2001

The Role of Immunity in Resistance of Gulf Coast Native Sheep to Haemonchus Contortus Infection.

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THE ROLE OF IMMUNITY IN RESISTANCE OF GULF COAST NATIVE SHEEP TO *HAEMONCHUS CONTORTUS* INFECTION

A Dissertation

Submitted to the Graduate Faculty of
Louisiana State University and
Agriculture and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Interdepartmental Program in
Veterinary Medical Sciences through the
Department of Epidemiology and Community Health

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May 2001

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ACKNOWLEDGMENTS

I would like to thank my major professor Dr. James Miller for his guidance and kindness throughout my graduate education. While working with him I not only acquired experience in the fields of Epidemiology and Parasitology, but I also learned how to work in a team. I greatly appreciate his unconditional encouragement and support at times when I mostly needed it. I am also grateful tothe members of my Committee: Dr. T. R. Klei, Dr. D. W. Horohov and Dr. M.E. Hugh Jones for the support and advice provided for my dissertation.

I would also like to thank the faculty and staff of the Department Epidemiology and Community Health including Drs. M.G. Groves, S. Shane, R. A. Thompson, D.T. Scholl, Mr. J.P.T. Roberts, Mr. M.T. Kearney, Mrs. B. Elbourne, Mrs. S. Barras, Mrs. J. Broussard, and Mrs. K. Harrington, for being like a family to me during my stay here. The help of Mrs. S. Porcieu is highly appreciated.

A special thank to the staff, graduate students and student workers in the lab, without their help I could not have accomplished my work. I am also grateful to Mr. T. Harding, staff and students working at the Central Research Station Sheep Unit for their kindly help with my research.

My studies and work would not have been possible without the support of my friends and family. I would like to give special thanks to my parents Dr. R.V. and M.T. Peña who guided me with happiness and unconditional love throughout my life.

I thank the Almighty God for giving me health and joy and to whom I am grateful for giving me the opportunity to study and work in such a nice environment.

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ABSTRACT

Haemonchus contortus is a serious constraint for sheep production in tropical and subtropical regions of the world. The use of genetically resistant animals is a promising alternative for controlling nematode infections without relying on the use of anthelmintics. Extensive epidemiological studies demonstrated that Gulf Coast Native sheep are naturally resistant to *H. contortus* infection, but the mechanism underlying this resistance is not well known. The main purpose of this research was to define the components of the immune response that may be involved in resistance of GCN to *H. contortus* infection. Three studies were conducted. In the first study, a group of neonate lambs was treated with dexamethasone for ten weeks while grazing on pasture with their dams. All lambs were monitored weekly for blood packed cell volume (PCV), white blood cell differential, and fecal egg count (FEC) for the duration of the study. The second study was similar to the first one but post-weaned lambs were used. They were kept in dirt floored pens and experimentally infected. Treated lambs showed higher FEC and nematode burden and lower PCV and antibody titer to *H. contortus* whole nematode antigen, compared to controls. These studies indicate that there is a component of the immune response that may play a role in the natural resistance of GCN sheep to *H. contortus* infection. To further characterize components of the immune response, a third study was conducted in which a group of GCN lambs was depleted of their $CD4^+$ T lymphocytes and challenge with *H. contortus* infective larvae. The lambs in the treatment group received serial injections of mouse monoclonal antibody to sheep $CD4^+$ T

lymphocytes. Lambs in the treatment group showed higher FEC and nematode burden than the controls. These results indicated that $CD4^+$ T lymphocytes were important in immunity of GCN sheep to *H. contortus* infection. This research has contributed to the better understanding of the mechanism(s) underlying natural resistance of GCN sheep to *Haemonchus contortus* infection.

CHAPTER 1

GENERAL INTRODUCTION

1.1. Background

Gastrointestinal (GI) nematode parasitism is a major problem in sheep production worldwide. In Pakistan, annual losses resulting from haemonchosis were estimated at US $781,945 (Javed *et al.*, 1992). Mcleod (1995) using a cost-benefit model estimated that nematodes cost the Australian sheep industry $222 million per year. In Kenya annual losses to the agriculture industry due to *Haemonchus contortus* were estimated at $26 million (Allonby, 1973). In a survey conducted in by USDA/APHIS (USDA, 1996), GI nematode parasitism was reported as of moderate to high concern to U.S. sheep producers (62% of the operations). In addition, GI nematode parasitism was a condition reported to be present in the previous 5 years in 49% of the operations (USDA,1996). Most parasite losses are subclinical, go unnoticed, are not measurable, and probably far exceed the estimates. Loss of production, costs of anthelmintics, and animal death are some of the major concerns associated with the widespread occurrence of infection with GI nematode parasites, particularly *H. contortus*. Chemical products are commonly used for the control of nematodes, but the evolution of anthelmintic resistance in nematode populations threatens the success of drug treatment (Craig, 1993; Prichard, 1990; Sangster, 1999; Sangster and Gill, 1999; Waller, 1987, 1994). In addition, there is an increasing awareness of environmental issues that may influence the use of anthelmintics as more consumers demand animal products and pastures that are free of chemical residues. Due to these facts, there is a need for alternative approaches for controlling GI nematode parasites.

One alternative is the use of vaccines against nematodes and such efforts have been directed against *H. contortus*. The first approach for vaccine development was the use of irradiation to attenuate infective larvae. Smith and Christie (1979) reported 98.9% protection with an irradiated larvae vaccine against *H. contortus*. Vaccines made from excretory-secretory products from adult *H. contortus* worms induced protective immune responses that resulted in 68% reduction in the number of worms (Schallig and Van Leeuwen, 1997). Finally, the most recent finding is that proteins present at the surface of the parasite's gut, known as hidden antigens, have been shown to confer protection (Andrews *et al.*, 1995, Smith *et al.*, 1994). Protection levels reached by hidden antigen vaccines were reported to be higher than 90% reduction in FEC's and 75% reduction in nematode burdens (Newton, 1995). Although high levels of protection are conferred by the different vaccines there is still no commercial vaccine available.

Biological control such as nematode trapping fungi, is another approach for controlling GI nematodes. Several studies demonstrated that larval populations of nematodes were significantly reduced in feces and pasture grazed by fungus-treated animals (Nansen *et al.*, 1995; Wolstrup *et al.*, 1994; Larsen *et al.*, 1998; Larsen *et al.*, 1994; Fernandez *et al.*, 1999; Faedo *et al.*, 1997; Gronvold *et al.*, 1993). Although these results are promising, there are some concerns to be considered such as feasibility of using the same isolates in different climatic conditions, delivery methods, and the negative effects that certain anthelmintics may have when used together in an integrated control strategy (some benzimidazoles have anti-fungal activity).

The use of genetically resistant animals is another method for controlling parasitic infections. The evidence for genetic variation in resistance to infection with nematodes comes from three sources: variation within breeds, variation among breeds,

and the identification of genes contributing to the variation (Stear and Murray, 1994). Examples of within breed variations include genetic variation in resistance to infection with *H. contortus* in Australian Merinos (Gray *et al.*, 1990; Gray *et al.*, 1992; Gray and Gill, 1993), and with *Teladorsagia circumcincta* in Scottish Blackface (Stear and Murray, 1994). There is substantial evidence that supports variations in susceptibility to *H. contortus* infections between breeds. Breeds with high resistance to *H. contortus* include the Scottish Blackface (Abbott *et al.*, 1985a,b; Altaif and Dargie, 1978), Red Massai (Preston and Allonby, 1978, 1979; Bain *et al.*, 1993; Baker *et al.*, 1993, 1994), Romanov (Gruner *et al.*, 1986), Barbados Blackbelly, Florida Native, Saint Croix (Bradley *et al.*, 1973; Yazwinski *et al.*, 1979; 1980, Courtney *et al.*, 1985a,b; Gamble and Zajac, 1992), and Gulf Coast Native (Lemarie *et al.*, 1987; Lemarie, 1988, Miller *et al.*, 1993; Miller *et al.*, 1998; Bahirathan, 1994; Bahitaran *et al.*, 1996).

Gulf Coast Native (GCN) is a breed of sheep raised throughout the Gulf Coast region which has survived without benefit of much intensive management effort such as deworming. These sheep are extremely hardy, have excellent mother instincts, and are considered to be parasite and foot rot resistant. The ability for them to overcome the hot and humid conditions characteristic of the subtropical Gulf Coast region makes the breed a prime candidate for production throughout the southern states. This breed was derived form the first sheep brought to the area by Spanish and French explorers and settlers beginning in the 1500s. Merino and Rambouillet were the first breeds introduced whereas Southdown, Hampshire, Dorset Horn and Cheviot were introduced more recently. Crossbreeding these breeds and natural selection played a major role in the development of the GCN breed. Parasite resistance is one of the most important features of this breed and has been demonstrated by extensive epidemiological studies

(Lemarie *et al.*, 1987; Lemarie, 1988; Miller *et al.*, 1993; Bahirathan, 1994; Bahitaran *et al.*, 1996; Bradley *et al.*, 1973; Amarante *et al.*, 1999).

1.2. The Parasite, Life Cycle and Pathogenicity.

Haemonchus contortus (Rudolphi, 1803) is a nematode that is included in the Phylum Nematoda, Class Secernentea, Subclass Rhadbitia, Order Strongylida, Superfamily Trichostrongyloidea, and the Family Trichostrongylidae. This nematode is in the abomasum of sheep and goats and *Haemonchus placei* is found in cattle and other ruminants (Soulsby, 1982). The life cycle is composed by a preparasitic and a parasitic phase and is a direct life cycle. The adult parasites are located in the abomasum and lay eggs that are excreted in the feces. Under satisfactory environmental conditions development to infective larvae (L_3) is reached in four to six days. Following ingestion of L_3, exsheatment occurs in the rumen and the L_3 migrate to the abomasum and penetrate the gastric epithelial cells from which they emerge as fourth stage larvae (L_4) (Soulsby, 1982). The L_4 mature into adults in the abomasum and feed and produce eggs. The prepatent period in sheep is 15-18 days.

Haemonchus contortus is one of the most pathogenic parasites of sheep. The principal feature of *H. contortus* infection is anemia. Both the adult and the L_4 ingest blood and in addition, move and leave wounds which heamorrhage into the abomasum (Soulsby, 1982). The clinical signs of haemonchosis may be divided in three syndromes: hyperacute, acute and chronic. In the hyperacute syndrome animals are exposed to a massive infection that causes a severe anemia, dark color feces and sudden death from acute blood loss. Acute syndrome occurs in young susceptible lambs that become heavily infected. Anemia also develops but there is an expansion of the erythropoietic response of the bone marrow. The anemia is accompanied by

hypoproteinaemia and edema and deaths may occur. A common sign of this syndrome is a submandibular edema called 'bottle-jaw' (Taylor *et al.*, 1990). Chronic hemonchosis is due to low numbers of parasites and is characterized by high morbidity and low mortality. Parasitized animals are weak, unthrifty and emaciated and anemia may or may not be severe depending on the erythropoietic capacity of the animal.

1.3. Immune Response to Gastrointestinal (GI) Nematodes

Protective responses to GI nematodes involve cellular and humoral immune responses of the adaptive or acquired immunity. Four main consequences of the adaptive immune response against GI nematodes are recognized. First, rapid expulsion, which is directed against infective larvae as they enter the GI tract not allowing establishment and subsequently larvae are expelled in the feces within 24-48 hrs of challenge. Secondly, immune responses are directed against established developing larvae or pre-adults that are expelled before they reach adulthood (Miller, 1984). Thirdly, depression of fecundity characterized by decreased egg output by adult female worms. Finally, the self-cure phenomenon as described by Stewart (1955) where established adult worms were expelled over several days after challenging sheep with L_3. Several studies indicate that local hypersensitivity reactions occurred in response to the elimination of GI nematodes (Stewart, 1955; Smith *et al.*, 1984; Yakoob *et al.*, 1983). These local reactions (Type I or immediate hypersensitivity) are composed of two phases: the first (sensitization phase) occurs 1-2 days after challenge whereas the second (effector phase) occurs between 5-10 days after challenge. In the first phase, parasite antigen taken up by M cells overlying Peyer's patches or diffusing through the gut epithelium activates antigen-specific B and T cells. T cells produce cytokines that act on B cells inducing activation and differentiation and leading to antibody production

(IgE). IgE binds the antigen and IgE receptors located on a variety of cell types (eosinophils, mast cells and basophils). Once antigen-specific IgE is generated and bound to mast cells, subsequent parasite antigen exposure causes crosslinking of bound IgE resulting in degranulation and release of pre-formed (histamine) and secondary formed mediators (leukotrienes and prostaglandins). Histamine causes dilatation and increased permeability of blood vessels whereas leukotrienes and prostaglandins induce contraction of smooth muscle. In addition there is an increased mucus secretion that together with muscle contraction and increased permeability results in paralysis and disorientation of larvae and subsequent expulsion. Lee and Ogilvie (1982) demonstrated that the mucus layer acts as a barrier to establishment of *Trichinella spiralis* in immune rats, and does so in the presence either of specific antibody or a heat-sensitive component, presumably complement, in normal serum. Douch et al. (1984, 1986, 1996) showed that the presence of larval migration inhibitory compounds in the GI mucus of sheep was associated with resistance to GI nematodes demonstrated that mucosal mast cells and globule leukocytes were the source of these substances. The association of mast cells and resistance was demonstrated by Huntley *et al.* (1992) when steroid treatment of immune sheep resulted in inhibition of mastocytosis with concomitant reduction in the mucosal content of sheep mast-cell proteinase, and loss of resistance to larval challenge. In accordance Winter *et al.* (1997a) observed a lower tissue mast cell and eosinophil count that resulted in higher *Nematodirus battus* worm burdens and higher fecundity of the nematodes in lambs treated with dexamethasone that in untreated lambs.

Eosinophils have also been implicated in the immune response to GI nematodes. Mast cells products are chemotactic to eosinophils that are attracted to sites of nematode

invasion. Because eosinophils have Fc receptors, they can bind antibody-coated parasites. Once bound they degranulate and release their granule contents including products of the respiratory burst generated by eosinophil peroxidase and lytic enzymes such as lysophospholipase and phospholipase D. Major basic protein, the crystalline core of the specific granules, can damage the cuticle of nematodes. Eosinophil cationic protein is a ribonuclease that is lethal for nematodes. Eosinophil neurotoxin is also a ribonuclease that is toxic for nematode parasites causing reversible paralysis of larval stages (Miller, 1984). The release of eosinophil-derived neurotoxin will restrict motility of the larvae allowing adhering eosinophils to discharge their granule content directly on the larval surface. Rainbird *et al.* (1998), observed that eosinophils obtained from mammary washes of sheep immobilized and killed *H. contortus* larvae in vitro in the presence of antibody, complement and IL-5. Dawkins *et al.* (1989) observed that lambs selected for resistance to *Trichostrongylus colubriformis* infection based on fecal egg counts (FEC) were capable of mounting a marked eosinophilia. Accordingly, Thamsborg *et al.* (1999) showed that genetically resistant Merino ewes had higher eosinophil counts that their random-bred counterparts. Rothwell *et al.* (1993) demonstrated that there was a negative correlation between eosinophil numbers in tissues and blood and responsiveness to *T. colubriformis* infection. Eosinophils and mast cells were also related to nematode expulsion in *N. battus* infections (Winter *et al.*, 1997b).

Humoral immunity has been shown to be involved with protection against GI nematodes. The majority of the antibodies directed against the different parasitic stages are locally produced. Gill *et al.* (1992, 1994) demonstrated that there was an increase of antibody-containing cells of the abomasal mucosa after *H. contortus* challenge.

Accordingly, Smith (1977) using a radioimmonoassay technique to follow the anti-larval antibody response in the serum of sheep subjected to repeated dosing with *H. contortus* demostrated that IgA was derived from the abomasal mucosa whereas IgG was derived from blood. Smith and Christie (1978) vaccinated lambs with irradiated *H. contortus* larvae and showed that resistance to challenge was conferred by mucosal IgA and IgG. In another study, Huntley *et al*, (1998) demonstrated an increase in IgE concentrations during primary and secondary challenge with *T. circumcincta*. The evaluation of total protein and IgE in serum compared with gastric lymph confirmed that IgE was produced in the regional lymph nodes. IgE B-cell proliferation in germinal centers of GI lymph nodes indicates that IgE production against GI parasites occurs predominantly in the gut-associated lymph node tissues (Kooyman *et al.*, 1997; Mayrhofer *et al.*, 1976).

Baker and Gershwin (1993) demonstrated that IgE levels were inversely correlated with numbers of *Ostertagia ostertagi* suggesting that circulating IgE levels declined as a result of mast cell binding. Charley-Poulain *et al.* (1984) demonstrated a temporal relationship between the rise in local anti-worm IgA antibodies and the fall in the FEC. Stear and Murray (1994) found a negative correlation of *T. circumcincta* burdens with concentration of IgA plasma cells in the abomasal mucosa. Nematode length of adult *T. circumcincta* female is another feature controlled by local IgA response and specific IgA against L_4 is the major component (Stear *et al.*, 1999). Kooyman *et al.* (1997) found a negative correlation between *H. contortus* burden and serum total IgE levels, and suggested that Ig E is involved in anti-*Haemonchus* immune mechanisms. In addition to IgA and IgE isotypes, IgG1 and IgM have also been implied in the immune response to nematodes in sheep (Bisset *et al.*, 1996).

Cellular immunity is another important feature of the immune response to GI nematode infections. Several approaches have been used to analyze the role of T lymphocytes in laboratory animals. Studies that used neonatally thymectomized or athymic animals showed that GI nematodiasis was prolonged in these animals indicating that T lymphocytes are centrally involved in the acquired immune response against infection (Wakelin, 1978; Mitchel, 1979). Another approach for defining the role of lymphocytes in protection against nematodes includes adoptive transfer of protection with immune cells harvested from donors at various stages of infection. Grencis and Walkelin (1982) observed that lymphoblasts were more effective than resting lymphocytes in transferring immunity to *T. spiralis* in mice. Accordingly, Smith *et al.* (1984) demonstrated that the transfer of lymphoblasts caused a marked reduction in the worm burden of the recipient sheep. They suggested that some of the lymphoblasts and IgA containing cells transferred from the donor, selectively migrated to the gut of the recipient where, after maturation and proliferation, those present in the abomasal mucosa synthesized large amounts of IgA some of which discharged into the gastric lymph. Adoptive transfer of lymphocytes and/or bone marrow cells to an x-irradiated host has also been used to study the importance of T lymphocytes in the response to nematode infections. Ogilvie *et al.* (1977) showed that T lymphocyte-enriched populations of immune thoracic duct lymph were capable of expelling damaged worms from lethally irradiated rats. Different approaches were used in ruminants to define the role of T lymphocyte in the immune response to GI nematodes. Haig *et al.* (1989), using cell proliferation assays, observed that T lymphocyte lines consisting of greater than 90% T helper lymphocytes were generated from the peripheral blood mononuclear cells of sheep following primary and challenge *H.*

contortus infections. Furthermore, the proliferation of cell lines tested was restricted by MHC Class II but not MCH Class I molecules, further evidence that the lines were helper T lymphocytes. $CD4^+$ T helper lymphocytes can be subdivided into subsets (Th1 and Th2) according to the profile of cytokines produced. The Th1 subset, characterized by interferon-γ and IL-2 production, is involved in monocyte/macrophage mediated immune responses and is associated with cellular immunity to intracellular parasites. The Th2 subset produces IL-4, IL-5 and IL-10 and is involved in antibody production (including IgE) mast cell and eosinophil proliferation and function, and is associated with humoral immune responses to nematode parasites (Finkelman and Urban, 1992). One particular cytokine (IL-4) is responsible for causing the isotype switch from IgM to IgE. The study of sequential responses in the abomasal mucosa with the use of biopsy has also proved that T lymphocytes are involved in the immune response to GI nematodes. Using immunochemistry stained with monoclonal antibodies for $CD4^+$ and $CD8^+$, Pfeffer *et al.* (1996) showed that there was an increase of $CD4^+$ and $CD8^+$ T lymphocyte populations in the abomasal mucosa following infection with *T. colubriformis*. Mouse monoclonal antibodies have also been successfully used in studies where lambs depleted of their $CD4^+$ T lymphocyte were rendered highly susceptible to *H. contortus* infections (Gill *et al.*, 1993). In this study, lines of Merino sheep selected for resistance reverted to susceptibility when they were treated with monoclonal antibodies to deplete CD4 cells. Whether the same effect will occur in a naturally selected breed such as Gulf Coast Native remains to be determined.

Evidence previously presented indicates that the immune response to GI nematodes is an association of cellular and humoral immunity with local immunity.

Whether this is the mechanism responsible for the higher resistance of GCN sheep to *H. contortus* is yet to be elucidated.

1.4. Research Objectives

The main purpose of this research was to define components of the immune response that may be involved in resistance of GCN sheep to *H. contortus* infection.

1.5. Hypothesis Testing

1. Corticosteroid treatment of neonatal GCN lambs will render them susceptible to *H. contortus* infection.
2. Corticosteroid treatment of post-weaned GCN lambs that have been previously exposed to *H. contortus* infection will suppress acquired immunity that will result in increased susceptibility to further challenge.
3. Treatment with mouse anti-CD4 monoclonal antibodies will deplete CD4 T lymphocyte in GCN lambs and will result in higher susceptibility to *H. contortus* infection.

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CHAPTER 2

PILOT STUDY: DOSE TITRATION OF DEXAMETHASONE

2.1. Introduction

Dexamethasone is a synthetic glucocorticosteroid used as an anti-inflammatory or immunosuppressive agent. Glucocorticosteroids are naturally occurring hormones that prevent or suppress inflammation and immune responses when administered at pharmacological doses. At the molecular level, glucocorticosteroids bind with high affinity to specific cytoplasmic receptors. The hormone-receptor complex is then transported to the nucleus, where it binds to DNA sequences alters gene expression. This binding results in an inhibition of leukocyte infiltration at the site of inflammation, interference in the function of mediators of inflammatory response, and suppression of immune responses. Steroids contribute to the maintenance of normal circulation and cell membrane stability. The steroids act by stimulating the formation of lipocortin in damaged cells that suppresses the release of the enzyme phospolipase A 2 which in turn cleaves phospholipids in cell membranes to release arachidonic acid. Arachidonic acid is a substrate for cyclo-oxygenase and lypoxygenase that are precursors of prostaglandins and leukotrienes, respectively. Prostaglandin E is found in high concentrations in inflammatory exudates, it acts as a vasodilator, increases capillary permeability, and sensitizes local pain receptors. Thromboxane causes platelet aggregation and vasoconstriction, and PGI_2 causes vasodilation and inhibition of platelet aggregation, the thromboxane-PGI_2 interaction is a balance system to maintain circulatory stability and homeostasis. Leukotrienes act as major chemotactic agents attracting polymorphs to the site of inflammation. Biological actions include increased leukocyte adhesion, chemotaxis, and degranulation, increased vascular permeability,

bronchoconstriction and vasoconstriction (Barragry, 1994). The suppressive effect of the glucocorticoids on the release of arachinonic acid will result in minimization and abolition of local inflammatory processes and inhibition of chemotaxis.

Glucocorticoids suppress both the number of cells and the actions of the immune system. Cell-mediated immunity is more affected by suppressive effects of the glucocorticoids than humoral immunity. Antibody production is inhibited at higher dosages and longer-term therapy with glucocorticoids than it is used to suppress cellular immunity. The capacity of the lymphocytes to process antigens is diminished, and activation of lymphocytes previously sensitized to an antigen is inhibited (Barragry, 1994). Glucocorticoids inhibit virus-induced interferon synthesis and diminish the functional capacity of monocytes, macrophages, and eosinophils through inhibition of the formation of interleukines such as IL-1 (macrophages), IL-2 (lymphocytes), IL-3, and IL-6 and other chemotactic factors (Ferguson and Hoenig, 1995). Studies have suggested that corticosteroids may inhibit T-lymphocyte proliferation, T-lymphocyte dependent immunity, and the expression of genes encoding cytokines (IL-1, IL-2, IL-6, IFN-α, and TNF-α) (Knudsen *et al.*, 1987; Zanker *et al.*, 1990; Araya *et al.*, 1984; Vacca *et al.*, 1992). It has been shown that many genes encoding cytokines have a glucocorticoid response element in the 5′ regulatory sequence that is a target for the complex formed by the association of the corticosteroid with its intracellular receptor protein. The binding of the complex to the glucocorticoid response element blocks the transcription of the IL-2 gene (Vacca *et al.*, 1992). It has also been shown that dexamethasone suppressed IL-13 gene expression by PBMCs in a dose-dependent manner (Fushimi *et al.*, 1998).

Hamid and Aldeen (1992) showed that dexamethasone treatment in sheep, resulted in a significant increase in neutrophils and decrease in lymphocyte, together with a net increase in the total leukocyte count. According to Panaretto and Wallace (1978), the increase in the neutrophils is a result of stimulation of their release from the bone marrow and inhibition of their migration outside the capillaries. On the contrary, the reduction in lymphocytes is a consequence of the redistribution of these cells outside the circulatory system and concentration in the lymph nodes and bone marrow (Cohen, 1972). They also showed that there was a significant reduction in the eosinophil numbers, significant increase in the monocyte numbers, and a non-significant reduction in the number of basophils.

The purpose of this study was to determine the effective dose of dexamethasone capable of suppressing the immune response in Gulf Coast Native sheep.

2.2. Materials and Methods

2.2.1. Animals

Six yearling Gulf Coast Native (GCN) ewes were removed from pasture and dewormed to remove existing nematode infection. All animals were kept in dirt floor pens at the Central Station Sheep Farm, Louisiana Agricultural Experimental Station, Baton Rouge, LA. They were fed a maintenance ration and water was available at all times. The ewes were randomly allocated to three treatment groups (2 ewes per group). The ewes were treated with dexamethasone (Azium ® 2 mg/ml, injectable IM) three times a week receiving 0.5 mg/kg (Group 1), 1 m/kg (Group 2), and 2 mg/kg (Group 3) for a ten week period.

2.2.2. Hematology

Peripheral blood was collected weekly for white blood cell (WBC) and leukocyte differential determinations. WBC was obtained by an automated hematology analyzer with Cap Piercer (INC. Baker System 9110 + Plus). Differential leukocyte counts were made on cover glass smears stained with modified Wright's stain.

2.2.3. Lymphoproliferation Assays

Lymphoproliferation assays were run on peripheral blood mononuclear cells at weeks 3 and 7. Lymphocytes were isolated from whole blood using Ficoll-Plaque. After 3 washes with PBS, 1 ml of RPMI-1640 (see Appendix) was added, and cell concentration was determined with a haemocytometer using Trypan Blue to obtain a final suspension of $2x10^6$ cells/ml. Phytohemagglutinin (PHA), Concanavalin A (ConA), and Pokeweed (PW) were used to test T lymphocyte function. ConA and PW were used at 4, 2 and 1 μg/ml and PHA was used at 8, 4 and 2 μg/ml. Mitogens were diluted in RPMI-1640 and added to respective wells in 100 μl volumes. RPMI-1640 was added to the control wells, and then 100 μl of the cell suspension was added to the plates. All cultures were done in triplicate. The plates were incubated at 39°C in a humidified incubator with 5% CO_2 for 3 days. The plates were then pulsed with 0.5 μCi [^{3}H] thymidine/well for 4 hours and then harvested for liquid scintillation counting.

2.2.4. Enzyme-linked Immunosorbent Assay (ELISA) for Antibodies to *Haemonchus contortus* Whole Worm Antigen (WWA)

The ELISA test used was a modification of the procedure described by Smith *et al.* (1999). Microtitre plates were coated with 50 μl of WWA obtained from mature *H. contortus* worms. The antigen was diluted in carbonate (pH 9.6) coating buffer to 10 μg/ml. The plates were incubated at room temperature overnight and then washed with PBS containing 0.05% Tween-20 (washing buffer). Serum samples diluted 1/500 in

serum diluent were added to the plates and then incubated for 2 hrs at room temperature. Serum from a mature ewe with a high infection level was used as a positive control and was also diluted to 1/500 dilution in serum diluent and incubated for 2 hrs at room temperature. All serum samples (test and positive control) were done in triplicate. After another wash, 50 μl of rabbit anti-sheep IgG alkaline phosphatase conjugate (Kirkegaad and Perry, MD, U.S.A) diluted to 1:1000 in blocking buffer was added and incubated for 2 hrs incubation. The plates were then washed and dried, and 75 μl of pNPP substrate (Kirkegaad and Perry, MD, U.S.A.) was added. The plates were then incubated in the dark for one hour and the color reaction was stopped with 75 μl of 5% EDTA. The plates were read at 405 nm with an automatic ELISA plate reader.

2.3. Results

2.3.1. WBC Differential

Mean lymphocyte count decreased from week 6 to the end of the study in the three groups (Table 2.1). At week 10 the mean lymphocyte in the three groups was lower than the mean at week 0 and these means were lower than what is reported as being the normal (5000) in sheep (Jain, 1986). Mean eosinophil count showed a marked decrease from week 5 to the end of the study. At week 10 mean eosinophil count of the three groups was lower than what is reported as being the normal (400) and lower than the mean at week 0. Mean neutrophil count increased from week 4 and remained higher than what is reported as being the normal (2400) to the end of the study in the three groups.

2.3.2. Lymphoproliferation Assays

Lymphoproliferation assays were run on weeks 3 and 7 of dexamethasone treatment. The results of ConA stimulation are presented in Table 2.2.

There was a decrease in mean count per minute (CPM) in response to ConA stimulation from week 3 to week 7 in all the groups except for group 1 (1μg/ml) and the responses were relatively equal for groups 2 and 3 at all ConA concentrations.

Table 2.1.Weekly mean white blood differential counts for ewes given 0.5 mg/kg (Group 1, n=2), 1.0 mg/kg (Group 2, n=2), and 2.0 mg/kg (group 3, n=2) dexamethasone.

			Weeks of treatment											
Group	Dose	Type[1]	0	1	2	3	4	5	6	7	8	9	10	Mean
1	0.5	L	2945	2542	4808	2996	2976	2211	2383	4637	2769	4345	2595	3201
2	1.0	L	4072	2134	3211	4227	3436	3549	2379	3972	2797	2610	2707	3190
3	2.0	L	6151	3107	2546	4131	4060	4171	3088	2404	3858	2942	2655	3556
1	0.5	E	366	407	582	180	205	72	0	251	281	0	74	220
2	1.0	E	1508	1132	243	230	515	186	24	358	28	131	287	422
3	2.0	E	704	339	128	146	445	108	37	150	22	0	45	93
1	0.5	N	1726	5469	3786	1533	8744	3526	2942	2655	2868	3707	2827	3617
2	1.0	N	1844	1969	5060	1376	5324	2640	2632	4279	2100	2076	2480	2889
3	2.0	N	2307	4478	3269	2640	4297	3857	2270	3101	2678	3044	3459	3218
1	0.5	M	110	60	195	135	162	147	111	216	169	235	131	152
2	1.0	M	199	94	35	209	128	160	51	179	127	51	236	134
3	2.0	M	92	33	94	227	170	364	76	89	205	124	86	142
1	0.5	B	76	143	391	77	205	147	89	216	145	235	134	169
2	1.0	B	77	86	35	112	160	202	76	155	155	178	145	126
3	2.0	B	94	0	95	146	144	263	169	85	68	95	86	113
1	0.5	T	5223	8650	9762	4884	12209	6104	5525	7917	6276	8523	5663	7339
2	1.0	T	7699	5415	8634	5893	9659	6738	5163	8942	5180	5045	5856	6748
3	2.0	T	9348	8003	6131	7290	9169	8763	5639	5802	6831	6204	6355	7230

[1] L: lymphocytes, E: eosinophils, N: neutrophils, M: monocytes, B: basophils, T: total white blood count.

Lymphoproliferation in response to stimulation with PHA at 8, 4, and 2 μg/ml, is presented in Table 2.3.

There is a decrease in mean CPM in response to PHA stimulation from week 3 to week 7 for the three groups across doses and the 1.0 mg/kg dose appeared to have the greatest effect.

Lymphoproliferation in response to stimulation with PW at 4, 2, and 1 μg/ml, is presented in Table 2.4. Groups 2 and 3 only showed a decrease in mean CPM in response to PW stimulation from week 3 to week 7 and the responses were relatively equal at all PW concentrations.

2.3.3. ELISA for *H. contortus* WWA

ELISA tests were determined on weeks 2, 5 and 10 of the study. The results expressed as percent optical density (OD) are shown in Table 2.5. The three groups showed a decrease in antibody production to *H. contortus* WWA and group 2 showed the greatest decrease.

Table 2.2. Mean lymphocyte proliferation (counts per minute) in response to Concavalin A stimulation for ewes given 0.5 mg/kg (Group 1, n=2), 1.0 mg/kg (Group 2, n=2) and 2.0 (Group 3, n=2) dexamethasone on weeks 3 and 7 of treatment.

Group	Dose	Media		ConA (4 μg/ml)		ConA (2 μg/ml)		ConA (1μg/ml)	
		Week 3	Week 7	Week 3	Week 7	Week 3	Week 7	Week 3	Week 7
1	0.5	196	256	52091	28764	43785	32748	29194	35846
2	1.0	320	365	48585	8359	55528	19488	45551	20790
3	2.0	1015	102	54230	2347	57959	24797	43714	31905

Table 2.3. Mean lymphocyte proliferation (counts per minute) in response to Phytohemagglutinin stimulation for ewes given 0.5 mg/kg (Group 1, n=2), 1.0 mg/kg (Group 2, n=2) and 2.0 (Group 3, n=2) dexamethasone on weeks 3 and 7 of treatment.

Group	Dose	Media		PHA (8 μg/ml)		PHA (4 μg/ml)		PHA (2 μg/ml)	
		Week3	Week 7	Week 3	Week 7	Week 3	Week 7	Week 3	Week 7
1	0.5	148	346	40818	20703	44085	30677	28062	24147
2	1.0	349	261	44940	10336	47761	11779	43896	8379
3	2.0	1121	243	42602	30063	44968	27682	44345	27800

Table 2.4. Mean lymphocyte proliferation (counts per minute) in response to Pokeweed stimulation for ewes given 0.5 mg/kg (Group 1, n=2), 1.0 mg/kg (Group 2, n=2) and 2.0 (Group 3, n=2) dexamethasone on weeks 3 and 7 of treatment.

Group	Dose	Media		PW (4 μg/ml)		PW (2 μg/ml)		PW (1 μg/ml)	
		Week 3	Week 7	Week3	Week 7	Week 3	Week 7	Week 3	Week 7
1	0.5	130	360	41781	39108	44614	41323	33779	41516
2	1.0	320	376	37739	21614	42526	18463	40523	18143
3	2.0	779	113	52037	19986	52480	18876	51200	16216

Table 2.5. Total antibodies to *H. contortus* whole worm antigen expressed as mean percent OD for ewes (n=2 per group) given 3 dose levels of dexamethasone.

			Weeks of treatment			
Group	Dose	N	2	5	10	Mean
1	0.5	2	27.7	13.8	7.2	16.2
2	1.0	2	19.4	6.8	2.7	9.6
3	2.0	2	23.8	14	3	13.6

2.4. Discussion

Dexamethasone is a synthetic glucocorticosteroid used as an antinflammatory and immunesupressive agent. It is one of the longest acting corticosteroids used in clinical medicine. The effect of dexamethasone on the leukogram is mainly lymphopenia and eosinopenia due to cell redistribution and lysis, and increase in neutrophil counts due to the stimulation of their release from the bone marrow and inhibition of their migration outside the capillaries as well (Panaretto and Wallace, 1978). Hamid and Aldeen (1992) demonstrated that dexamethasone treatment resulted in an increase in neutrophils and decrease in lymphocytes. Similar results were observed in this study as all the treatment groups showed a decrease in lymphocytes and eosinophils counts and an increase in neutrophils counts. Basophils and monocytes counts remained unchanged. The hematology parameters led us to conclude that

dexamethasone given at 1mg/kg (IM) effectively reduced the numbers of lymphocytes in peripheral blood of sheep.

Lymphocyte proliferation assays are used as indicators of T lymphocyte activity. A number of agents can be employed to induce T lymphocyte proliferation. Lectins such as PHA, ConA, and PW are carbohydrate-binding proteins derived from plants and bacteria that activate T lymphocytes by indirectly cross-linking the T-cell receptor and are known as mitogens (Roitt *et al.*, 1998). Pruett *et al.* (1987) observed that dexamethasone suppressed both cellular and humoral aspects of the bovine immune response. They concluded that the immunesuppressive effect of dexamethasone depends on the dose of dexamethasone administered, the pharmacological level of dexamethasone in vivo, physiological adaptation of the host, mitogen dose used in evaluation, and time of evaluation post drug administration. In this study, dexamethasone treatment affected T lymphocyte functionality as was shown by low CPM across all mitogens and doses from about weeks 5-6 to the end of the study. Although all three doses of dexamethasone decreased the CPM with all mitogens tested, 1 mg/kg was more effective than 0.5 mg/kg and was as effective as 2 mg/kg. We concluded from these results that 1mg/kg was effective in decreasing T lymphocyte functionality.

Antibody production is another aspect of the immune response that can be affected by dexamethasone treatment. Pruett *et al.* (1987) demonstrated a decrease in the antibody production to KLH in dexamethasone treated steers. In this study, antibody production to *H. contortus* WWA declined throughout the study. Even though all dexamethasone doses reduced antibody production, the greater effect was seen with 1 and 2 mg/kg and both were better than 0.5 mg/kg.

We can conclude from the results of this study that dexamethasone is an effective immunesuppressive agent, and that 1 mg/kg was appropriate as a minimal dose that adequately suppressed factors involved in the immune response in GCN sheep.

2.5. References

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CHAPTER 3

EFFECT OF CORTICOSTEROID IMMUNESUPPRESSION OF GULF COAST NATIVE NEONATAL LAMBS ON RESISTANCE TO *HAEMONCHUS CONTORTUS* INFECTION

3.1. Introduction

Epidemiological studies have shown that Gulf Coast Native (GCN) sheep are more resistant to gastrointestinal (GI) nematode infection (especially *H. contortus*) than Suffolk sheep (Bahirathan *et al.*, 1996; Miller *et al.*, 1998).

Several studies demonstrated that resistance to GI nematode infection is related to a local inflammatory reaction involving different cells, antibodies, GI mucus, and inflammatory mediators. This local response, known as immune exclusion, in which incoming larvae fail to establish has been documented in sheep against *H. contortus* infection (Jackson *et al.*, 1988; Miller *et al.*, 1983; Miller *et al.*, 1985). Immune exclusion can be affected by corticosteroid treatment as several studies have demonstrated (Jackson *et al.*, 1988; Miller *et al.*, 1983; Adams, 1982, Mattews *et al.*, 1979; Huntley *et al.*, 1992). Mast cells and globule leukocytes are the cell types involved in immune exclusion of GI nematode infection (Presson *et al.*, 1988; Miller *et al.*, 1985; Huntley *et al.*, 1992; Douch *et al.*, 1986, 1996; Miller, 1984). Studies of repeated infection over prolonged periods with nematode larvae have consistently shown that there is a massive infiltration of the mucosa with mast cells and globule leukocytes (reviewed in Miller, 1984). Presson et al. (1988) observed that resistance to *H. contortus* in a resistant genotype of Merino sheep was abrogated by treatment with corticosteroids. It was demonstrated that the loss of resistance was associated with a decline in mast cell and globule leukocyte density

and decline in concentrations of sheep mast-cell proteinase (SMCP) in abomasal mucosal tissues.

Self-cure is another important mechanism involved in resistance to GI nematode infection. This phenomenon, described by Stewart (1955) is characterized by the expulsion of established adult nematodes after challenging sheep with larvae. It was also demonstrated that hypersensitivity reactions were involved in the expulsion of the established nematodes and this was evidenced by a transient rise in blood-histamine, the development of skin reactivity to an antigen prepared from larvae and the development of edema in the GI tract. Charley-Poulain (1984) demonstrated that local IgA was involved in inducing self-cure of *H. contortus* and indicated that the rise in IgA was highest when tested against L_3 and L_4 antigens. Duncan *et al.* (1978) vaccinated sheep with irradiated *H. contortus* larvae and demonstrated that protection against challenge was associated with the production of abomasal mucus IgA and serum IgG antibodies. Accordingly, Gill et al. (1992, 1994) demonstrated that there was an increase of antibody-containing cells of the abomasal mucosa after *H. contortus* challenge. Stear *et al.* (1999) indicated that there was a negative correlation of *Teladorsagia circumcinta* burdens and of nematode length and specific IgA levels. In another study, (Baker and Gershwin, 1993) showed that serum IgE levels were inversely correlated with numbers of *Ostertagia ostertagi* in calves.

T lymphocytes are also involved in the local inflammatory response to GI nematode infection. In a secondary nematode challenge, helper T lymphocytes that were sensitized after a first exposure are directed to the GI mucosa and produce cytokines that will act on another cells such as B lymphocytes to produce antibodies

and mast cells and eosinophils to release preformed and secondary formed mediators. The role of these helper T lymphocytes have been demonstrated in studies where sheep were became highly susceptible to *H. contortus* challenge after being depleted of these (Gill *et al.*, 1993; Karanu *et al.*, 1997).

The mechanisms involved in the resistance to Gulf Coast Native sheep have not being characterized yet. Whether local immune responses involving mast cells, globule leukocytes, antibody production and helper T lymphocytes are also important in the natural resistance to *H. contortus* infection in GCN sheep remain to be elucidated. The purpose of this study was to suppress the immune response (including cellular and humoral immunity) of GCN neonatal lambs with dexamethasone and assess the effect of the immunesuppression on their natural resistance to *H. contortus* infection.

3.2. Materials and Methods

3.2.1. Animals

At four weeks of age, eight (GCN) neonatal lambs were randomly allocated into either a treatment or a control group (four lambs each). Lambs in the treatment group were treated with dexamethasone (Azium ® 2 mg/ml, injectable IM) at 1 mg/kg of body weight three times a week for a period of 9 weeks. Four Suffolk neonatal lambs (susceptible breed) were maintained with and monitored similar to the GCN lambs throughout the study. The lambs were maintained on ryegrass pasture with their dams during the study. All lambs were bled and fecal sampled on a weekly basis and the GCN lambs were necropsied at the end of the study.

3.2.2. Fecal Egg Counts (FEC)

Individual fecal samples were collected from the rectum and 2 grams were processed to determine FEC using the modified McMaster technique and reported as eggs per gram (EPG) (Whitlock, 1948) (see appendix).

3.2.3. Necropsy

At the end of the study, GCN lambs were euthanized (Beuthanasia®-D, 1 ml/10lb of body weight) and necropsied to recover the GI tract for nematode recovery, enumeration and identification. Abomasal lymph nodes were also taken to collect lymphocytes for lymphoproliferation assay. As soon as the abdominal cavity was exposed the abomasal lymph nodes were taken aseptically and immersed in PBS. The abomasum was isolated from the rest of the GI tract and opened throughout the greater curvature and contents were emptied into a 10 liter tub. The abomasal mucosa was thoroughly rinsed and the abomasum was then transferred to another 10 liter tub and left to soak overnight in warm water. Water was added to the abomasal contents to bring the volume to 5 liters. The contents were thoroughly stirred and a 500 ml aliquot was transferred to a labeled plastic bottle. The small intestine was open on its entire length and the contents were emptied into a 10 liter tub. Water was added to bring the volume to 5 liters in which the SI was thoroughly rinsed and then discarded. The contents were thoroughly mixed and a 500 ml aliquot was transferred to a labeled plastic bottle. The large intestine was stripped along the caecum and proximal colon and the contents were emptied into a 10 liter tub. A 500 ml aliquot was collected following the same procedure described for the SI. The bottles were left to settle for 1 hour and 100 ml was poured off and 100 ml of

and discarded and water was added to bring the volume to 5 liters and a 500 ml aliquot was transferred to a labeled plastic bottle.

3.2.4. Nematode Enumeration and Identification

After inverting the bottle several times to thoroughly mix contents, 100 ml was poured off into a 200 mesh sieve and was washed. Contents were then washed into a beaker and iodine was added. Small amounts were poured in a petri dish and scanned using a dissecting scope. Nematodes were transferred to a slide in a drop of lactophenol and were then identified to species under a microscope. The total nematode burden in each GI compartment was obtained by multiplying the number of nematodes found in 100 ml by 50.

3.2.5. Peripheral blood tests

3.2.5.1. Hematology

Peripheral blood was collected in 7 ml EDTA vaccutainer tubes via jugular venipuncture. White blood cell leukocyte differential (WBC), and pack cell volumes (PCV) were determined on a weekly basis with the same procedures detailed in Chapter 2.

3.2.5.2. Lymphoproliferation Assays

Lymphoproliferation assays were run on peripheral blood mononuclear cells (PBMC) at weeks –1, 6 and 9 following the same procedures described in Chapter 2. A final concentration of $2x10^6$ cells/ml was used and T lymphocyte proliferation was measured by thymidine incorporation (see Materials and Methods, Chapter 2).

3.2.5.3. Enzyme-linked Immunosorbent Assay (ELISA) for Antibodies to *H. contortus* Whole Worm Antigen (WWA).

ELISA tests were run with the same procedure described in Chapter 2. The antigen used to coat the microtitre plates was *H. contortus* WWA diluted in

carbonate coating buffer to give 10 μg/ml solution (pH 9.6). Serum test samples were diluted to 1/500 in serum diluent. Serum from a mature ewe with a high infection level was used as a positive control and was also diluted to 1/500 dilution in serum diluent. The conjugate used was also rabbit anti-sheep IgG alkaline phosphatase conjugate (Kirkegaad and Perry, MD, U.S.A) diluted to 1:1000 in blocking buffer. And finally pNPP (Kirkegaad and Perry, MD, U.S.A.) was used as the substrate. The plates were read at 405 nm with an automatic ELISA plate reader.

3.2.6. Lymph Node Lymphoproliferation Assays

Abomasal lymph nodes were transferred to plastic petri dishes, cut and mashed through a strainer. The strainer was then rinsed into another petri dish with PBS to wash out the cells and the remained liquid was transferred to a 15 ml centrifuge tube. The cell suspension was brought to 14 ml with PBS and centrifuged for 30 min at 1800 rpm. The supernatant was discarded and the sediment was resuspended in PBS and centrifuged again for 10 min at 800 rpm. After two more washes with PBS and subsequent centrifugations, the sediment was resuspended with 1ml of RPMI-1640 media and cells were counted in a hematocytometer using trypan blue. The plates were set up and incubated using the same procedure described in Chapter 2 for lymphoproliferation assays on peripheral blood but only for PHA and ConA mitogens.

3.2.7. Brucellosis Card Test

At week 9, all GCN lambs were vaccinated with a killed *Brucella abortus* strain 19 (1 mg in 1 ml of saline solution per animal) vaccine. Serum samples were

on the day of vaccination and 10 days after vaccination with the Brucellosis card test (Brewer Diagnostic Kit) that is an antigen-antibody agglutination test.

3.2.8. Statistical Analysis

Statistical analysis was done with raw data except for FEC and total nematode counts which were log transformed to stabilize variance. The means of the groups were compared using PROC MIXED and PROC GLM for repeated measures in SAS. Tukey test was used to compare differences in nematode count between groups. Differences were considered statistically significant when $p<0.05$.

3.3. Results

3.3.1. Fecal Egg Count (FEC)

The overall mean FEC was 6365 EPG, 3110 EPG, and 10977 EPG for Suffolk, Control and Treated groups, respectively (Figure 3.1). The mean FEC of the T group was significantly higher than the mean of the C group from week 6 to the end of the study.

3.3.2. Total Nematode Counts at Necropsy

At necropsy, *H. contortus* was the predominant nematode recovered (Table 3.1). The mean number of *H. contortus* in T lambs was significantly higher than C lambs.

Table 3.1. Mean nematode burden in abomasum, small intestine, and large intestine of dexamethasone treated (T) and untreated (C) Gulf Coast Native neonatal lambs.

		Abomasum	Small Intestine		Large Intestine	
	N	*Haemonchus contortus*	*Trichostrongylus spp.*	*Cooperia spp.*	*Oesophagostomum spp.*	Total
C	4	238[a]	38[a]	0[a]	25	301[a]
T	4	2325[b]	225[b]	125[b]	0	2675[b]

Means with unlike superscript are significantly different ($p<0.05$).

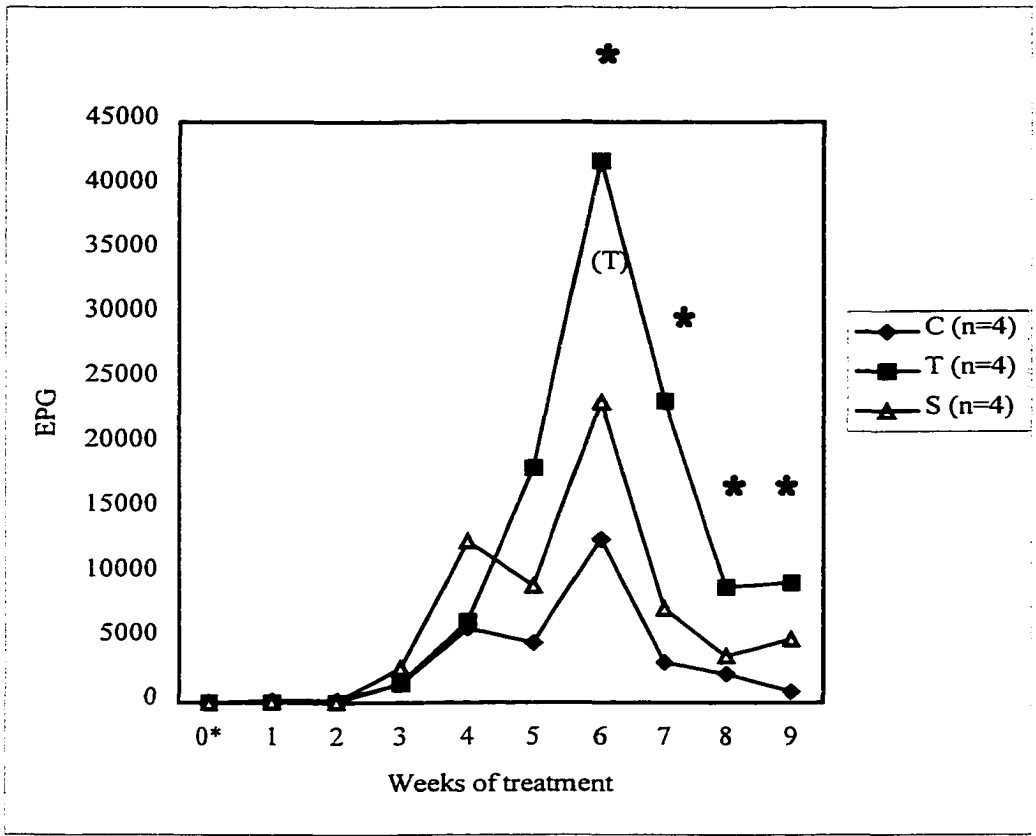

Figure 3.1. Weekly mean fecal egg count comparing dexamethasone treated (T) Gulf Coast Native (GCN) neonatal lambs with untreated (C) Gulf Coast Native and Suffolk (S) neonatal lambs. ✱ Significant differences ($p<0.05$). * At week 0, lambs were 4 weeks of age. (T) Suffolk lambs were dewormed.

3.3.3. Peripheral Blood Tests

3.3.3.1. Blood Packed Cell Volume

The mean PCV of the dexamethasone treated GCN group was similar to that of the untreated Suffolk group and both groups were significantly lower than the mean of the untreated GCN group from week 6 to the end of the study (Figure 3.2).

3.3.3.2. White Blood Cell Differential

Mean lymphocyte count in the treated group was significantly lower than that of the control group form week 7 to the end of the study, however the lower ytrend was present from week 3 (Table 3.2). The overall mean lymphocyte was also

significantly lower in the treated lambs compared to that of the control lambs. There was no significant difference in mean neutrophil count between treatment groups but there was a trend of higher counts in Dexamethsone treated lambs from week 6 to the end. Mean eosinophil count was consistently higher in the treatment group but this difference was not significant, and no differences in mean basophil or monocyte counts were seen between groups.

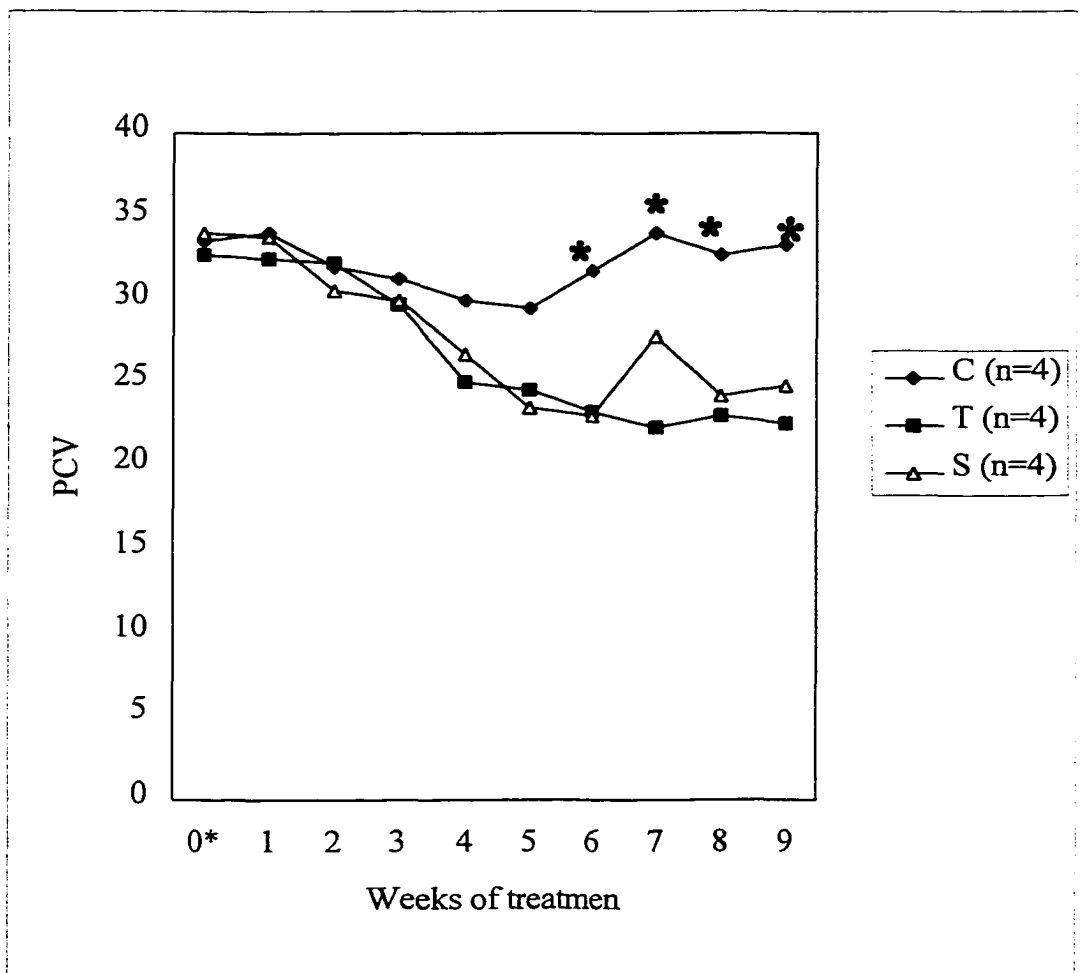

Figure 3.2. Weekly mean blood packed cell volume for dexamethasone treated (T) Gulf Coast Native neonatal lambs compared to untreated (C) Gulf Coast Native and Suffolk (S) neonatal lambs. ✱ Significant differences ($p<0.05$). *At 0 week, lambs were 4 weeks of age.

3.3.3.3. Lymphoproliferation Assays

Lymphoproliferation assays on PBMC were done on weeks –1, 6, and 9 of dexamethasone treatment.

ConA was used at 4, 2, and 1 μg/ml and the results expressed as count per minute (CPM) are presented in Table 3.3.

PHA was used at 8, 4 and 2 μg/ml and the results expressed as CPM are presented in Table 3.4.

Lymphoproliferation in response to stimulation with PW at 4, 2, and 1 μg/ml is presented in Table 3.5.

Dexamethasone treatment did not affect T lymphocyte functionality as evidenced by the lymphoproliferation results.

Table 3.2. Weekly mean white blood differential counts for dexamethasone treated (T, n=4) Gulf Coast Native neonatal lambs compared to untreated (C, n=4) Gulf Coast Native neonatal lambs.

	Type of cell											
	Lymphocyte		Eosinophil		Neutrophil		Monocyte		Basophil		Total	
Week	C	T	C	T	C	T	C	T	C	T	C	T
0	2768	2527	150	910	2166	1377	175	197	160	71	5391	4760
1	3140	2744	465	258	3461	2658	249	205	197	85	7513	5908
2	3614	3806	225	798	3369	3245	257	267	178	196	7574	8074
3	4311	2528	118	487	3592	3076	250	207	86	140	8243	6437
4	3944	2575	158	562	2096	3027	129	175	101	151	6425	6449
5	3400	2842	95	187	2545	2245	157	158	75	74	6272	5493
6	4449	2362	38	99	1638	1940	180	135	68	135	6330	4646
7	5392[a]	2700[b]	82	149	2622	3411	277	356	77	106	8450	6850
8	6164[a]	2293[b]	69	63	2702	4702	518	400	24	18	9475	7475
9	5612[a]	2836[b]	105	502	2158	2608	316	280	160	109	8350	6700
Mean	4279[a]	2721[b]	150	401	2628	2829	251	238	113	109	7515	6154

Table 3.3. Mean lymphocyte proliferation (counts per minute) in response to Concavalin A stimulation for dexamethasone treated Gulf Coast Native neonatal lambs compared to untreated Gulf Coast Native neonatal lambs on weeks –1, 6, and 9 of dexamethasone treatment.

		Media			ConA (4 μg/ml)			ConA (2 μg/ml)			ConA (1 μg/ml)		
		Weeks of treatment			Weeks of treatment			Weeks of treatment			Weeks of treatment		
Treat.	N	-1	6	9	-1	6	9	-1	6	9	-1	6	9
None	4	153	1363	519	12557	46637	29540	19954	48018	39631	18458	56502	35299
Dexa	4	2400	174	837	41675	40224	36033	44608	39058	41880	39263	38047	40212

Table 3.4. Mean lymphocyte proliferation (counts per minute) in response to Phytohemagglutinin stimulation for dexamethasone treated Gulf Coast Native neonatal lambs compared to untreated Gulf Coast Native neonatal lambs, on weeks –1, 6, and 9 of dexamethasone treatment.

		Media			PHA (8 μg/ml)			PHA (4 μg/ml)			PHA (2 μg/ml)		
		Weeks of treatment			Weeks of treatment			Weeks of treatment			Weeks of treatment		
Treat.	N	-1	6	9	-1	6	9	-1	6	9	-1	6	9
None	4	153	373	519	17618	40366	47622	15320	33476	35113	12636	31795	28332
Dexa	4	2399	173	837	34585	26558	62551	37894	29297	41618	36614	29059	31904

Table 3.5. Mean lymphocyte proliferation (counts per minute) in response to Pokeweed stimulation for dexamethasone treated Gulf Coast Native neonatal lambs compared to untreated Gulf Coast Native neonatal lambs, on weeks –1, 6, and 9 of dexamethasone treatment.

		Media			PW (4 μg/ml)			PW (2 μg/ml)			PW (1 μg/ml)		
		Weeks of treatment			Weeks of treatment			Weeks of treatment			Weeks of treatment		
Treat.	N	-1	6	9	-1	6	9	-1	6	9	-1	6	9
None	4	432	611	1866	9067	34128	34056	7636	30827	43933	5703	23265	34199
Dexa	4	3590	1906	469	34695	35331	39363	25837	37721	35541	25917	35523	29822

3.3.3.4. ELISA for *H. contortus* WWA

The antibody levels started high in both groups and steadily declined through week 5 of dexamethasone treatment due to depletion of colostral antibody

(Table 3.6.). At this time antibody levels increased in both groups and remained elevated in untreated lambs whereas antibody levels declined continually in the treated group.

3.3.4. Proliferation Assays on Lymph Node Lymphocytes

No differences were seen in the mean CPM of lymphocyte proliferation in response to PHA or ConA stimulation of abomasal lymph node lymphocytes (Table 3.7.), therefore, dexamethasone treatment did not affect T lymphocyte functionality. of the groups.

Table 3.6. Mean percent OD to *H. contortus* whole worm antigen for dexamethasone treated Gulf Coast Native neonatal lambs compared to untreated Gulf Coast Native neonatal lambs

		Weeks of treatment										
Treatment	N	0*	1	2	3	4	5	6	7	8	9	Mean
None	4	9.0	7.9	5.8	5.4	1.4	0.9	5.2	5.6	4.7	5.0	5.1
Dexa	4	16.9	14.2	11.0	8.8	3.4	0.9	4.9	3.6	2.8	1.5	7.0

* At week 0, lambs were 4 weeks of age.

Table 3.7. Mean lymphocyte proliferation (counts per minute) in response to Concavalin A and Phytohemagglutinin stimulation of abomasal lymph node lymphocytes of dexamethasone treated Gulf Coast Native neonatal lambs compared untreated Gulf Coast Native neonatal lambs

			ConA			PHA		
Treat	N	Media	4 μg/ml	2 μg/ml	1 μg/ml	8 μg/ml	6 μg/ml	4 μg/ml
None	4	640	21267	12733	6557	14912	17894	15320
Dexa	4	454	27270	19948	18369	19600	19754	15518

3.3.5. Brucellosis Card Test

On week 8 of the study, GCN lambs were vaccinated with a killed *B. abortus* strain 19 vaccine. The brucellosis card test showed that all lambs were negative prior to vaccination and all treated lambs remained unresponsive to the

vaccination, where as untreated lambs had a strong response to vaccination (Table 3.8.).

Table 3.8. Brucellosis card test results of dexamethasone treated (T) Gulf Coast Native neonate lambs compared to untreated (C) Gulf Coast Native neonatal lambs on vaccination (week 8 of dexamethasone treatment) and 10 days after vaccination (week 10 of dexamethasone treatment).

	Group	Week 8	Week 10
9052	C	(-)	3(+)
9058	C	(-)	3(+)
9059	C	(-)	3(+)
9081	C	(-)	3(+)
9064	T	(-)	(-)
9080	T	(-)	(+/-)
9084	T	(-)	(+/-)
9085	T	(-)	(+/-)

3.4. Discussion

In this study dexamethasone treatment resulted in naturally resistant GCN neonatal lambs becoming more susceptible to *H. contortus* infections as evidenced by higher FEC and nematode burden and lower PCV than non-treated GCN lambs. Previous studies by others demonstrated that sheep were rendered more susceptible to *H. contortus* challenge after corticosteroid treatment (Miller et al., 1985; Huntley et al., 1992; Mathews et al., 1979; Jackson et al., 1988). Two of those studies showed that treatment with corticosteroids was associated with a decrease in the mast cell and globule leukocyte population (Miller et al., 1985; Huntley et al., 1992). These two cell types play a role in immune exclusion which is one of the most important effector mechanisms acting in resistance to GI nematode infection and specially against *H. contortus* infections (Miller, 1984; Huntley et al., 1998;

Jackson et al., 1988). Other studies indicated that lymphocytes are also important in resistance to GI nematode infection (Kambara and McFarlane, 1996; Pfeffer et al., 1996; Gill et al., 1992, 1993; Karanu et al., 1997). It has also been demonstrated that corticosteroid treatment affected the number and functionality of lymphoctes (Mathews et al., 1979; Chun et al., 1986; Cohen, 1972; Pruett et al., 1987; Cohen et al., 1984; Hamid and Aldeen, 1992). Similar to those studies, in this study, GCN lambs treated with dexamethasone had a consistent depression in lymphocyte numbers from week 3 to the end of the study and the depression became significant at week 7. Chung et al. (1986) treated mice with glucocorticosteroids and found that the capacity of normal lymphocytes to localize within tissue sites of antigen challenge was severely decreased. They also found that the lymphocyte binding capacity of high endothelial venules from the lymph nodes was significantly decreased. They therefore suggested that lymphocytopenia was the consequence of enhanced sequestration of mature lymphocytes to the bone marrow due to changes in the circulating lymphocyte receptiveness of the bone marrow compartment. This study also observed a marked lymphocytopenia after dexamethasone treatment that might have been a consequence of lymphocyte sequestration in bone marrow compartment.

Presson *et al.* (1988) treated a resistant genotype of Merino sheep with dexamethose that resulted in reversion to susceptibility as evidenced by no differences with the susceptible genotype in FEC, nematode burdens, nematode weights, thymus weights, and globule leucocyte numbers in response to infection with *H. contortus*. In this study, GCN neonatal lambs treated with dexamethasone showed higher FEC and nematode burdens, and lower PCV and lymphocyte

numbers than the untreated lambs. The lambs treated with dexamethasone became susceptible to *H. contortus* infection and this susceptibility was similar to the Suffolk lambs. Whether the mechanisms responsible for the resistance between breeds are the same as within breed resistance remains to be elucidated, but it seems that there is a component of the immune system that is involved in both between breed and within breed resistance.

The results of the ELISA test showed that there was a continual reduction of antibodies to *H. contortus* WWA through week 5 of the study and there were no significant differences between groups. This decline coincides with depletion of colostral transferred antibodies to combat infection. After week 5, antibody production increased in each group indicating that lambs were capable of mounting an independent immune response. Again there was no significant difference between groups but the response in dexamethasone treated lambs consistently decreased and remained lower than untreated lambs through to the end of the study. These results concur with Mattews et al. (1979) and Michel and Sinclair (1969) where corticosteroid treatment reduced but did not totally suppress production of antibodies. Even though we did not find differences in the antibody titers to *H. contortus* WWA between groups, there was a marked reduction of antibodies to *B. abortus* vaccine in the dexamethasone treated group which indicated that the dexamethasone treatment did suppress antibody production. These results suggest that humoral antibodies may not be that important in resisting initial *H. contortus* infections in neonatal GCN lambs.

No significant differences were seen in lymphocte responses to T-lymphocyte mitogens PHA, ConA, and PW between control and dexamethasone

treated lambs. These results are consistent with Eckblad et al. (1984) where no differences were seen between control and dexamethasone-treated animals in lymphocyte transformation responses to PHA and ConA when dexamethasone was used at a dose rate of 0.2 mg/kg injected three times a week for 26 days. Pruett et al (1987) observed that dexamethasone treatment had a suppressive effect on lymphocyte functionality that was more notable at suboptimal dilutions of the mitogens. Suppression was minimal or not observable at optimal or greater concentrations of mitogens. They suggest that certain subpopulations of T lymphocytes may exist that have different sensitivities to the mitogens and dexamethasone. In this study, steroid resistant lymphocyte might have been circulating and collected for lymphoproliferation assays resulting in a similar response to mitogen stimulation in the groups. Muscoplat et al (1975) suggested that recovery of responsiveness to PHA was possibly the result of changes in the ratio of two populations of T lymphocytes, one steroid sensitive and one steroid resistant. Initial suppression in response to high mitogen was the result of a higher ratio of steroid-sensitive cells. However, with the continued administration of dexamethasone, elimination of steroid-sensitive cells from circulation would occur. Recovery of responsiveness would result with accompanying recruitment of steroid-resistant cells into circulation, thereby favoring a responsive ratio. In this study, the lack of a difference in T lymphocyte response to mitogens between dexamethasone treated and control lambs might suggest that the prolonged treatment with dexamethasone changed the ratio of the sub-populations of T lymphocytes resulting in a higher proportion of steroid-resistant cells.

Although lymphocyte response to mitogen stimulation was similar in both groups, there was a significant depression in lymphocyte numbers in the treated group compared to the untreated group. Dexamethasone treatment might have also resulted in inhibition of homing of T lymphocytes to the abomasal mucosa and therefore, fewer B lymphocytes were stimulated to produce antibodies. A decrease in antibody production (which could have been IgE) might have resulted in less IgE bound to mast-cells and consequently a lower inflammatory response associated to a higher nematode establisment in treated lambs. It might also be suggested that dexamethasone decreased mast cells and globule leukocyte numbers as was shown in several studies (Huntley *et al.*, 1992; Winter *et al.*, 1997; Miller *et al.*, 1985). Although this cannot be confirmed in this study, the higher FEC and nematode counts seen in the treated lambs may suggest that dexamethasone treatment was effective in suppressing the inflammatory response allowing higher nematode establishment in the treated lambs compared to the control lambs. The results of this study suggest that some component(s) of the immune response were affected by dexamethasone treatment that may be important in the natural resistance of GCN neonatal lambs to *H. contortus* infection.

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CHAPTER 4

EFFECT OF CORTICOSTEROID IMMUNESUPPRESSION OF GULF COAST NATIVE POST-WEANED LAMBS ON RESISTANCE TO *HAEMONCHUS CONTORTUS* INFECTION

4.1. Introduction

There is a substantial body of evidence that supports variation among breeds in resistance to *Haemonchus contortus*. Breeds with superior resistance to *H. contortus* include the Scottish Blackface (Abbott *et al.*, 1985 a,b), Red Massai (Preston and Allonby, 1978, 1979; Bain *et al.*, 1993), Barbados Blackbelly, Gulf Coast Native (GCN), and St. Croix (Loggins *et al.*, 1965; Bradley *et al.*, 1973; Yazwinski *et al.*, 1979, 1980; Courtney *et al.*, 1985a,b; Gamble and Zajac, 1992; Bahirathan *et al.*, 1996; Miller *et al.*, 1998). Most of the evidence that shows that the immune system as involved in resistance to nematode infections is related to variation within breed (Presson *et al.*, 1988; Stear and Murray, 1994; Gill *et al.*, 1993; Bisset *et al.*, 1996). The mechanisms responsible for the superior resistance of these breeds have not yet been determined. It has been demonstrated that Gulf Coast Native (GCN) sheep have a higher resistance to *Haemonchus contortus* infection than Suffolk sheep (Bahirathan *et al.*, 1996; Miller *et al.*, 1998). This higher resistance is characterized by lower fecal egg count (FEC) and nematode burden in GCN lambs than in Suffolk lambs. The major difference found between breeds was that *H. contortus* was predominant in Suffolk lambs and *Trichostrongylus colubriformis* was predominant in GCN lambs. Suffolk lambs consistently had higher infections, required substantially more anthelmintic treatments, and deaths were only attributed to haemonchosis.

The mechanisms involved in the immune response to nematode infections have been reviewed in Chapter 3. Different effector mechanisms have been resported for between-breed and within-breed variations in resistance to nematode infection. Resistant Red Massai sheep and Scottish Blackface sheep show strong peripheral eosinophil responses that in turn are associated with high levels of globule leucocytes and of *H. contortus*-specific mucus IgA (Stear and Murray, 1994). An example of within-breed variations can be described by the differences in susceptibility to *Teladorsagia circumcincta* in Scottish Blackface sheep. Resistant sheep show high eosinophil, globule leucocyte and IgA plasma cell response with low nematode burden. Susceptible sheep have high IgG_1 response and high nematode burden. Bendixsen *et al.* (1995), demonstrated that the release of sheep mast cell protease by mucosal mast cell degranulation is intimately associated with the development of immunity to some GI nematodes such as *T. colubriformis* and *H. contortus*. This mechanism known as immune exclusion, in which incoming larvae are rapidly rejected at the site of parasite establishment can be inhibited by dexamethasone treatment (Emery and McClure, 1995; Huntley *et al.*, 1992).

The objective of this study was to determine the role of immunity in post-weaned GCN lambs that are naturally resistant to *H. contortus* infection after previous infection during the neonatal period.

4.2. Materials and Methods

4.2.1. Experimental Design

Fifteen five-month-old GCN lambs were removed from pasture and denematodeed to remove existing nematode infection. Lambs were maintained in dirt floor pens at the Central Station Sheep Farm, Louisiana Agricultural

Experimental Station, Baton Rouge, LA. They were fed a growing ration and water was available at all times. Lambs were randomly allocated to treatment (n=8) and control (n=7) groups and the lambs assigned to the treatment group were treated with dexamethasone (Azium ® 2 mg/ml, injectable IM) at 1 mg/kg of body weight three times a week throughout the study period of 14 weeks. Fecal and blood samples were collected on a weekly basis. Feces were collected directly from rectum and blood was collected by jugular venipuncture into 7 ml EDTA vaccutainer tubes. At week 6, all lambs began receiving trickle infections with 500 *H. contortus* L_3 given orally three times a week for 9 weeks. At week 8, all lambs were vaccinated with a killed *Brucella abortus* strain 19 (1 mg in 1 ml of saline solution per animal) vaccine. Pre and 10 days post vaccination serum samples were tested with the brucellosis card test (Brewer Diagnostic Kit). All lambs were euthanized (Beuthanasia®-D, 1 ml/10 kg of body weight) at the end of the study to recover nematodes found in the GI tract and collect abomasal lymph nodes.

4.2.2. Fecal Egg Counts (FEC)

Individual fecal samples were processed to determine FEC using the modified McMaster technique with 2 grams of feces (Whitlock, 1948) (see appendix). FEC was reported as egg per gram (EPG) of feces.

4.2.3 Necropsy

The protocols for necropsy processing are described in Chapter 3.

4.2.4. Peripheral Blood Tests

4.2.4.1. Hematology

Peripheral blood was processed for white blood cell differential and packed cell volume (PCV) as described in Chapter 2.

4.2.4.2. Lymphoproliferation assays

Lymphoproliferation assays were run on peripheral blood mononuclear cells at weeks –1, 5 and 14 as described in Chapter 2.

4.2.4.3. Enzyme-linked Immunosorbent Assay (ELISA)

ELISA tests to determine antibody response to *H. contortus* whole worm antigen were run on weeks 1, 3, 5, 7, and 9 post- initial experimental infection as described in Chapter 2.

4.2.5. Proliferation Assays on Lymph Node Lymphocytes

Abomasal lymph nodes were aseptically removed at necropsy and processed as described in Chapter 3.

4.2.6. Statistical Analysis

Statistical analysis was done with raw data except for FEC and total nematode counts that were log transformed to stabilize variance. The means of the groups were compared using PROC MIXED and PROC GLM for repeated measures in SAS. Tukey test was used to compare differences in nematode count between groups. Differences were considered statistically significant when $p<0.05$.

4.3 Results

4.3.1. Fecal Egg Count (FEC)

The mean FEC remained low through week 3 after experimental infection, increased slightly at week 4 and then increased dramatically in the treated group while the control group remained relatively low for the duration of the study (Figure 4.1). The difference in mean FEC was significant at weeks 5-9. The overall mean FEC for control and treated group were 497 EPG and 3722 EPG, respectively, and this difference was significant.

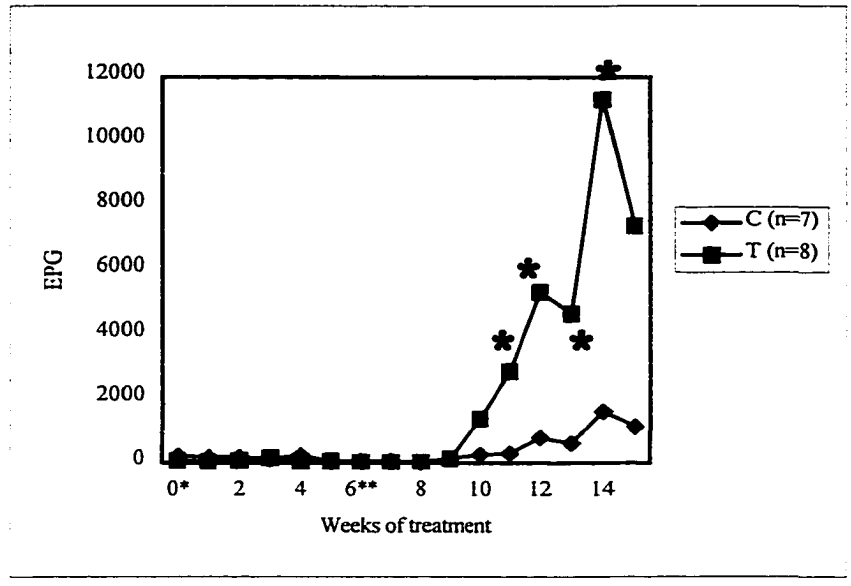

Figure 4.1. Weekly mean fecal egg count comparing dexamethasone treated (T) Gulf Coast Native (GCN) post-weaned lambs with untreated (C) Gulf Coast Native post-weaned lambs.* Significant differences ($p<0.05$). * Week 0, lambs were five-month-old. ** Lambs started receiving trickle infections of 500 infective larvae 3 times a week

4.3.2. Nematode counts

H. contortus was the predominant species (>98%) recovered from the abomasum of control and treated lambs (Table 4.1). Other species found in the GI tract were *Trichostrongylus spp.* and *Cooperia spp.* The mean number of total nematodes and *H. contortus* recovered from the abomasum of the treated group was significantly higher than that of the control group.

Table 4.1. Mean nematode burden in the abomasum and small intestine of dexamethasone treated and untreated Gulf Coast Native post-weaned lambs.

		Abomasum		Small Intestine		
Treat.	N	*Haemonchus contortus*	*Trichostrongylus spp.*	*Trichostrongylus spp.*	*Cooperia spp.*	Total
None	7	617[a]	8	42	0	667[a]
Dexa	8	1356[b]	31	29	13	1429[b]

Means with different superscript are significantly different ($p<0.05$)

4.3.3. Peripheral Blood Tests

4.3.3.1. Blood Packed Cell Volume

The mean PCV of the treated group was significantly lower than that of the control group from week 7 (1 week after experimental infections) to the end of the study (Figure 4.2).

4.3.3.2. White Blood Cell Count Differential

Lymphocyte counts did not vary that much between groups, but treated lamb counts had a trend of being lower than control lamb counts, being significant at weeks 5, 15, and 16 (Table 4.2). The overall mean lymphocyte counts were 4910 and 4341 in control and treated lambs, respectively, and this difference was significant. Mean eosinophil count of the treated group was consistently lower than that of the control group from week 3 and significantly lower at weeks 3, 8, and 11-13. The overall mean eosinophil counts were 108 and 46 in control and treated lambs, respectively, and this difference was significant. Mean neutrophil count was consistently higher in the treated group than that of the control group from week 2 and significantly higher at weeks 3, 7, 9, 10, and 12-15. The overall mean neutrophil counts were 2133 and 4297 in control and treated lambs, respectively, and this difference was significant. The mean monocyte count was significantly lower in the control group than that in the treatment group at weeks 2, 8, and 10. The overall mean monocyte counts were 165 and 274 in control and treated lambs, respectively, and this difference was significant. The mean basophil count of the treated group was significantly higher than the mean in the control group at weeks 7 and 10. The overall mean basophil counts were 44 and 53 for control and treated, respectively.

4.3.3.3. Lymphoproliferation Assays

Lymphocyte proliferation assays were run a week prior to the initial dexamethasone treatment, and at weeks 5 and 14 of treatment.

Lymphoproliferation in response to stimulation with ConA at 4, 2 and 1 μg/ml is presented in Table 4.3.

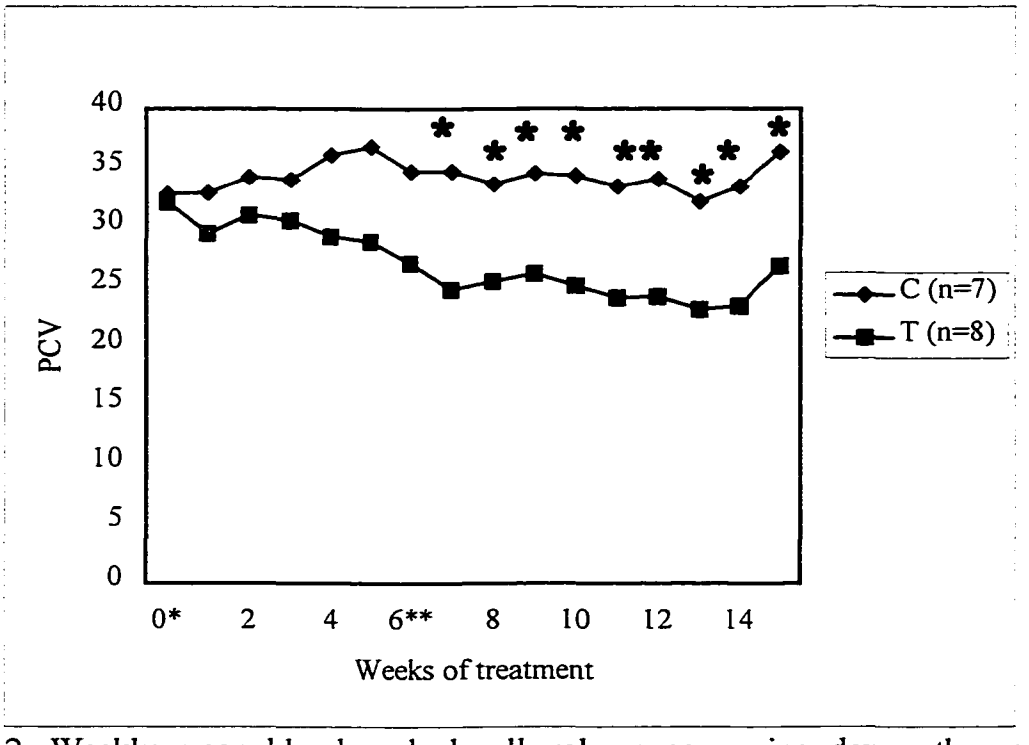

Figure 4.2. Weekly mean blood packed cell volume comparing dexamethasone treated (T) Gulf Coast Native (GCN) post-weaned lambs with untreated (C) Gulf Coast Native post-weaned lambs. * Significant difference ($p<0.05$). *At week 0, lambs were five-month-old. **Lambs started receiving trickle infections of 500 infective larvae 3 times a week.

Lymphoproliferation in response to stimulation with PHA at 8, 4 and 2 μg/ml is presented in Table 4.4.

Lymphoproliferation in response to stimulation with PW 4, 2 and 1 μg/ml is presented in Table 4.5.

Dexamethasone treatment did not appeared to affect T lymphocyte functionality as evidenced by similar lymphoproliferation responses to mitogen stimulation found between treated and control group.

Table 4.2. Weekly mean white blood cell differential comparing dexamethasone treated (T, n=8) Gulf Coast Native post-weaned lambs with untreated (C, n=7) Gulf Coast Native post-weaned lambs.

	Cell Type[1]											
	Lymphocyte		Eosinophil		Neutrophil		Monocyte		Basophil		Total	
Weeks[2]	C	T	C	T	C	T	C	T	C	T	C	T
0[3]	5644	6240	0	0	3872	3175	55	98	0	0	9571	9513
1	6046	6156	68	63	3028	3106	257	311	16	44	9843	9650
2	5650	5539	115	114	2426	4074	277[a]	730[b]	47	56	8514	10513
3	6000	5293	153[a]	47[b]	1786[a]	3949[b]	135	220	30	26	8300	9525
4	5122[a]	3567[b]	82	40	2506	2829	193	194	39	8	7943	6638
5	4509	5001	71	17.9	2097	2990	149	165	32	23	6857	8200
6[4]	4805	4149	49	48	1659	3387	226	294	38	11	6757	7875
7	4955	4783	153	75	1986[a]	4300[b]	110	280	11[a]	113[b]	7214	9538
8	4581	3929	112[a]	6[b]	2234	4165	102[a]	403[b]	90	60	7129	8563
9	4699	4565	180	98	1787[a]	5753[b]	253	364	86	58	7029	10838
10	4334	2944	102	20	1120[a]	6086[b]	96[a]	350[b]	47[a]	125[b]	5700	9525
11	4452	3606	163[a]	52[b]	1604	4335	132	97	119	72	6471	8163
12	4339	4416	164[a]	10[b]	2047[a]	5427[b]	159	146	34	77	6743	10075
13	4593	3360	190[a]	16[b]	1635[a]	4102[b]	228	314	39	95	6686	7888
14	4705[a]	3216[b]	79	81	2191[a]	6691[b]	58	171	69	67	7129	10225
15	4116[a]	2688[b]	53	51	2142[a]	4386[b]	201	243	11	6	6514	7375
Mean	4910[a]	4341[b]	108[a]	46[b]	2133[a]	4297[b]	165[a]	274[b]	44	53	7400	9006

[1] Differences within cell type with unlike superscript are significantly different (p<0/05)
[2] Weeks of treatment.
[3] At week 0, lambs were five-month-old.
[4] Lambs started receiving trickle infections with 500 infective larvae 3 times a week.

4.3.3.4. ELISA for *H. contortus* WWA

At the time of experimental infection, serum antibody levels (expressed as percent optical density OD) were similar in both groups (Table 4.6). Serum antibody levels remained low in the treated group and increased in the control group and this

difference was significant from week 5 after initial experimental infection. The overall mean percent ODs were 12.7 and 3.3 for control and treated groups, respectively, and this difference was significant.

Table 4.3. Mean lymphocyte proliferation (counts per minute) in response to Concavalin A stimulation for dexamethasone treated Gulf Coast Native post-weaned lambs compared to untreated Gulf Coast Native post-weaned lambs on weeks –1, 6, and 9 of treatment.

		Media			ConA (4 μg/ml)			ConA (2 μg/ml)			ConA (1 μg/ml)		
		Weeks of treatment			Week of treatment			Week of treatment			Week of treatment		
Treat	N	-1	5	14	1	5	14	-1	5	14	-1	5	14
None	7	525	3065	700	24211	40482	26544	25831	39798	24275	17970	39794	20832
Dexa	8	856	3877	171	19074	33094	19350	19441	35191	16946	16512	27745	10048

Table 4.4. Mean lymphocyte proliferation (counts per minute) in response to Phytohemagglutinin stimulation for dexamethasone treated Gulf Coast Native post-weaned lambs and untreated Gulf Coast Native post-weaned lambs on weeks –1, 6, and 15 of treatment.

		Media			PHA (8 μg/ml)			PHA (4 μg/ml)			PHA (2 μg/ml)		
		Weeks of treatment			Weeks of treatment			Weeks of treatment			Weeks of treatment		
Treat.	N	-1	5	14	-1	5	14	-1	5	14	-1	5	14
None	7	525	3065	700	23523	26572	30953	18712	28659	30513	19756	27301	30943
Dexa	8	856	3877	171	16871	15953	25372	17026	15077	24649	14483	13088	22991

Table 4.5. Mean lymphocyte proliferation (counts per minute) in response to Pokeweed stimulation in dexamethasone treated Gulf Coast Native post-weaned lambs and untreated Gulf Coast Native post-weaned lambs on weeks –1, 6, and 15 of treatment.

		Media			PW (4 μg/ml)			PW (2 μg/ml)			PW (1 μg/ml)		
		Weeks of treatment			Weeks of treatment			Weeks of treatment			Weeks of treatment		
Treat.	N	-1	5	14	-1	5	14	-1	5	14	-1	5	14
None	7	444	3877	514	20989	30953	22265	23648	30513	11968	23496	30943	16645
Dexa	8	318	3644	323	18099	25372	16304	18194	24649	15134	17549	22991	10526

4.3.4. Proliferation Assays on Lymph Node Lymphocytes.

Lymphocytes were stimulated with ConA, PHA, and PW. No significant differences were found in lymphocyte response to stimulation between the groups and across the different mitogens (Table 4. 7).

Table 4.6. Mean percent OD to *Haemonchus contortus* whole worm antigen in dexamethasone treated Gulf Coast Native post-weaned lambs and untreated Gulf Coast Native post-weaned lambs.

		Weeks after Initial Experimental Infection					
Treatment	N	1	3	5	7	9	Mean
None	7	4.6	7.9	14.2[a]	19.1[a]	17.5[a]	12.7[a]
Dexa	8	2.4	2.6	4.1[b]	3.5[b]	3.8[b]	3.3[b]

Means with unlike superscripts are significantly different ($p<0.05$).

Table 4.7. Mean lymphocyte proliferation (counts per minute) in response to Concavalin A, Phytohemagglutinin, and Pokeweed stimulation in abomasal lymph node lymphocytes of dexamethasone treated Gulf Coast Native post-weaned lambs and untreated Gulf Coast Native post-weaned lambs.

		ConA (μg/ml)				PHA (μg/ml)				PW (μg/ml)			
Treat.	N	Media	4	2	1	Media	8	4	2	Media	4	2	1
None	7	1362	16476	15724	14343	1362	14738	15654	15687	912	24503	22158	19935
Dexa	8	254	11648	11821	10588	254	10382	12041	10872	529	21441	19236	17581

Dexamathesone treatment did not affect functionality of T cells in the lymph nodes as evidenced by the similar responses treated and control groups.

4.3.5. Brucellosis Card Test

On the week of the brucella vaccination (week 8) all lambs were negative in the card test except for one animal in the treated group with a 2+ reading and one in the control group with a + reading (Table 4.8). Treated lambs remained negative at week 12 and became slightly positive on week 16. All but 1 control lamb showed strong seroconversion on weeks 12 and 16.

Table 4.8. Brucellosis card test results of dexamethasone treated (T) Gulf Coast Native post-weaned lambs compared to untreated (C) Gulf Coast Native post-weaned lambs on vaccination (week 8 of dexamethasone treatment), 10 days after vaccination (week 12), and at the end of the study (week 16).

ID	Group	Week 8	Week12	Week 16
9055	T	(-)	(-)	(-)
9070	T	(-)	(-)	(+)
9073	T	(-)	(-)	(+)
9088	T	(-)	(-)	(+)
9089	T	(-)	2(+)	(+)
NT1	T	2(+)	(-)	2(+)
NT3	T	(-)	(-)	(-)
NT5	T	(-)	(-)	(+)
9066	C	(-)	(+)	2(+)
9068	C	(-)	2(+)	2(+)
9071	C	(-)	(-)	(+)
9075	C	(-)	2(+)	2(+)
9077	C	(-)	2(+)	2(+)
9087	C	(-)	2(+)	2(+)
NT4	C	(+)	(+)	2(+)

4.4. Discussion

Similar to what was observed in the previous study using neonatal GCN lambs, post-weaned GCN lambs treated with dexamethasone became susceptible to *H. contortus* infection as evidenced by higher FEC and nematode count and lower PCV than control lambs. The results of this study also concur with Presson *et al.* (1988), where a resistant genotype of Merino sheep was rendered susceptible to *H. contortus* challenge after corticosteroid treatment. Several studies have demonstrated that corticosteroid (dexamethasone) treatment resulted in abolishment

of acquired but not innate immune responses to *H. contortus* infection (Adams, 1982; Adams and Davies, 1982; Adams, 1988; Huntley *et al.*, 1992; Jackson *et al.*, 1988; Miller *et al.*, 1985). In this study, dexamethasone treatment appeared to have an effect on acquired immunity of post-weaned lambs that were previously exposed to *H. contortus* infection during the neonatal period.

The importance of lymphocytes in resistance to GI nematodes and the effect of dexamethasone treatment on lymphocyte count has already been reviewed in Chapter 3. In this study, dexamethasone treatment resulted in consistently lower lymphocyte count in treated lambs compared to control lambs. Increases in blood and mucosal eosinophils have also been associated with the development of resistance to nematode parasites (Thamsborg *et al.*, 1999; Rainbird *et al.*, 1998; Winter *et al.*, 1997; Douch *et al.*, 1986; Douch & Morum, 1993; Gorrell *et al.*, 1988; Gill, 1991; Buddle *et al.*, 1992; Pfeffer *et al.*, 1996). Eosinophils have been observed to accumulate around the invasive L_3 of *H. contortus* (Gorrell *et al.*, 1988) and *T. colubriformis* (Douch *et al.*, 1986), and eosinophil potentiating activity in efferent lymph was shown to be inversely correlated with nematode burden challenge infection with *Teladorsagia circumcincta* (Stevenson *et al.*, 1994). Several studies have indicated that dexamethasone treatment resulted in a decreased number of eosinophils (Buddle *et al.*, 1992; Hamid & Mohi Aldeen, 1992; Eldestone *et al.*, 1978; Winter *et al.*, 1997). In this study, GCN lambs treated with dexamethasone showed consistently fewer eosinophils than the control lambs. These results suggest that lymphocytes and eosinophils (components of the cellular immune response) may be important in the resistance of GCN sheep to *H. contortus* infection.

Antibody production has also been associated with the immune response to GI nematode infection. Gill *et al.* (1993) found significantly higher anti-*H. contortus* IgA and IgG_1 antibody levels in a resistance genotype of Merino sheep compared with random-bred Merino sheep and that there was a negative correlation between FEC and the levels of IgA and IgG_1. Stear and Murray (1994) reported that sheep with the fewest IgA containing plasma cells had the highest *Teladorsagia circumcincta* burdens. Huntley *et al.* (1998) showed that there was an increase in IgE concentrations during primary or secondary *T. circumcincta* challenge, and the source of this IgE was mucosal or associated lymph nodes. In this study, treated lambs showed a significantly lower production of total antibodies to *H. contortus* WWA than the control lambs from week 5 to the end of the study. Treated lambs also showed a lower response to *B. abortus* than the control lambs. Dexamethasone treatment affected antibody production as evidenced by lower antibody production to *H. contortus* and this was associated with a higher susceptibility to this nematode infection in treated lambs compared to the control lambs. It may therefore be suggested that humoral immunity may be important in the resistance of GCN sheep to *H. contortus* infection.

Similar to that observed with neonatal GCN sheep, no significant differences were seen in lymphocte responses to T-cell mitogens PHA, ConA, and PW between control and dexamethasone treated lambs. It has been suggested two subpopulations of T lymphocytes exist: steroid-susceptible and steroid-resistant (Muscoplat *et al.*, 1975; Pruett *et al.*, 1987). A shift in the subpopulation of T lymphocytes from steroid susceptible to resistant may explain the similar response to mitogen stimulation between dexamethasone treated and control groups. On the other hand,

even though dexamethasone treatment affected lymphocyte numbers, the dose used might not have affected T functionality as evidenced by the similar responses seen in the lymphoproliferation assays for treated and control group.

GCN lambs treated with dexamethasone were rendered susceptible to *H. contortus* infection as evidenced by higher FEC and nematode counts than control lambs. Dexamethasone treatment might have affected the inflammatory response that is generated in GI nematode infection resulting in this difference. Treated lambs showed a decrease in lymphocyte numbers that might have been a consequence of the redistribution of these cells outside the circulatory system and concentration in the lymph nodes and bone marrow as suggested in previous studies (Panaretto and Wallace, 1978; Cohen, 1972). The redistribution of lymphocytes to the lymph nodes and bone marrow might have resulted in decrease or absence of directing of lymphocytes to the site of the infection (GI mucosa) and consequently a reduced number of stimulated B lymphocytes with less antibody production. A decrease in IgE production might have resulted in reduced binding of IgE bound to mast-cells and consequently reduced release of preformed and newly formed mediators that are responsible of the inflammatory response characterized by increased vascular permeability and dilatation, increased mucus secretion and increased peristalsis. The consequence of the inflammatory response is the creation of a hostile environment for the incoming larvae or the established nematodes that are expelled from the preference sites. Although this cannot be proven in this study, treated lambs did have a higher number of nematodes than control lambs and that might have been a consequence of a lower inflammatory response due to dexamethasone treatment. The consequence of dexamethasone treatment was an increased susceptibility to *H.*

contortus infection of treated lambs than control lambs and this suggested that immunity may play some role in the higher resistance of GCN to *H. contortus* infection.

4.5. References

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CHAPTER 5

EFFECTS OF CD4$^+$ T LYMPHOCYTES DEPLETION ON RESISTANCE OF GULF COAST NATIVE SHEEP TO *H. CONTORTUS* INFECTION

5.1. Introduction

Several studies have demonstrated the importance of CD4$^+$ T lymphocytes in the immunity to gastrointestinal nematode (Koyama *et al.*, 1995; Katona *et al.*, 1988; Urban *et al.*, 1991; *Gill et al.*, 1993, 1992; Karanu *et al.*, 1997).

Monoclonal antibodies (mAb) to CD4$^+$ T lymphocytes have been shown to be invaluable in characterizing and defining the role of these lymphocytes in local immune responses to gastrointestinal (GI) nematodes. Using immunocytochemistry and mAb antibodies to CD4$^+$ and CD8$^+$ T lymphocytes, Kambara and McFarlane (1996) examined T lymphocyte populations from the intestinal lymph duct and blood of sheep infected with *Trichostronglus colubriformis*. They indicated that parasite antigen-primed CD4$^+$ T lymphocytes were associated with protective immunity particularly in older animals.

Several laboratory animal models have established that CD4 T lymphocytes are required for immunity to GI nematode infections. Koyama *et al.* (1995) demonstrated that in vivo depletion of CD4$^+$ T lymphocytes resulted in the suppression of the expulsion of *Trichinella muris* in BALB/c mice. In a similar study, Katona *et al.* (1988) depleted mice of CD4$^+$ T lymphocytes which resulted in prevention of spontaneous cure of *Nippostrongylus brasiliensis* infection and inhibition of CD4$^+$ T lymphocyte function including induction of IgE response and intestinal mucosal mast cell hyperplasia. They also demonstrated that both the

persistence of increased serum IgE levels in mice infected with *N. brasiliensis* after the peak period of IgE secretion and the development of a secondary IgE response to re-infection are dependent on the continuing presence of $CD4^+$ T lymphocytes. Urban *et al.*, (1991) indicated that $CD4^+$ T lymphocytes are also required for the induction and maintenance of an IgE response following *Heligmosomoides polygyrus* infection in mice. In addition, adult nematode fecundity was increased in the depleted mice compared to the control non-depleted. This indicated that $CD4^+$ T lymphocytes play a critical role in the stability and fecundity of gastrointestinal nematode population.

Mouse mAb antibodies have also been successfully used to deplete $CD4^+$ T lymphocytes in cattle and sheep. Naessens *et al.* (1998) effectively depleted cattle for $CD4^+$ and $CD8^+$ T lymphocytes using mAb to bovine T lymphocyte antigens. They observed that when depletion was effected by intravenous injections of murine antibody isotypes that activate complement (IgG2a) the targeted cells disappeared from peripheral blood in less than 1 hour. In contrast, when non-complement binding antibody (IgG1) was used, the target cells remained in circulation for several days coated with mAb and were slowly removed until their near-total disappearance more than 1 week after the treatment. Gill *et al.* (1992) successfully depleted sheep of their $CD4^+$ T lymphocytes and showed that depleted lambs did not mount an antibody response to ovalbumin (OVA) and they did not show a skin reaction to T-lymphocytes mitogens. When anti-$CD4^+$ T lymphocyte mAb was administrated to a genetically selected line of Merino lambs resistant to *H. contortus*, their expression of resistance was abrogated as indicated by significantly higher fecal egg counts (FEC) and nematode burdens found in the $CD4^+$ T-

lymphocyte-depleted lambs compared with those of controls (Gill *et al.*, 1993). In addition, host responses associated with resistance to *H. contortus* including mucosal mast cell hyperplasia, tissue eosinophilia, and antibody responses to *H. contortus* were also significantly suppressed in the lymphocyte depleted lambs. Based on the lack of antibody response they concluded that T lymphocyte help is required for the generation of anti-parasite antibody in *Haemonchus*-infected sheep. Karanu *et al.* (1997) indicated that $CD4^+$ T lymphocyte depletion partially abrogated immunity induced by gut antigen immunization against challenge infection with *H. contortus*. These findings are consistent with Howard *et al.* (1989) where calves depleted of their $CD4^+$ T lymphocytes showed a reduced ability to mount an antibody response to human 0 RBC and OVA. Accordingly, Wofsy *et al.* (1985) demonstrated that mice treated with monoclonal antibody to L3T4 cells were unable to generate an IgG response to either bovine serum albumin or OVA.

The objective of this study was to determine the role of $CD4^+$T lymphocytes in the natural resistance of GCN sheep to *H. contortus* infection.

5.2. Materials and Methods

5.2.1. Animals

Ten five-month-old GCN lambs raised on pasture at the Central Station Sheep Farm, Louisiana Agricultural Experimental Station, Baton Rouge, LA., were randomly assigned to a treatment (n=5) or control (n=5) group. All animals were denematodeed with albendazole (Valbazen, Pfizer, 10 mg/kg) and levamisole (Levasole, Schering-Plough, 8.8 mg/kg) at the beginning of the study and kept in dirt floor pens for the duration of the study. Lambs were fed a growing ration and water was available at all times.

5.2.2. Monoclonal Antibody Treatment

Mouse anti-ovine $CD4^+$ monoclonal antibodies from the 44.97 hybridoma cell line provided by the Centre of Animal Biotechnology, the University of Melbourne, Melbourne, Australia, were produced in tissue culture at the LSU Medical Center Core Laboratory. Lambs in the treated group were injected IV with anti-$CD4^+$ mAb as shown in Table 5.1.

Table 5.1. Monoclonal antibody treatment schedule in Gulf Coast Native post-weaned lambs.

		Days of mAb treatment								
		-3	-1	1	3	5	7	10	14	17
Group	N									
None	5	-	-	-	-	-	-	-	-	-
$CD4^+$ mAb	5	2mg	2mg	2mg	4 mg	4 mg	4 mg	4 mg	2mg	2mg

5.2.3. Experimental Infections

All lambs received an experimental infection of 10,000 *H. contortus* L_3 on day 0 by oral inoculation.

5.2.4. Fecal and Blood Samples

Individual fecal samples were collected directly from rectum on a weekly basis and processed to determine FEC using the modified McMaster technique with 2 grams of feces (Whitlock, 1948) (see appendix). Results were reported as egg per gram (EPG).

Peripheral blood was collected in 7 ml EDTA vaccutainer tubes via jugular venipuncture. White blood cell (WBC) differential and packed cell volume (PCV) were run as described in Chapter 2.

5.2.5. Necropsies

The necropsies were done at day 28 after experimental infection where abomasum, small intestine and large intestine were collected for nematode enumeration and identification following the procedure described in Chapter 3.

5.2.6. Flow Cytometry

Peripheral whole blood was collected biweekly via jugular venipuncture into 7 ml heparin vaccutainer tubes and $CD4^+$ lymphocytes were stained by an indirect procedure to be enumerated on a FACScan Becton Dickison flow cytometer. The indirect staining procedure was done as follows: 50 µl of blood was incubated for 30 min at room temperature and in the dark with 50 µl of mouse anti-CD4 mAb diluted 1/50 in PBS. Cells were then washed with 2 ml volumes of 1X PBS and were centrifuged for 5 min at 1400 rpm. After centrifugation the supernatant was decanted and 50 µl of 1:200 diluted anti-IgG FITC conjugate was added to the tubes. The samples were then vortexed and incubated for 30 min at room temperature in the dark. After the second incubation 1X NH4Cl lysis buffer (see Appendix) was added to the tubes to lyse the erythrocytes and the samples were centrifuged again at 1400 rpm for 5 min. Once the supernatant was decanted the cells were washed with 1X PBS and centrifuged. The supernatant fluid was decanted and the pellet was vortexed and the cells were fixed with 200 µl of 1% paraformaldehyed-PBS (see Appendix) for flow cytometer analysis.

5.2.7. FACS of $CD4^+$ Lymphocytes from Mesenteric Lymph Nodes

Mesenteric lymph nodes were removed at necropsy and transferred to plastic petri dishes, cut and mashed through a strainer. The strainer was then rinsed into another petri dish with PBS to wash out the cells and the remaining liquid was transferred into a 50 ml centrifuge tube. The cells were then centrifuged at 1500 rpm for 5 min and after decanting the pellet was resuspended in PBS and centrifuged again. After three more washes and centrifugations in PBS the cells were stained using the same procedure described previously for peripheral whole blood cells for flow cytometer analysis.

5.2.8. ELISA for *H. contortus* WWA

ELISA tests were run on weeks: -1, 1, 2, 3, 4, and 5 of treatment as described in Chapter 2.

5.2.9. Lymphoproliferation Assays.

Lymphoproliferation assays were run on peripheral blood on day +7 as described in Chapter 2.

5.2.10. Statistical Analysis

Statistical analysis was done with raw data except for FEC and total nematode count which were log transformed to stabilize variance. The means of the groups were compared using PROC MIXED and PROC GLM for repeated measures in SAS. Tukey test was used to compare differences in nematode count means between groups. Differences were considered statistically significant when $p<0.05$.

5.3. Results

One of the animals in the treatment group did not respond to the treatment and showed normal $CD4^+$ T lymphocyte numbers throughout the study, therefore, it was not included in the statistical analysis of any of the variables measured.

5.3.1. Fecal Egg Counts

The FEC of the lambs increased at week 3 and after experimental infection being more marked in the treated lambs than in the control lambs. The overall means were 325 EPG and 1431 EPG for controls and treated, respectively, and this difference was significant (Figure 5.1).

5.3.2. Nematode Counts

Haemonchus contortus was the only species identified in the abomasum and more than 99% were adults. No nematodes were found in the small or large intestine of either of the groups. The mean nematode count in the control group (698) was significantly lower than the mean count (1743) in the treated group.

5.3.3. FACS

The effect of CD4 depletion was not seen until 10 days after the initial treatment. Figures 5.2. and 5.3. show the histogram of the number of $CD4^+$ stained cells in a normal lamb (control) and a depleted lamb (treated), respectively. The mean percent of $CD4^+$ stained remained similar through day 6, and from day 9 to the end of the study $CD4^+$ lymphocytes dropped significantly and were essentially gone from peripheral circulation (Table 5.1.).

5.3.4. FACS on Mesenteric Lymph Node Lymphocytes

There was no significant difference in $CD4^+$T lymphocytes percent between treated (Mean: 41.9%, Range: 31.7-50.6%) and controls (Mean: 48.9%, Range:

43.1-53.1) which indicates that $CD4^+$T lymphocytes in the lymph nodes of the treated lambs were not depleted .

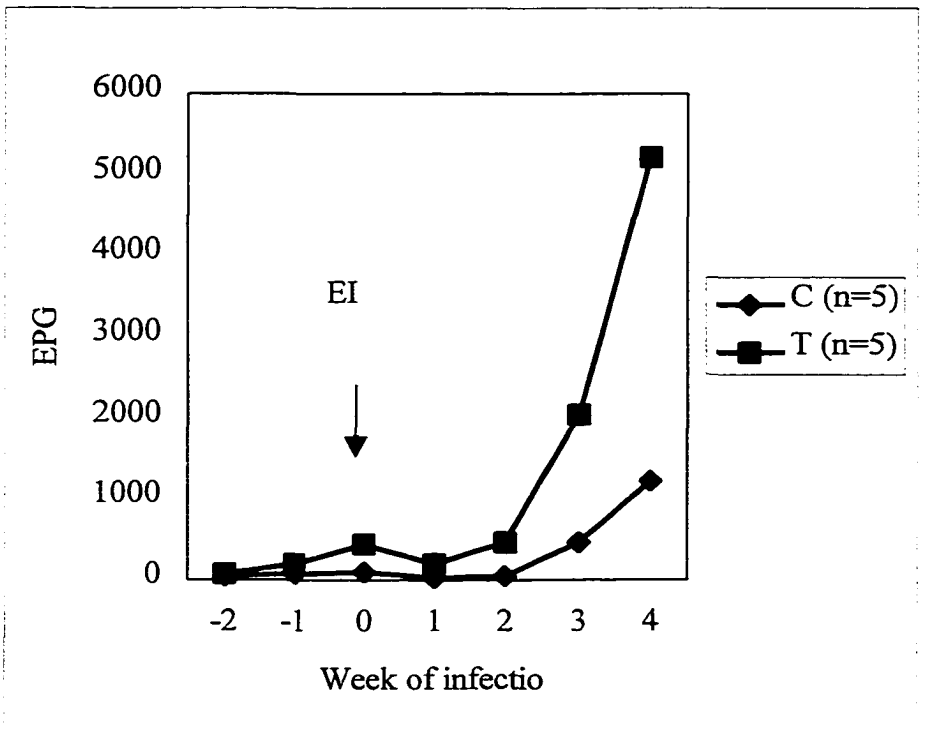

Figure 5.1. Weekly mean fecal egg counts comparing $CD4^+$ depleted (T) Gulf Coast Native post-weaned lambs to undepleted (C) Gulf Coast Native post-weaned lambs.
EI: experimental infection of 10,000 *H. contortus* L_3

Table 5.2. Percent of $CD4^+$ lymphocytes stained in $CD4^+$ depleted (T) Gulf Coast Native post-weaned lambs compared to undepleted (C) Gulf Coast Native post-weaned lambs.

		Day of treatment								
Treatment	N	-1	2	6	9	13	16	20	27	Mean
None	5	34.2	29	28	29.5^a	28.9^a	29.4^a	31^a	31.1^a	30.1^a
$CD4^+$ mAb	5	27.4	27.5	28.6	0.10^b	0.09^b	0.07^b	0.05^b	0.06^b	10.5^b

Means with unlike superscripts are significantly different ($p<0.05$).

5.3.5. Peripheral Blood Tests

5.3.5.1. Blood Packed Cell Volume

There was no difference in PCV between groups at any time during the study (Figure 5.5).

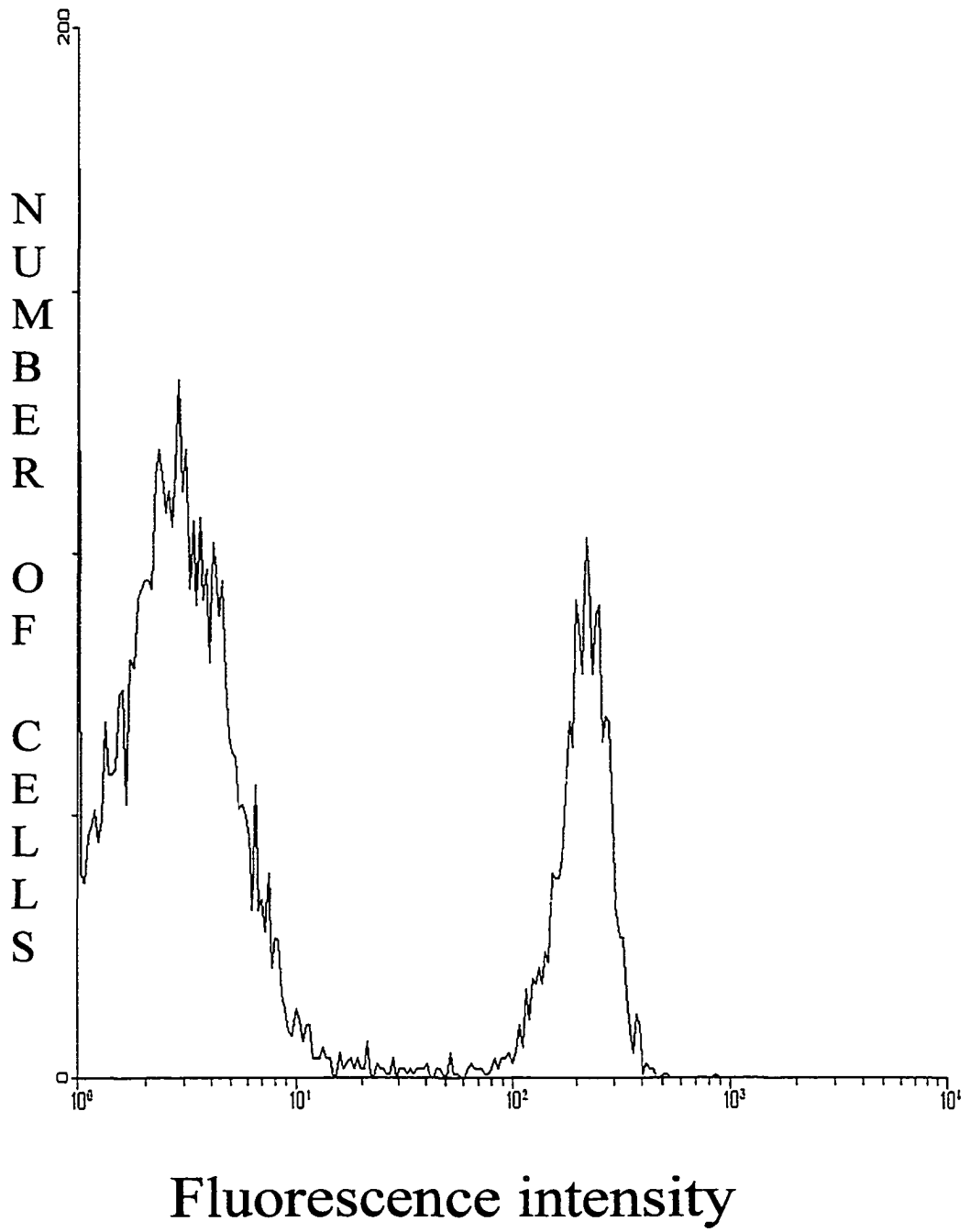

Figure 5.2. Histogram of number of $CD4^+$ stained cells in a normal Gulf Coast Native post-weaned lamb

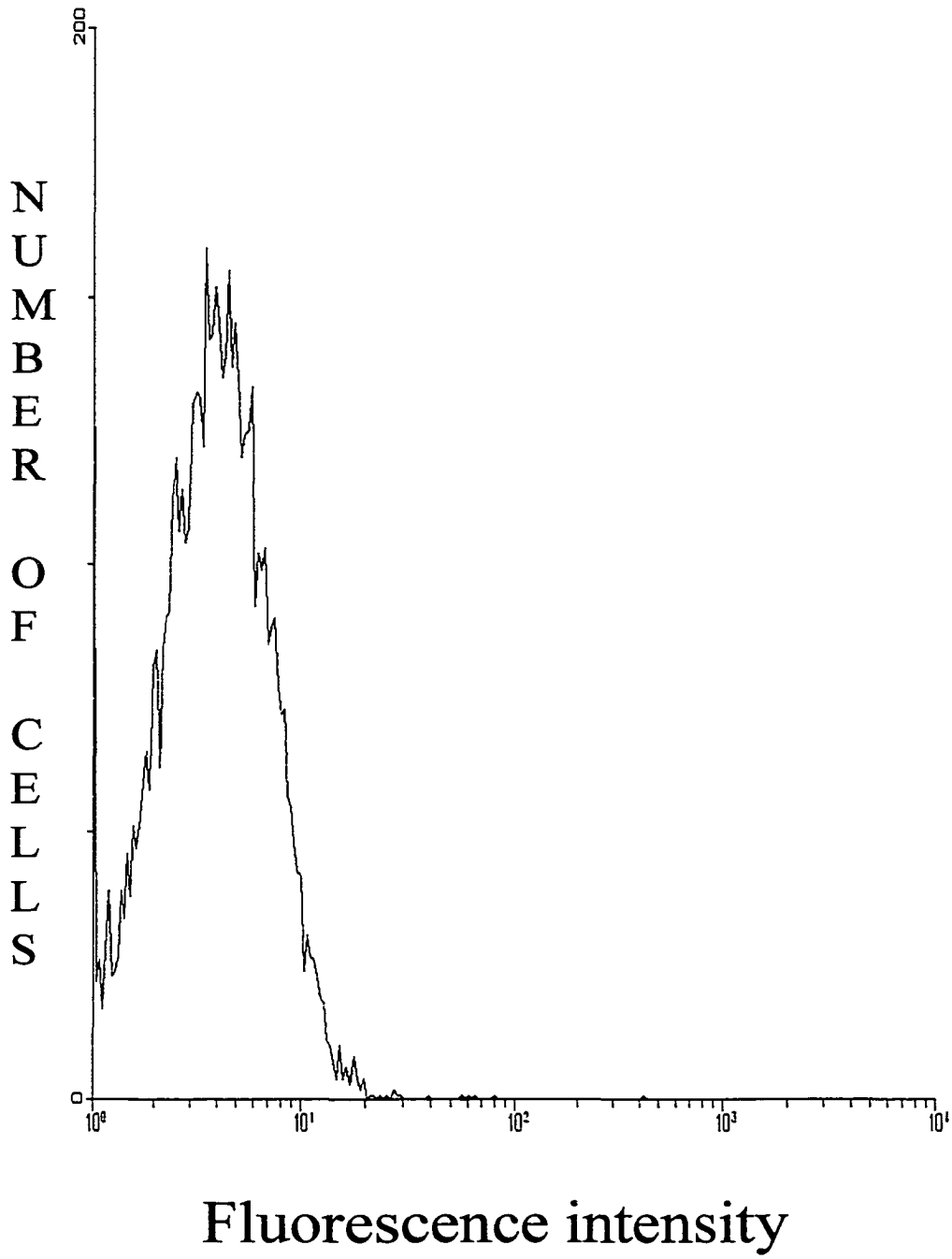

Figure 5.3. Histogram of number $CD4^+$ stained cells in a depleted Gulf Coast Native post-weaned lamb

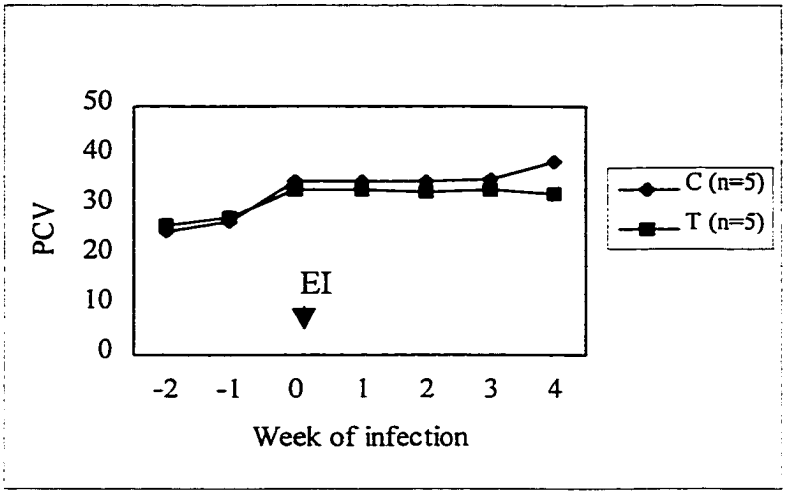

Figure 5.4. Weekly mean packed cell volume in $CD4^+$ depleted (T) Gulf Coast Native post-weaned lambs to undepleted (C) Gulf Coast Native post-weaned lambs. EI: Experimental infections of 10, 000 *H. contortus* L_3.

5.3.5.2. White Blood Count Differential

No differences were seen in white blood count differential except for basophil mean count that was significantly higher in the treatment group compared to the control group at weeks 4 and 5 of treatment (Table 5.2).

5.3.5.3. ELISA for *H. contortus* WWA

ELISA tests to determine antibody titer to *H. contortus* WWA were done weekly. No differences were found in the mean antibody titer between groups (Table 5.9.).

5.3.5.4. Lymphoproliferation Assays

Because $CD4^+$ lymphocytes were still circulating on day 6, proliferation assays were run on day 7 to test functionality of the lymphocytes. PHA, ConA, and PW were used at the same doses described in Chapter 2. Results of the lymphoproliferation assays showed that the treatment did not affect $CD4^+$ lymphocyte functionality as evidenced by similar responses seen to mitogen stimulation in both groups.

Table 5.3.Weekly mean white blood count differential in $CD4^+$ depleted (T) Gulf Coast Native post-weaned lambs to undepleted (C) Gulf Coast Native post-weaned lambs

	Type of cells[1]											
	Lymphocyte		Eosinophil		Neutrophil		Monocyte		Basophil		Total	
Weeks[2]	C	T	C	T	C	T	C	T	C	T	C	T
1	4769	4207	313	118	2422	1718	92	136	25	21	7620	6200
2	5127	4552	720	1970	1680	1004	153	112	60	113	7740	7750
3	5090	5021	1074	1598	1578	1545	114	94	64	72	7920	8350
4	5799	4822	535	165	1725	1416	104	153	57[a]	195[b]	8220	6750
5	4909	4788	236	267	1438	1238	149	131	28[a]	126[b]	6760	6550
Mean	5139	4678	576	824	1769	1384	122	125	47[a]	105[b]	7652	7120

[1] Differences within cell type with unlike superscripts are significantly different ($p<0.05$).
[2]Weeks of treatment

Table 5.4. Weekly mean OD to *Haemonchus contortus* whole worm antigen in $CD4^+$ depleted (T) Gulf Coast Native post-weaned lambs to undepleted (C) Gulf Coast Native post-weaned lambs

	Weeks of treatment						
Treatment	-1	0	1	2	3	4	Mean
None	0.3434	0.7094	0.5814	0.5252	0.6694	0.6312	0.58
$CD4^+$mAb	0.4244	0.6858	0.4974	0.601	0.6768	0.6102	0.58

Table 5.5. Mean stimulation index in response to Phytohemagglutinin, Concavalin A, and Pokeweed stimulation in $CD4^+$ depleted Gulf Coast Native post-weaned lambs to undepleted Gulf Coast Native post-weaned lambs

		PHA (µg/ml)			ConA (µg/ml)			PW (µg/ml)		
Treatment	N	8	4	2	4	2	1	4	2	1
None	5	87.5[a]	82.2[a]	73.5[a]	140.2[a]	129.9[a]	112.3[a]	87.7[a]	83.7[a]	75.6[a]
$CD4^+$ mAb	5	17[b]	13.8[b]	8.7[b]	22.5[b]	20.4[b]	17.7[b]	17.3[b]	17.3[b]	16.5[b]

Means with unlike superscript are significantly different ($p<0.05$).

5.4. Discussion

Several studies have established the importance of $CD4^+$ T lymphocytes in immunity to GI nematodes (Koyama *et al.*, 1995; Katona *et al.*, 1988; Urban *et al.*,

1991; Gill *et al.*, 1993, 1992; Karanu *et al.*, 1997). Similar to what was reported by Gill *et al.* (1993) for genetically derived resistant Merino lambs, naturally resistant GCN lambs that were depleted of $CD4^+$ T lymphocytes became susceptible to infection with *H. contortus*. In both studies, lambs treated with anti-$CD4^+$ mAb showed a significantly higher FEC and nematode burden than the control lambs. In contrast with Gill *et al.*, (1992, 1993), antibody titers did not differ between groups. In that study, the anti-$CD4^+$ mAb used was complement fixing (IgG_{2a}) and $CD4^+$ T lymphocytes were depleted within 48 hrs. (before experimental infection). In this study, a non complement binding anti- $CD4^+$ mAb (IgG_1) was used and $CD4^+$ T lymphocyte depletion was not seen until 9 days after initial treatment. Therefore $CD4^+$ T lymphocytes were still circulating when the lambs were challenged. It may be suggested that during this period circulating $CD4^+$ T lymphocytes were providing help to B lymphocytes to produce immunoglobulins to *H. contortus* WWA resulting in a normal antibody response in treated lambs. In a previous study, (Miller, unpublished observations) GCN lambs that were given anti-$CD4^+$ complement fixing T lymphocyte mAb there was a substantial decrease ranging from 50-80% in antibody titer for IgA, IgM, and IgG_2 compared to control non-treated, and no difference was found in IgG_1 levels. In this study, total IgG was determined, therefore, normal IgG_1 titers might have obscured other isotypes. On the other hand, the results are consistent with Howard *et al.* (1992) where calves depleted of their $CD4^+$ T lymphocytes produced an antibody response to bovine virus diarrhoea virus similar to that of controls.

In this study there were no differences in the PCV between treated and controls. Even though the treated group had a significantly higher nematode burden

than the control group the difference in the number of nematodes might not have been enough to affect the PCV. On the other hand, lambs were experimentally infected after the second depletion when they still have circulating $CD4^+$ T lymphocytes and therefore, will still reject some of the incoming larvae not allowing establishment and subsequent blood feeding. Lastly, this study had a short infection period (4 weeks) that might have not been enough time for anemia to develop.

Because depletion was not achieved until day 12 of depletion, lymphoproliferation assays were done on day 10 to determine the effect of mAb on T lymphocyte functionality. The results of these tests showed that there was a decreased functionality of T lymphocyte functionality of the lambs in the treatment group compared to those of the controls. These results concur with Howard *et al.* (1989) where the responses of PBMC preparations to ConA, PHA, and PW were significantly reduced in BoT4 ($CD4^+$) depleted calves. These results may suggest that the coating of the lymphocytes by the mAb might have affected their functionality by altering the surface membrane. Considering the role of $CD4^+$ T lymphocytes in resistance to *H. contortus* infection, the early on decreased functionality of $CD4^+$ T lymphocytes observed might explain the higher establishment of nematodes in the treated group compared to the controls.

The results of this study suggest that $CD4^+$ T lymphocytes are associated with the natural resistance of GCN sheep to *H. contortus* infection.

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CHAPTER 6

GENERAL DISCUSSION

6.1. Discussion of the Results

Breeding for parasite resistance is one approach to overcome *Haemonchus contortus* infection because resistance has developed to almost every anthelmintic in the market. Epidemiological studies demonstrated that GCN sheep are relatively resistant to nematode infection and in particular to *H. contortus* (Lemarie *et al.*, 1987; Lemarie, 1988, Miller *et al.*;1993, Bahirathan, 1994; and Bahirathan *et al.*, 1996). In order to apply the benefits of a resistant breed in a successful breeding program, an understanding of the mechanism underlying resistance or susceptibility to infection is essential. One approach for determining what mechanisms are involved is the use of corticosteroids to suppress acquired immunity in resistant sheep. In a number of studies, sheep treated with a corticosteroid (dexamethasone) lost resistance to further GI nematode challenge (Presson et al., 1988; Miller et al., 1985; Huntley et al., 1992; Mattews et al., 1979; Winter et al., 1997). In accordance with these studies, our studies demonstrated that neonatal and post-weaned lambs treated with corticosteroids lost their natural resistance to *H. contortus* infection. This loss of resistance was mainly characterized by higher FEC and nematode burdens, and lower PCV in the treated lambs compared to the control lambs after natural (neonatal) or experimental (post-weaned) *H. contortus* challenge. Gill (1991) demonstrated that a resistance genotype of Merino sheep to *H. contortus* infection was attributable to an acquired immune response. In that study, after a secondary infection, resistant lambs had significantly lower FEC and nematode

burdens than random-bred susceptible lambs. In our study, post-weaned lambs were previously exposed to *H. contortus* infection, therefore, it may be suggested that resistance of GCN lambs to *H. contortus* infection can also be attributed to an acquired immune response. The effector mechanisms that participate in an acquired immune response are complex including activation of parasite-specific T lymphocytes, antibodies and mast cells and eosinophils. The T lymphocytes do not act directly on the parasite but exert their influence by providing help for antibody production and by mediating a variety of inflammatory reactions and physicochemical changes in the gut. Initiation, regulation, and expression of these responses is under tight genetic control and aberration in any one of these responses could impair the ability of the host to mount an effective immune response against parasitic infection (Wakelin, 1985). Immediate hypersensitivity reactions involving mucosal mast cells, globule leucocytes and eosinophils have been considered an important effector mechanism in the rejection of incoming larvae, as well as established nematodes following a challenge infection (Miller, 1984). Corticosteroid treatment has been shown to reduce the number of these cells with the subsequent loss of resistance to nematode infections (Jackson *et al.*, 1988; Miller *et al.*, 1985; Presson *et al.*, 1988; Winter *et al.*, 1997). Winter *et al.* (1997) demonstrated that dexamethasone treatment resulted in decreased peripheral eosinophilia and loss of resistance to *Nematodirus battus* infection in lambs. In the post-weaned lamb study, dexamethasone treated lambs also had significantly lower eosinophil counts compared with the control group. Whether the loss of resistance to *H. contortus* infections in this group was due to decreased eosinophil numbers cannot be determined.

Dexamethasone treatment also resulted in a decreased number of lymphocytes in both neonates and post-weaned lambs compared to the controls. Cohen and Duke (1984) indicated that glucocorticosteroids such as dexamethasone cause lymphocyte death by activating a calcium-dependent endonuclease that rapidly and extensively degrades the DNA. The substantial decreased in the lymphocyte count seen in the treatment groups might have been a result of the toxic effect that dexamethasone has on the lymphocytes. T lymphocytes proliferate within the mesenteric lymph nodes in response to GI nematode antigens during infection and release a variety of lymphokines that are involved in amplification, recruitment and differentiation of inflammatory cell types, and antibody producing cells. Grencis *et al.* (1985) demonstrated that immunity to *Trichinella spiralis* in the mouse is a strongly thymus-dependent phenomena. In another study, Katona *et al.* (1988) indicated that the induction of polyclonal IgE reponse, intestinal mucosal mast hyperplasia and spontaneous cure of *Nippostrongylus brasiliensis* infection are all dependent on T lymphocytes of the helper phenotype. Accordingly, Miller *et al.* (1985) reported a profound reduction in the number of mast cells and globule leukocytes in normal and hyperimmune sheep treated with corticosteroid. That was associated loss of resistance to *H. contortus* infections. This study showed that GCN lambs treated with dexamethasone had a decline on their lymphocyte counts and were rendered suceptible to *H. contortus* infection. It might be suggested that the decrease of lymphocyte numbers affected the mucosal mast cell population and subsequent immune exclusion of incoming *H. contortus* infective larvae. The result of this would be a higher establishment of nematodes seen in the dexamethasone treated groups compared to the control groups. Results then suggest that T

lymphocytes appear be associated with the natural resistance to *H. contortus* infection in GCN sheep.

There are a number of studies that have indicated that protective immunity against GI nematode infection is $CD4^+$ T lymphocyte dependent. Urban *et al.* (1991) showed that $CD4^+$ T lymphocytes regulate host protective immunity, nematode fecundity, and IgE levels in *Heligmosoides polygyrus* infection. Vos *et al.* (1983), demonstrated that athymic mice was unable to mount a humoral immune response against *T spiralis* and the subsequent expulsion of adult *T. spiralis* was impaired, indicating a T lymphocyte dependency in the immune response to this nematode. Protective immunity against *H. contortus* has also been demonstrated to be $CD4^+$ T lymphocyte dependent (Gill *et al.*, 1993; Karanu *et al.*, 1997). To further confirm that $CD4^+$ T lymphocytes were involved in resistance of GCN sheep to *H. contortus* infection, GCN lambs were depleted of their $CD4^+$ T lymphocytes. Gill *et al.* (1993) indicated that $CD4^+$ T lymphocytes are responsible for the generation of mucosal mast cell hyperplasia, tissue eosinophilia and anti-*Haemonchus* antibody. In that study, administration of anti-$CD4^+$ lymphocyte monoclonal antibody to genetically resistant Merino sheep completely abrogated their expression of genetic resistance. The results of this study concur with that study as GCN sheep depleted of their $CD4^+$ T lymphocytes, were rendered susceptible *to H. contortus* infection as indicated by higher FEC and higher nematode burden in the depleted lambs compared with those of controls. Although host responses associated with resistance to *H. contortus* were significantly decreased in the treated group, there was no difference in antibody titer to *H. contortus* WWA between groups. One explanation may be that the dose of monoclonal used in our study was not enough to eliminate T

lymphocytes in the lymphoid organs and these lymphocytes can still provide help to B cells to produce antibodies. Naessens *et al.* (1998) indicated that the dose of 2 mg anti-$CD4^+$ mAb/kg body weight was necessary for efficient elimination of T lymphocytes in the lymphoid organs of cattle. The dose (2 mg total) used in this study did not deplete $CD4^+$ T lymphocytes in the lymph nodes as shown by the high percentage of $CD4^+$ T lymphocytes present in the mesenteric lymph nodes at necropsy of the treated lambs. Lastly, the restoration of memory function might have influenced the antibody responses in treated lambs. The return of immunological memory function in peripheral blood after cell depletion and recovery might be the result of: 1) survival of some memory $CD4^+$ T lymphocytes at sequestered sites of the body, 2) persistence of antigen in a form that could stimulate naïve $CD4^+$ T lymphocytes, and 3) the memory cells are less prone to elimination than naïve cells (Naessens *et al.*, 1998). Regardless which of these could have occurred, treated lambs showed similar antibody production to *H. contortus* WWA than control lambs. Whether these antibodies are important in resistance of GCN to *H. contortus* infection cannot be concluded from this study. Gill (1993) showed that depleted lambs had lower antibody titer than control lambs and this was associated with abrogation of resistance to *H. contortus* infection. These antibodies were detected with *H. contortus* larval antigens and it may be suggested that antibodies to larval antigens but not antibodies to adult nematodes (as used in this study) are important in resistance of GCN to *H. contortus* infection. In conclusion, GCN lambs depleted of their $CD4^+$ T lymphocytes were rendered more susceptible to *H. contortus* infection as evidenced by higher FEC and nematode burden seen in the depleted lambs compared to the control lambs. The results indicated that T

lymphocytes and in particular $CD4^+$ T lymphocytes are associated with the natural resistance of GCN sheep to *H. contortus* infection.

6.2. Future Studies

Another depletion study needs to be done using a complement-binding mAb in order to address the slow clearance of CD4 T lymphocytes observed in this study. It would be expected that abrogation of the resistance of GCN sheep to *H. contortus* infection after treatment with such a mAb will also support the findings.

Evidence presented in this study indicated that $CD4^+$ T lymphocytes are associated with the immune response of GCN sheep to *H. contortus* infection. $CD4^+$ T lymphocytes, also known as helper T cells, produce different cytokines according to the pathogen involved. Th1 cytokines, including IL-2 and IFN-γ, are involved in protection against intracellular pathogens, whereas Th2 cytokines, including IL-4 and IL-10, are required for protection against extracellular pathogens. A Th2 type response is a common feature in GI nematode infection and is characterize with the production of IL-4, IL-10, IL-13 and IL-5 that in turn will promote B lymphocyte differentiation, mastocytosis and eosinophilia creating an environment hostile to nematode survival (Else *et al.*, 1998). The generation of inflammatory responses in the GI tract, alterations in the physiology and antibody mediated interference of feeding, will result in damage of nematodes and rejection from their niche within the host. Canals *et al.* (1997) indicated that calves infected with *Ostertagia ostertagi* showed a substantial reduction in the transcription of IL-2 and IFN-γ and an elevation of the transcription of IL-4, which are consistent with a Th2 immune response. Whether the same cytokines are the ones present in the immunity to *H. contortus* in sheep has not yet been characterized. Further research will be focused

on the assessment and characterization of cytokine profiles that are presented in immunity of GCN sheep to *H. contortus* infection.

6.3. Conclusions

The results of this research have contributed to the better understanding of the mechanisms underlying natural resistance of GCN sheep to *H. contortus* infection. Understanding the mechanisms associated with resistance will help to improve progress in vaccine and drug development and will possibly help in the identification of genes that are involved in resistance. The identification of these genes and the use of genetic manipulation will then allow improvement of breeds with good meat production traits and with parasite resistance for tropical and subtropical regions where *H. contortus* is a serious constraint.

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APPENDIX: TECHNIQUES AND REAGENTS

- Modified McMaster Technique

Two grams of feces were mixed with 30 ml of saturated salt solution using an electric mixer. Once the feces were mixed into the solution, a sample was pipetted out and put into one half of a McMaster slide. The sample was mixed again and another sample was pipetted out to the other side of the slide. The eggs on both sides of the slide were counted and the total count was multiplied by 50 to obtain the eggs per gram.

- Saturated salt solution

In a 3000 ml beaker, 2500 ml of water was added to 500 g of salt, water was added to make the final solution volume 3000 ml. The solution was stirred at low heat on a magnetic stirrer for 2-3 hours.

- ELISA Reagents

Blocking Buffer

10 g (1%) Bovine serum albumin + 0.5 ml (0.05%) Tween-20, bring to a liter with phosphate buffered saline (PBS, pH 7.4).

Serum diluent

58.44 g (1M) NaCl + 1ml (0.1%) Triton X-100 + 10 g (1%) bovine serum albumin, then bring to 1 liter with PBS. Store the solution to 4°C and use within two weeks.

- FACS Reagents

Lysing buffer

0.829 g NH_4Cl + 0.109 g $KHCO_3$ + 0.037 g Disodium EDTA bring to 100 ml distilled water. Adjust pH to 7.3 to 7.4. Store at room temperature and use within 24 hours.

Formaldehyde Fixative

A 2% formaldehyde stock solution is made by adding 2 g paraformaldehyde to 100 ml of 1 X PBS (Ca^{2+} and Mg^{2+} free). Heat to 70°C in a fume hood until the paraformaldehyde goes into solution. Allow the solution to cool to room temperature. Filter through a 0.45 μm filter. Adjust to pH 7.2-7.4 using 1 M NaOH or 1 M HCl as needed. Store in the refrigerator.

- Media for proliferation assays

RPMI-1640 + Sodium bicarbonate + HEPES + 2-mercaptoethanol + L-Glutamine + Penicillin + Streptomycin + 5% FBS.

VITA

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DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Maria Teresa Pena

Major Field: Veterinary Medical Sciences

Title of Dissertation: The Role of Immunity in Resistance of Gulf Coast Native Sheep to Haemonchus contortus Infection

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

December 7, 2000