BHV-1 (IBR) antibodies in Dr. Mattson's Laboratory at the Veterinary School at Oregon State University. Briefly, sera were heat-inactivated at 56° C for 30 min and diluted in flat bottom 96 well microtiter plates using 2-fold dilution steps (50 μL per well). An equal volume of virus (50 μL) containing 100 tissue culture median infectious doses ($TCID_{50}$) was added to the diluted serum. The plates were incubated 1 h at 25° C after which the appropriate cells were added at a concentration of $5x10^5$ cells/mL. Finally, one drop of sterile mineral oil was added to each well and the plates incubated at 37° C in a 2.5% concentration of CO_2. The plates were examined for cell growth and presence of cytopathic effect after 5 to 7 d. Tests were performed in duplicate and the serum end-point titer was defined as the last serum dilution which inhibited the cytopathic effect (CPE) of the virus.

Bovine sera with BVD and IBR antibody titers above 1:256 and up to 1:1024 were chosen to determine the sIL-2 receptor activity. Bovine sera with negative antibody to BVD and IBR were used as a Control.

Effects of Se, vitamin E, and Se + vitamin E in calves with scours and without scours

Forty adult cows for each group were supplemented with Se, vitamin E, and Se + vitamin E during pregnancy. After birth, calves was also supplemented with Se, vitamin E, and Se+vitamin E. The following table shows the treatment groups.

	Cows	calves
Treatment 1 =	Vitamin E & Se bolus	Vitamin E & Se injections
Treatment 2 =	Se bolus	Se injections
Treatment 3 =	Vitamin E	Vitamin E injections
Treatment 4 =	No vitamin E or Se	No vitamin E or Se injections

The calves of the cows got injections in the following intervals:

6cc vitamin E and Se (by birth wt.) at birth

3cc vitamin E and Se (by birth wt.) at 2 wks

3cc vitamin E at 4 wks

and the cows got 40cc of vitamin E and slow release Se boluses. Blood samples were taken from all calves at birth , 7, 14, 21, 28 d. Sera were isolated and stored at -20^{o} C.

All calves were checked for any sign of scours (diarrhea). Severity of scours was scored from 1 to 4 accordance to following list.

1 = Very mild, slightly discolored, loose stool

2 = Definitely scours

3 = Severe, but not near death

4 = Severe, dehydration, white watery stool

Sera from the calves with a scours score of 2 to 4 were assayed for sIL-2 receptor. Changes in serum sIL-2 receptor activity was also measured in calves without scours from birth to up to 4 wks.

Statistical analysis

Soluble IL-2 receptor activity were analyzed with repeated measures analysis of variance to test the effects of two factors at weekly interval from birth to 4 weeks using the general linear models (GLM) procedure in SAS, 1985. Treatments were assigned to the animals according to completely randomized design. The second factor consisted of those animals who developed diarrhea and those without diarrhea. P value < 0.05 was considered statistically significant.

Results and Discussion

Effect of time and serum volume

The binding of ^{125}I-rbIL-2 to serum sIL-2 receptor was time dependent as shown in Fig. 1. At 30^{o} C, a maximum response was observed at 6 h. Prolonging the incubation time up to 8 h resulted in a decline in ^{125}I-rbIL-2 binding to serum sIL-2 receptor. Specific binding of ^{125}I-rbIL-2 increased as the serum volume increased to 200 μL (Fig. 2). Therefore, in all subsequent experiments, a serum volume of 100 μL was assayed for 6 h incubation time.

Binding affinity and capacity

Scatchard analysis (Scatchard, 1949) of binding of ^{125}I-rbIL-2 to serum sIL-2 receptor suggested a single population of binding sites with a low binding affinity (Ka= 1.5 $\pm$ 0.7 x 10^{-7} M^{-1}) and moderate capacity (Bmax= 5.6 $\pm$ 1.2 fmol/mg). The displacement curve and Scatchard plot are shown in Fig. 3. Table 1 shows the comparison of the binding characteristics sIL-2 receptor in both ovine and bovine serum.

Effects of Se, vitamin E, and Se + vitamin E in calves

Treatment of cows and calves with Se, vitamin E, or Se + vitamin E did not modulate sIL-2 receptor activity compared with controls. Control calves showed

slightly lower sIL-2 receptor activity as the calves aged. However, this decline was not significant ($p > 0.8$) (Fig. 4A). Calves treated with Se showed declining activity of serum sIL-2 receptor from week-1 to week-4, but this decline was not significant ($p > 0.10$) (Fig. 4B). Figure 4C and 4D show the sIL-2 receptor activity in calves treated with vitamin E, and vitamin E + Se. For these treatments, there were no differences in serum sIL-2 receptor activity as the animals grew ($p > 0.9$). The control animals were on adequate vitamin E and marginal Se supplementation in their diets. Therefore, it is possible that the over supplementation of vitamin E, Se, and vitamin E + Se did not have significant effect on sIL-2 receptor activity in compare to those in control animals. Scours was associated with an increase ($p < 0.01$) in serum sIL-2 receptor activity from 1.8 % in healthy calves to 2.5 % in sick calves as shown in Fig. 5. In a tissue culture assay, Gogu and Blumberg (1993) had shown that CTLL cultures in log phase and growing in 10% IL-2 medium in the presence of 1-16 μg vitamin E/mL showed a dose-dependent 2- to 15-fold stimulation of growth (assessed by ^{3}H-thymidine incorporation) relative to control. We did not measure the white blood cell (WBC) number to see if vitamin E causes an increase in the WBC population. Meydani et al. (1990) have reported that a significant increase in IL-2 formation in response to ConA was observed after vitamin E supplementation in healthy elderly humans. This suggested that vitamin E probably increased IL-2 receptor and this in turn caused an increase in the soluble form of IL-2 receptor in serum.

However, in our experiment, vitamin E did not increase the sIL-2 receptor activity in serum in calves.

Effects of viral diseases in cattle

Sera from 12 cows with positive high titer antibody against BVD and IBR and 17 healthy (negative BVD and IBR antibody titer) cows were studied. Serum sIL-2 receptor activity was higher in cows with positive antibody titers against BVD and IBR than that in cows with negative antibody titers (Fig. 6). It should be considered that some of the positive animals might be sick with other type of infectious diseases. However, the animals were selected as possible to same complication. The IL-2 receptor is expressed on and released predominantly by activated T cells (Rubin *et al.*, 1985). The level of sIL-2 receptor in serum is considered an estimate T cell activation and has been reported to be elevated in a variety of diseases in human (Stegeman *et al.*, 1993). For example, Kawasaki disease, a virally induced autoimmune-like disease of childhood, results in higher sIL-2 receptor level at the acute stage than that at the convalescent stage or in controls. Elevated levels of sIL-2 receptor also have been reported in hematologic malignancies and in a wide variety of diseases believed to be associated with aberrant immune activation; including collagen vascular diseases, transplant rejection, sarcoidosis, and mycobacterial infections (Obara, 1992). In this study we demonstrated that sIL-2 receptor is a marker of disease activity, since it is present in higher activity in diseased animals. However, the secretion of sIL-2 receptor could

be variable during the course of the disease due to other concurrent pathological events not related to BVD, IBR and scours. Altogether, these data indicate that sIL-2 receptor is a useful parameter to monitor the evaluation of BVD, IBR, and scours, but it is not a specific diagnostic marker for these diseases.

In summary these results show that an assay of sIL-2 receptor activity in bovine serum has been developed. In calves, neither Se nor vitamin E treatment had an effect on soluble IL-2 receptor binding activity. Soluble IL-2 receptor activity was higher in calves with diarrhea than in those without diarrhea. Soluble IL-2 receptor activity was also higher in viral diseased than that in non-diseased cows. Serum sIL-2 receptor might serve as a marker for immune response to several kinds of infectious diseases.

Implications

Serum sIL-2 receptor could serves as a marker for an immune response to specific disorders. While the appearance of sIL-2 receptor alone can not be used to determine the specific cause of a disease, it provides a useful marker for monitoring ongoing immune activity and contributes to the profile of the animal's overall assessment of immune and disease status. Since increased sIL-2 receptor levels have been demonstrated in viral infections as well as bacterial infection as described above, the appearance of above average titers of the soluble receptor may be indicative of the condition of the immune system. Serum levels of sIL-2 receptor may also prove to be valuable as a means of monitoring and regulating the success of drug therapy.

Table V. 1. Binding characteristics of ^{125}I-rbIL-2 to soluble form of IL-2 receptor in ovine and bovine serum.

Species	KD [Mol]	Bmax [Mol]	Hill Co.
Sheep	$1.30 \pm 0.15 \times 10^{-7}$	$8.0 \pm 0.85 \times 10^{9}$	$0.90 \pm .06$
Cow	$1.46 \pm 0.67 \times 10^{-7}$	$5.56 \pm 1.20 \times 10^{9}$	$0.92 \pm .04$

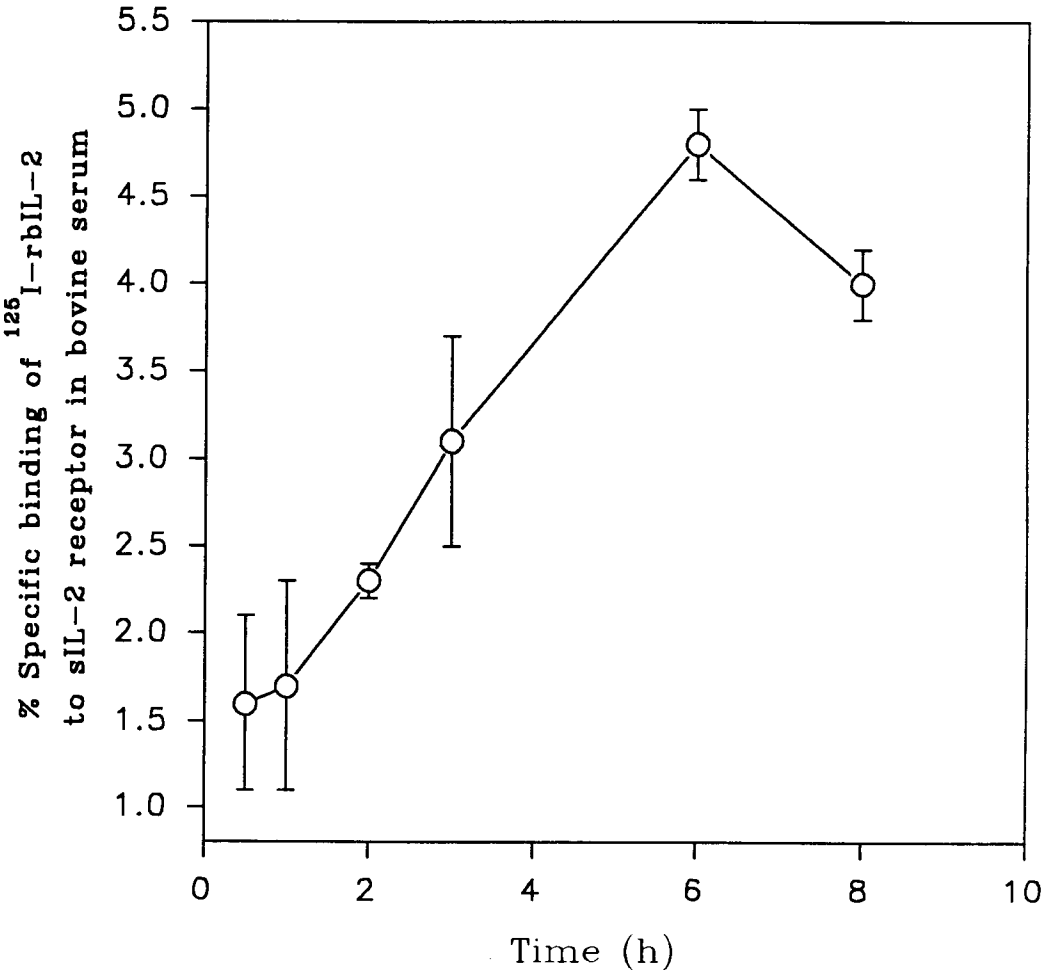

Figure V. 1. Effect of increasing incubation time on sIL-2 receptor activity using ^{125}I-rbIL-2.

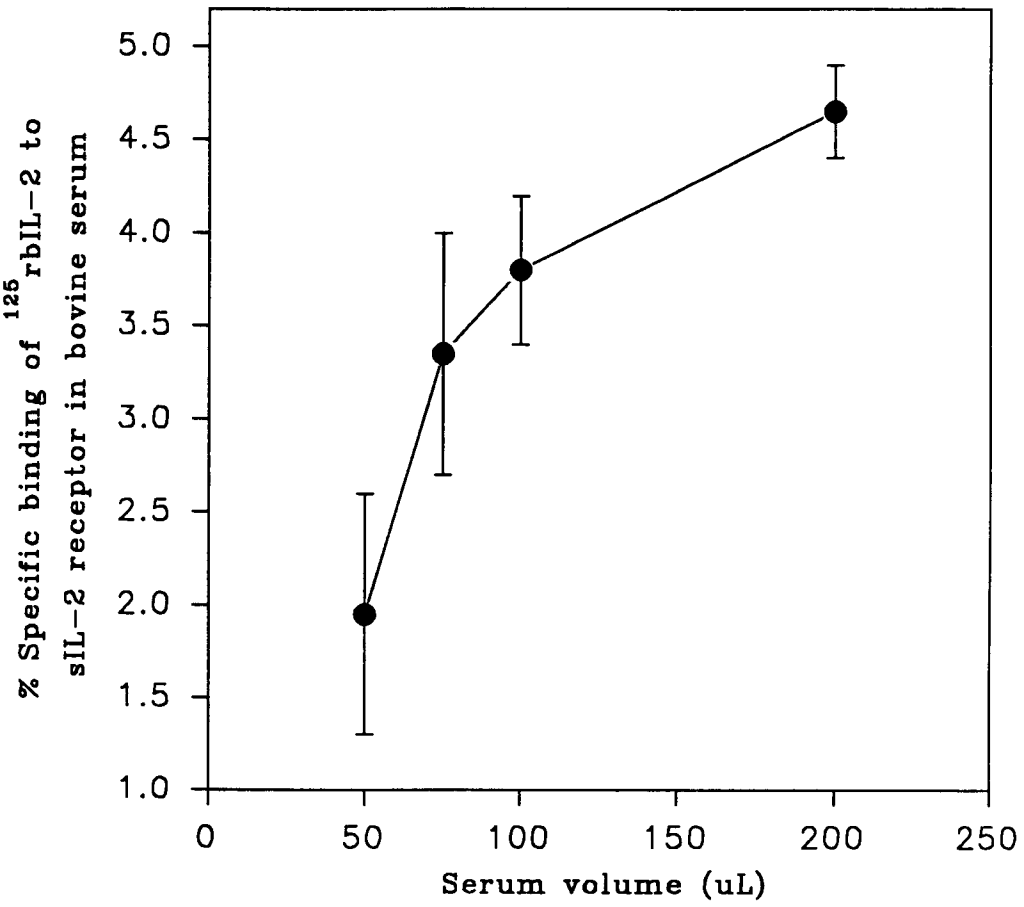

Figure V. 2. Effect of increasing serum volume on sIL-2 binding activity using ^{125}I-rbIL-2.

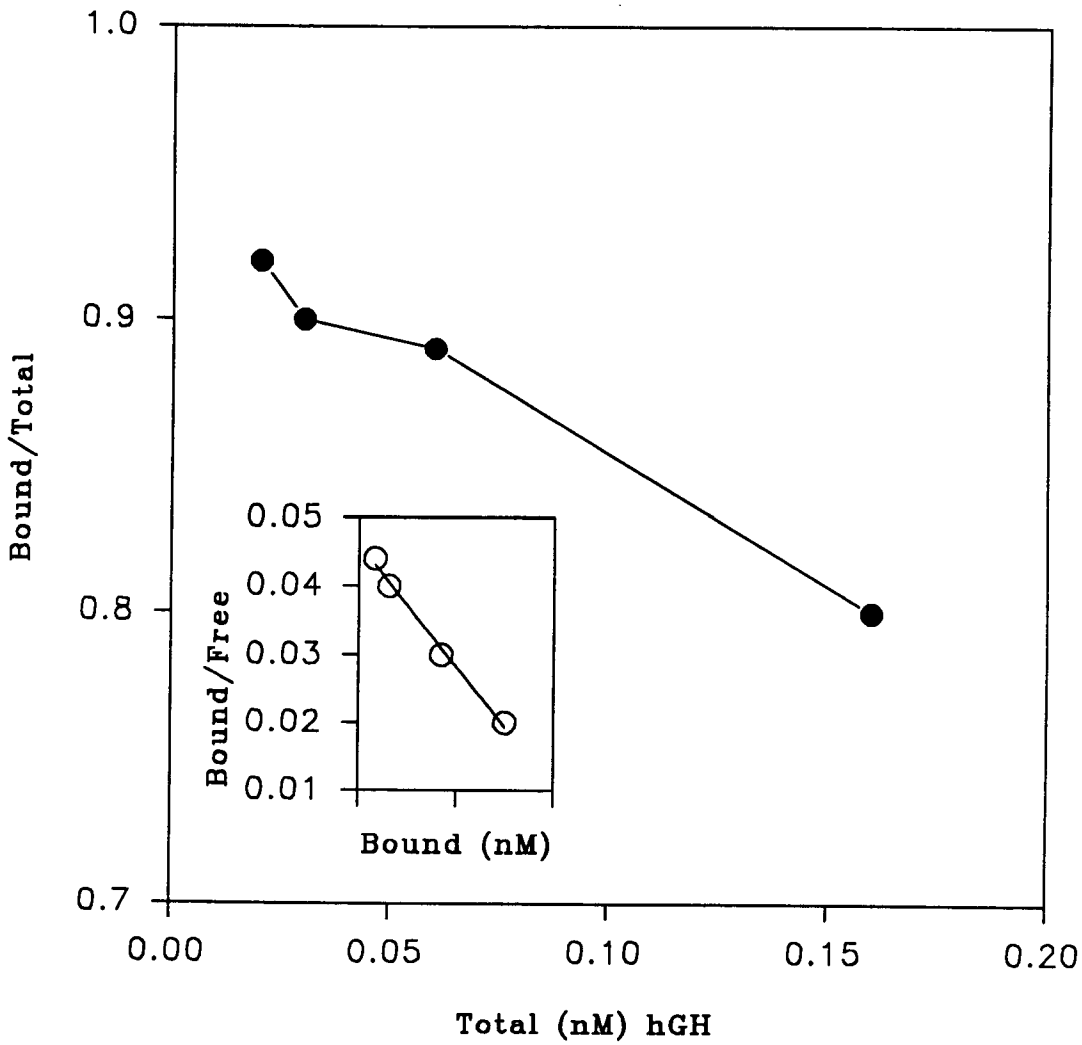

Figure V. 3. Ability of increasing dose of rbIL-2 to displace ^{125}I-rbIL-2 from serum sIL-2 receptor. Scatchard analysis of data is shown (insert).

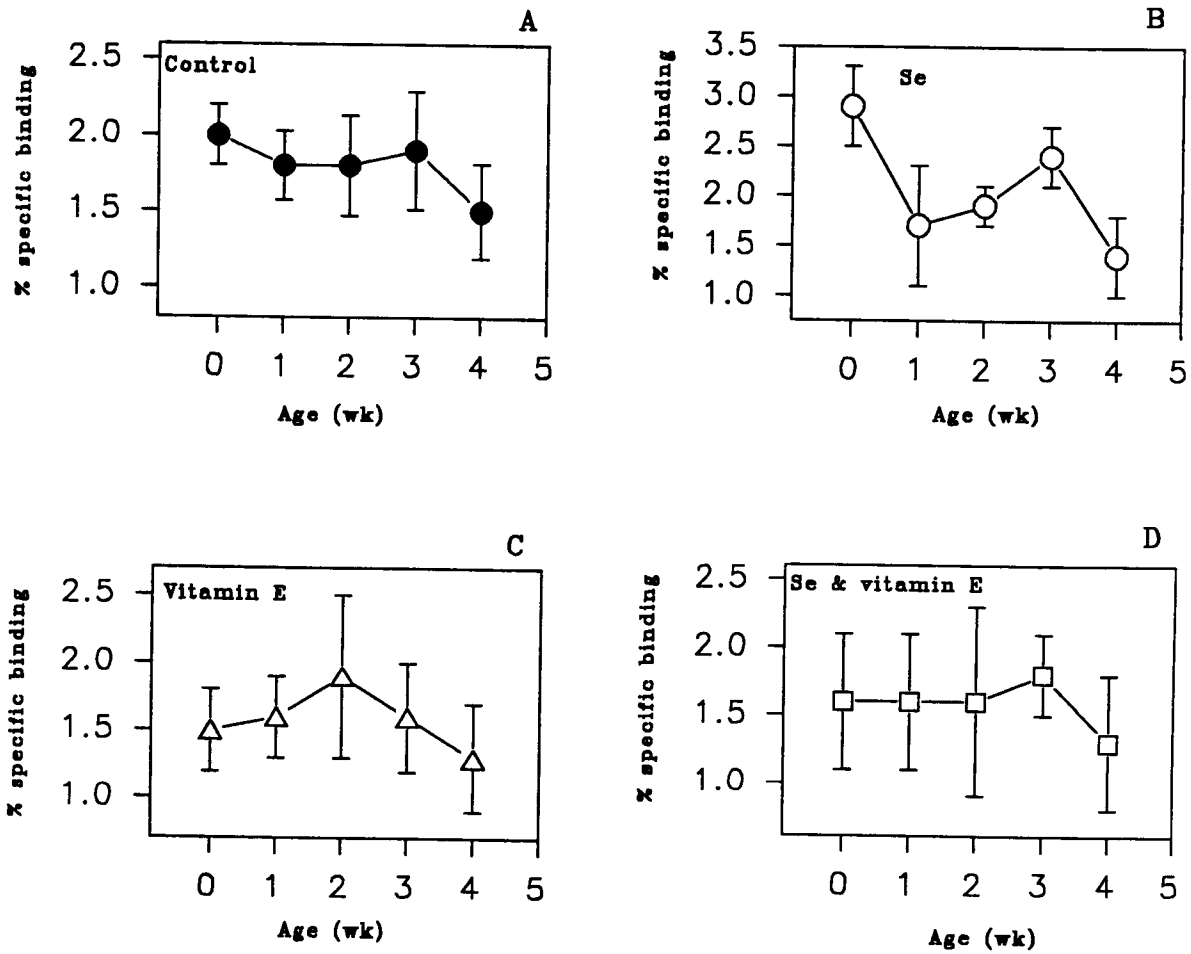

Figure V. 4. Effect of treatment with Se, vitamin E, and Se + vitamin E on the specific binding of ^{125}I-rbIL-2 to sIL-2 receptor in serum of calves without scours.

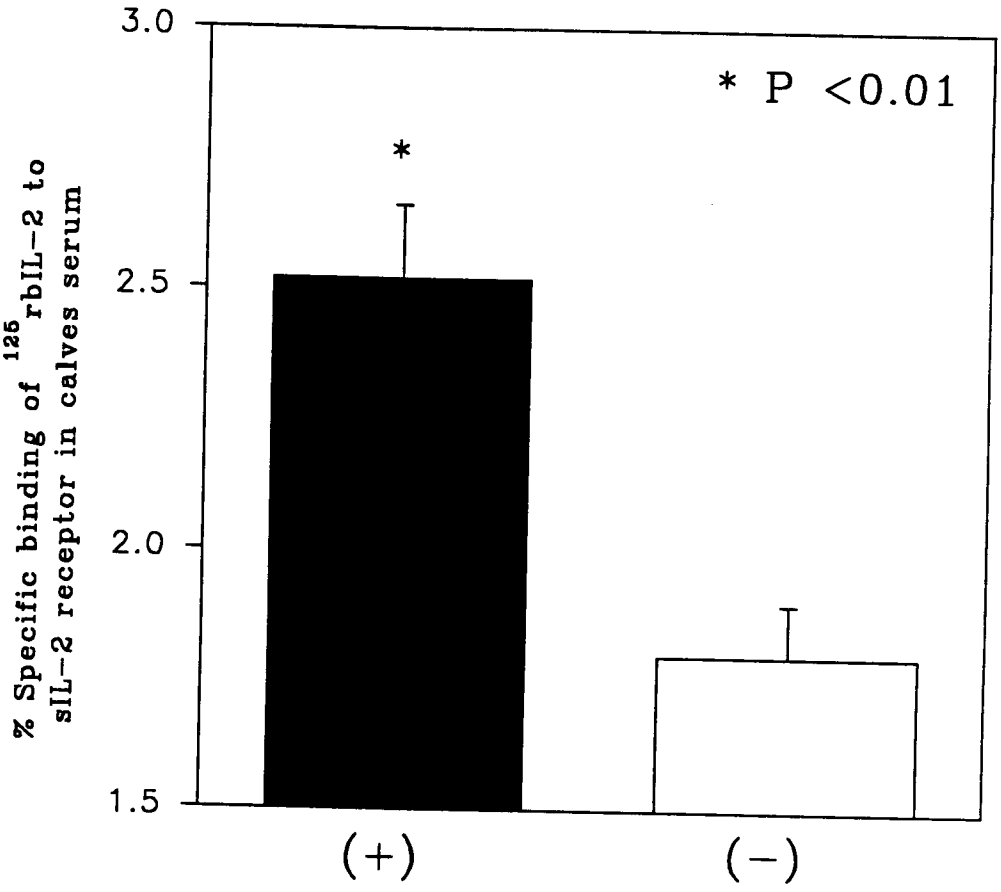

Figure V. 5. Serum sIL-2 receptor activity in calves with (+) and/or without (-) scours.

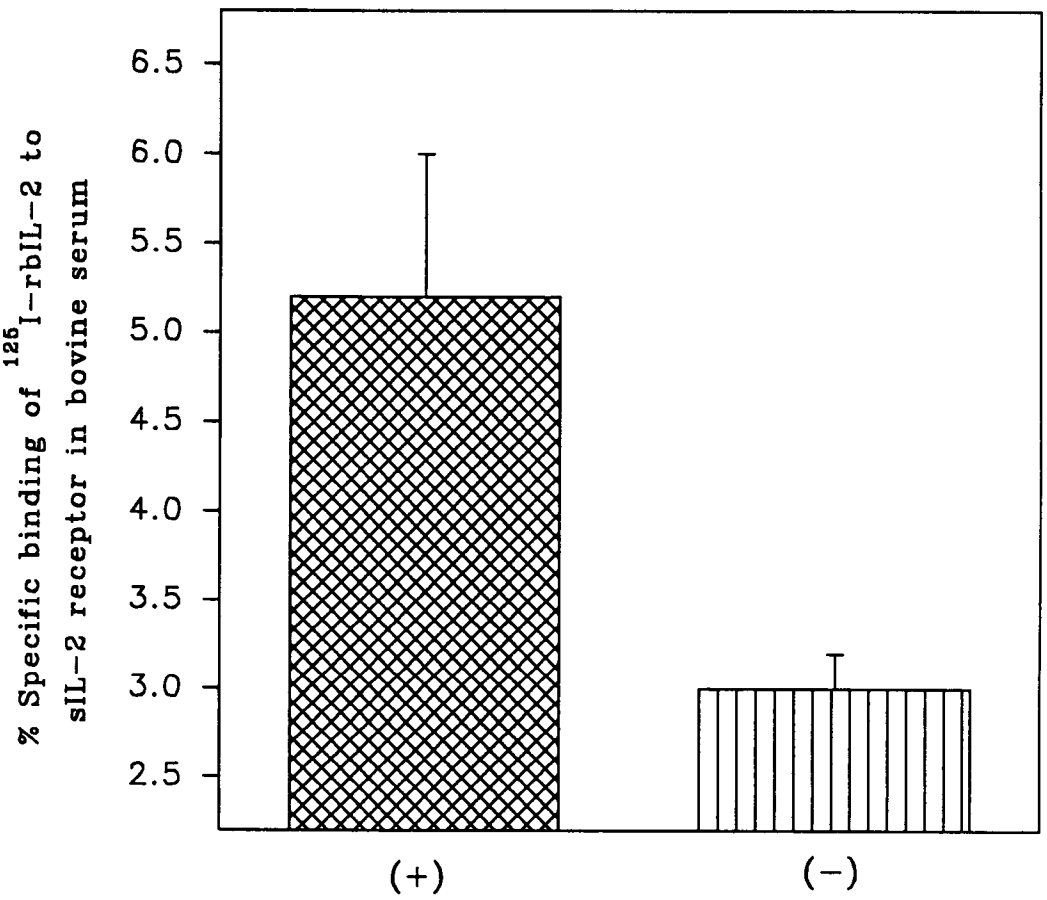

Figure V. 6. Serum sIL-2 receptor activity in cows with positive (+) antibody titer against BVD and IBR and non-positive (-) cows.

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Summary

Ovine PRL and oGH at 100 ng/mL have a stimulatory effect on the resting PBMN cell proliferation in vitro. However, when oGH is combined with IL-2, the response occurs at the higher dose of 1 or 2 μg/mL. These data suggest that PRL and GH might have regulatory effects on PBMN in sheep probably via specific receptors.

Specific binding of GH to PBMN cells increased from d 7 to d 150, but was at a very low level indicating that GH receptors are present, but at low levels. Plasma concentrations of GHBP were low during the first several weeks of age but increased ($p < 0.001$) from 30 to 150 d of age. There was a positive correlation between GH binding to PBMN cells and plasma GHBP ($r = 0.89$; $p = 0.049$).

A binding assay for serum sIL-2 receptor was developed for sheep and cattle. Using this assay, neither selenium nor vitamin E treatment had an effect on soluble IL-2 receptor binding activity in calves. Soluble IL-2 receptor activity was higher in calves with diarrhea than those without diarrhea. Soluble IL-2 receptor activity was higher in diseased than in non-diseased cows. Soluble IL-2 receptor may prove useful as a marker of immune status in future studies of hormonal treatment *in vivo*.

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APPENDIX

The following data provides more information regarding the studies described in previous chapter. The data presented in Appendix A is additional data for chapter IV. The data presented in the Appendix shows the concentration of thymulin in lambs from 7 to 150 days of age.

APPENDIX A

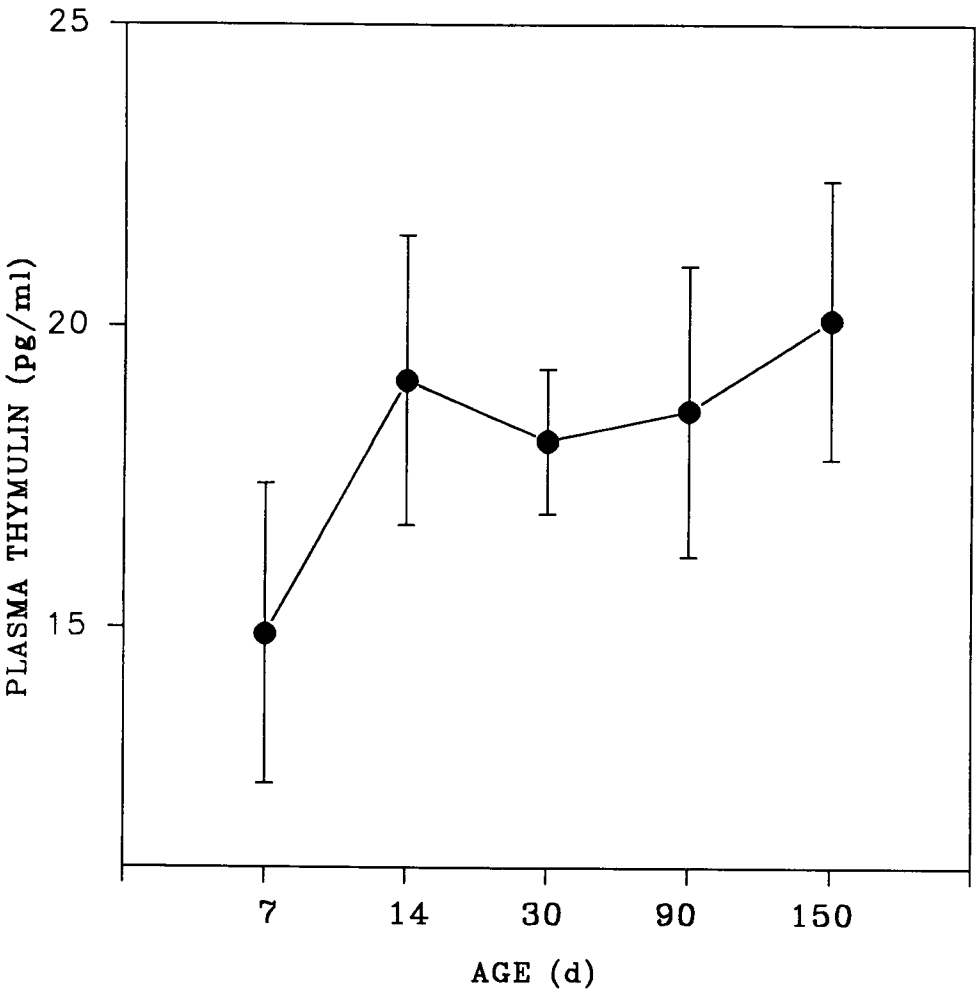

Figure A. 1. Plasma concentration of thymulin in lambs from 7 d to 150 d.

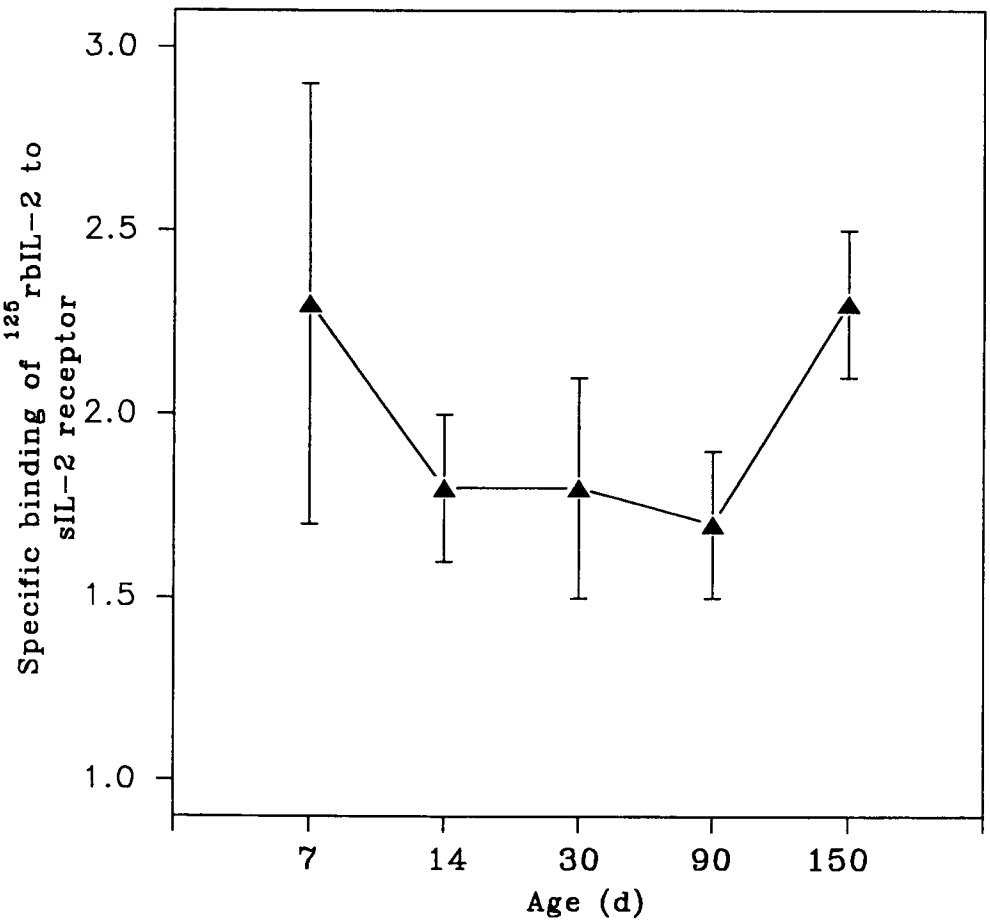

Figure A. 2. Plasma sIL-2 receptor activity in lambs from 7 d to 150 d.

Thymulin

Thymulin is produced in the thymus gland. Thymulin, a highly conserved nonapeptide, is synthesized within the thymus gland by two discrete populations of epithelial cells (Dardenne *et al.*, 1977). Thymic atrophy is seen in experimental hypophysectomy or advanced aging. Previously, investigators have tried to determine the deleterious consequences of aging on immune function by implanting GH and PRL secretory GH3 pituitary adenoma cells into aging rats (18-24 months old). This implantation resulted in partial regeneration of thymic tissue and partial restoration of T cell competence for proliferation (Kelly *et al.*, 1986). The production of IL-2 was significantly increased in these rats. Since previous observations indicated that GH3 implantation caused a dramatic rise in plasma GH and plasma PRL, the authors surmised that these hormones may have been responsible for the restoration of T cell responses seen in these animals. However, in support of these findings, exogenous GH injection of aging rats resulted in augmented spleen cell proliferation and NK cell activity (Davila *et al.*, 1987). Yet no morphologic alterations were seen in the thymus of the animals. Studies determining the combined effects of coadministration of exogenous GH and PRL on restoration of immune function would be of interest.

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