

**INDUCTION OF ANTI-ERGOTAMINE ANTIBODIES IN MICE AND STEERS
AND PROTECTION AGAINST FESCUE TOXICOSIS IN MICE**

by

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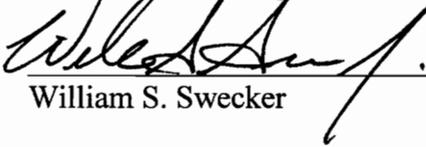
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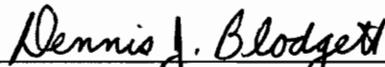
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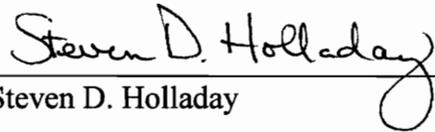
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(ABSTRACT)

Tall fescue (*Festuca arundinacea* Schreb.) is often infected by the endophytic fungus, *Acremonium coenophialum* (Morgan-Jones and Gams). The fungus produces ergopeptide alkaloids, especially ergovaline. Consumption of endophyte-infected (EI) fescue forage by cattle decreases serum prolactin and average daily weight gains, which results in economic losses for producers.

Anecdotal reports suggest cattle with fescue toxicosis may not respond to vaccination. Hypoprolactemia decreases cell-mediated and humoral immune responses in mice. Therefore, steers grazing EI or endophyte-free (EF) fescue forages were vaccinated to assess humoral immune responses. Steers grazing EI fescue exhibited classical clinical signs of fescue toxicosis, and mounted humoral immune responses to vaccination, despite hypoprolactemia.

Lymphocyte proliferation responses to mitogens in mice fed EI diets were similar to mice fed EF diets. Production of interferon gamma and interleukin-4 was higher by splenocytes from mice fed EI diets, whereas interleukin-2 tended to be lower. Fescue toxicosis may stimulate T helper cell 2 subset of CD4⁺ T cells. The T_H2 subset may augment humoral immune responses to vaccination.

Ergotamine, an ergot alkaloid structurally similar to ergovaline, was conjugated to BSA, concanavalin A, cholera toxin, and subunit B of cholera toxin (CTB) via the Mannich reaction. Parenteral vaccination of mice with protein-ergotamine conjugates induced IgG titers against ergotamine. Oral vaccination of mice with CTB-ergotamine conjugate induced mucosal sIgA and IgG against ergotamine. Parenteral vaccination of cattle with CTB-ergotamine conjugate in adjuvant induced anti-ergotamine titers. Vaccination of mice subcutaneously with BSA-ergotamine conjugate encapsulated into poly(DL-PLG) microspheres did not prolong anti-ergotamine titers.

Anti-ergotamine antibodies induced by parenteral vaccination with BSA-ergotamine conjugate were protective against adverse effects on weight gain in mice fed EI diet. Rate of weight gain was positively correlated to anti-ergotamine titers. Passive immunization with monoclonal antibodies against ergovaline and oral immunization with CTB-ergotamine conjugate tended to increase rate of weight gain. Prolactin concentrations tended to be increased in vaccinated mice fed EI diet and were positively correlated with titers.

In conclusion, further research is indicated to determine if anti-ergotamine titers in vaccinated cattle will protect against adverse effects on weight gains.

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List of Abbreviations

ADG	average daily gains
ALP	alkaline phosphatase
APC	antigen presenting cells
BALT	bronchus associated lymphoid tissue
BCA	bicinchoninic acid assay
BCGF	B-cell growth factor
BRDC	bovine respiratory disease complex
BSA	bovine serum albumin
BVD	bovine virus diarrhea
BW	body weight
°C	Celsius
cAMP	cyclic adenosine monophosphate
CMI	cell mediated immunity
Con A	concanavalin A
CPM	counts per minute
cRPMI	complete RPMI
CT	cholera toxin
CTB	cholera toxin subunit B
d	day
dL	deciliter
EF	endophyte-free
EG	ergotamine
EI	endophyte-infected
ELISA	enzyme-linked immunosorbent assay
EV	ergovaline
FCA	Freund's complete adjuvant
FFA	free fatty acids
FIA	Freund's incomplete adjuvant
g	gram
GALT	gut-associated lymphoid tissue
GH	growth hormone
h	hour
H ₂ O ₂	hydrogen peroxide
ha	hectare
HPLC	high performance liquid chromatography
HRPO	horseradish peroxidase
IBR	infectious bovine rhinotracheitis
Ig	immunoglobulin
IGF-I	insulin-like growth factor-I
IL-1	interleukin-1

IM	intramuscularly
INF- γ	gamma interferon
IP	intraperitoneally
KDa	kilodalton
kg	kilogram
L	liter
LPS	lipopolysaccharide
LTA	lymphocyte transformation assay
M	molar
MAF	macrophage activating factors
MALT	mucosal-associated lymphoid tissue
μ g	microgram
mg	milligram
MHC	major histocompatibility complex
min	minute
μ L	microliter
mL	milliliter
mM	millimolar
MW	molecular weight
n	number of observations
N	normal
NCR	National Cancer Research
ng	nanogram
nm	nanometer
OD	optical density
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline
PBST	phosphate buffered saline + Tween 80
pg	picogram
PG	prostaglandins
PGE ₂	prostaglandin E ₂
PHA	phytohemagglutinin
PI3	parainfluenza type 3
PLL	poly -L-lysine
poly (DL-PLG)	poly (DL-lactide-co-glycolide)
RIA	radio-immunoassay
SEM	scanning electron microscopy
SEM	standard error of the mean
SD	standard deviation
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sIgA	secretory immunoglobulin A
sp	specific gravity

SRBC	sheep red blood cells
T _H cell	T helper cell
TLC	thin layer chromatography
TMS	trace mineral salt
TRH	thyrotropin releasing hormone
U	units
v	volume
VMRCVM	Virginia-Maryland Regional College of Veterinary Medicine
w	weight
WBC	white blood cell

Chapter 1: General Introduction

Introduction

Tall fescue (*Festuca arundinacea* Schreb.) is a perennial grass, which covers an estimated 15 million hectares in the United States (Bacon, *et al.*, 1986). Tall fescue is often infected by the endophytic fungus, *Acremonium coenophialum* (Morgan-Jones and Gams, 1982). *Acremonium coenophialum* synthesizes ergopeptide alkaloids, such as ergovaline, within the fescue plant. The fungus imparts many desirable attributes to the plant. Desirable attributes of endophyte-infected (EI) tall fescue are its ease of establishment, wide adaptation, long grazing season, tolerance to environmental stresses, and pest resistance (Ball *et al.*, 1991). Evaluation of digestible dry matter, crude protein, and mineral concentrations of fescue forages indicated livestock grazing tall fescue should exhibit good performance (Ball *et al.*, 1991). Consequently, an estimated 8.5 million beef cattle and 700,000 horses are maintained on tall fescue pastures in the U.S.A. (Ball *et al.*, 1991).

However, despite the excellent forage quality of tall fescue, livestock grazing EI fescue often exhibit poor body weight gains, reproductive dysfunction, and decreased production that result in economic losses for producers in the horse and cattle industries. Consumption of EI fescue grass and hay is associated with summer slump syndrome,

fescue foot, and fat necrosis in cattle. The economic loss in the beef cattle industry alone was estimated to be greater than 800 million dollars annually (Hoveland, 1990).

An economically and environmentally sound solution to the fescue problem has not been forthcoming. Renovation of EI pastures is monetarily and environmentally unsound, and methods to ameliorate the deleterious effects need to be investigated. Preventative methods would be preferable to new therapeutic drugs, which may cause residue problems. Development of a vaccine to protect cattle against fescue toxicosis may be an environmentally safe and economical solution. A vaccine against fescue toxicosis may save producers millions of dollars, avoid environmental damage associated with pasture renovation, while allowing cattle to benefit from the nutritional value of EI fescue forage.

T-Cell Dependent Immune Responses and Antibody Production to Parenteral Vaccination

In 1879, Louis Pasteur established the general principle of vaccination while researching the resistance of chickens to fowl cholera, a disease caused by *Pasteurella multocida*. Pasteur discovered that aged cultures of *Pasteurella multocida* failed to induce fowl cholera in chickens. Subsequent experiments showed chickens inoculated with aged cultures of *P. multocida* also were resistant to disease when challenged by

fresh cultures of *P. multocida*. Previously in 1798, Edward Jenner prevented smallpox by inoculating humans with cowpox virus. Pasteur coined the word 'vaccination' (*vacca* is Latin for cow) based on results of his experiments and previous research by Jenner. Principles established by these early studies lead to production of many successful vaccines in the last century against a multitude of bacterial and viral pathogens in man and animals.

Since the early studies that established immunology as a science, many of the mechanisms of how the immune system responds to vaccination to induce humoral immunity have been elucidated. In 1959, Macfarlane Burnet postulated the clonal selection theory to explain generation of specific immune response against antigens (Tizard, 1987). The clonal selection theory stated that: 1) lymphoid stem cells differentiate randomly to produce clones of lymphocytes that are committed to single epitopes; 2) binding of antigen to lymphocyte receptors stimulates proliferation and differentiation of lymphocytes into effector cells and memory cells; 3) specificity of antibodies produced are identical to that of its antigen receptors; and 4) tolerance results when a clone of lymphocytes specific for an antigen is destroyed or suppressed. Since 1959, numerous studies have validated Burnet's postulates of the clonal selection theory.

As the immune system develops, the primitive omentum, yolk sac, and fetal liver produce lymphocyte stem cells. Lymphocyte stem cells develop and differentiate in primary lymphoid organs. In mammals, primary lymphoid organs are bone marrow, the thymus, and Peyer's patches. Later, lymphocytes migrate to secondary lymphoid organs,

such as the spleen, lymph nodes, tonsils, bone marrow, and mucosal-associated lymphoid tissue (MALT). Specific lymphocyte-antigen interactions occur in secondary lymphoid organs. Lymphocytes that mature in the thymus are called T-cells. B-cells develop from stem cells that populate the bone marrow.

T-cells can be categorized into $CD4^+$ and $CD8^+$ T-cells on the basis of their function and markers. The $CD8^+$ T-cells may function as either cytotoxic T-cells or suppressor T-cells. Cytotoxic T-cells function in cell mediated immunity (CMI) to destroy abnormal cells. Suppressor T-cells function in suppression of immune responses. The $CD4^+$ T-cells function to promote CMI and humoral immune responses. The $CD4^+$ or T helper (T_H) cells can be further divided into T_{H1} or T_{H2} subsets based on the lymphokines produced (Mosmann and Coffman, 1989).

Reactivities of T-cells to antigens are regulated by class I and class II histocompatibility antigens, which are proteins found on cell surfaces. Histocompatibility antigens of both classes are coded for by genes clustered on one chromosome. These genes form a gene complex known as the major histocompatibility complex (MHC). The reactivities of $CD8^+$ T-cells are regulated by class I histocompatibility antigens expressed on cell surfaces of potential cell targets. The activities of $CD4^+$ T-cells are regulated by class II histocompatibility antigens found on surfaces of cells that interact with T_H cells to promote immune responses. Class II histocompatibility antigens are expressed on cell surfaces of B-cells, on some activated T-cells, and, in general, on antigen presenting cells (APC). A variety of cell types may act as APC, such as macrophages, B-cells, dendritic

cells, and Langerhans' cells. T-cells can be cytotoxic to abnormal, infected or foreign cells. T-cells also function in helper activity to promote immune responses, and in suppressor activity to suppress immune responses. In most instances, T_H cells interact with B-cells and APC, which leads to production of specific antibodies.

Vaccination may stimulate either or both cell mediated and humoral immune responses. Humoral immune responses are mediated by mature B-cells or plasma cells that produce specific immunoglobulins or antibodies to interact with foreign proteins. Cell mediated immunity is mediated by T-cells that secrete a variety of soluble factors called lymphokines. In CMI, T-cells may act directly or indirectly through activation of other cells, such as macrophages, to destroy foreign material.

The first event to occur after parenteral administration of a vaccine is trapping and processing of the foreign vaccine material by APC. Antigen presenting cells engulf foreign proteins, after which intracellular enzymes process the protein into small peptide fragments of 10 to 20 amino acids. The small peptide fragment is expressed on the surface of the APC in association with MHC II protein for presentation to T_H cells. A T_H cell that recognizes the antigen fragment in association with MHC II protein binds the complex through its T-cell antigen receptor. Binding of T_H cell to the macrophage induces the macrophage to produce interleukin-1 (IL-1). Interleukin-1 then further stimulates the original activating T_H cell, as well as other T_H cells that have bound antigen, to proliferate and secrete IL-2, B-cell growth factor (BCGF) or IL-4, and to express IL-2 receptors. Interleukin-2 production promotes further activation,

differentiation, and proliferation of T_H cells, which produce soluble factors to perpetuate and enhance the immune response. Certain subpopulations of activated T_H cells also produce gamma interferon ($INF-\gamma$) after IL-2 exposure to enhance or activate macrophage function. Gamma interferon prevents viral replication and augments class II protein expression.

B-cells express immunoglobulin M (IgM) molecules on their cell surface early in development and, when mature, express both IgM and IgD. B-cell receptors are immunoglobulins attached to the B-cell membrane via the Fc region with the Fab sites exposed. B-cells are activated or sensitized after binding antigen to the membrane bound immunoglobulin (Ig) receptors, but usually do not proliferate and produce antibodies unless they receive help from T_H cells. In T-cell dependent immune responses, B-cells must interact with T_H cells to undergo proliferation and immunoglobulin production. However, helper activity of T_H cells is not required for B-cell proliferation and antibody production if the original antigen is T-cell independent. On the activated B-cell, surface receptors rearrange so that membrane-bound antigen is concentrated on a small area on the B-cell surface in a process called capping. The B-cell divides repeatedly and two B-cell populations develop that are morphologically and functionally discrete. One population of B-cells differentiates into plasma cells to produce large quantities of antigen specific antibodies.

Immunoglobulin M is the major immunoglobulin isotype produced upon initial exposure to antigen in a primary immune response. The other population of B-cells

remains structurally unchanged and functions as memory cells. Memory B-cells retain the ability to recognize and respond to a particular antigen when exposed to it again. More antigen sensitive B-cells are stimulated when memory cells are exposed to a specific antigen again. Thus, a secondary immune response is quantitatively greater than the primary response. Additionally, the lag time for antibody production is shorter in a secondary humoral response because of the memory cell population. In secondary immune responses, immunoglobulins predominately of the IgG isotype are produced because the isotype has switched from IgM to IgG. Immunoglobulin isotype switching occurs after antigen stimulation of a B-cell. Changes of immunoglobulin isotype occurs when recombination events on the constant region of the μ heavy chain gene, which codes for IgM production, switches to γ heavy chains of the γ gene to produce immunoglobulins of the IgG isotype (Tizard, 1987).

Chapter 2: Review of Literature

Extent of Endophyte-Infected Fescue Pastures and Impact on Animal Health

Most tall fescue pastures are endophyte-infected. Fescue pastures with endophyte infection levels as low as 20 percent may contain sufficient levels of ergot alkaloids to cause clinical signs of fescue toxicosis and decrease average daily gains (ADG) in beef cattle (Fribourg *et al.*, 1990). A survey of 200 fescue pastures from 42 counties in the Southeast found 97% of the pastures to be endophyte-infected at an infection level of 67% or greater (Siegel *et al.*, 1984). External signs of endophyte infection in fescue are not visible. Presence of the endophyte in fescue pastures is detected by staining stems, leaf sheaths, or seeds with aniline blue followed by light microscopy identification of the endophyte by trained personnel (Bacon *et al.*, 1977). An enzyme-linked immunosorbent assay (ELISA) was developed for the testing of the endophyte (Johnson *et al.*, 1982). However, presence of the endophyte is not always associated with high concentrations of ergovaline and ergopeptide alkaloids.

Effective measures to control the endophyte within the fescue plant or to alleviate adverse health effects in cattle to allow them to benefit from the excellent forage quality of fescue do not exist. Fungicides applied to EI fescue pastures do not destroy the

fungus. A definitive treatment does not exist for cattle with summer slump syndrome, fescue foot, or fat necrosis.

Pasture renovation of EI fescue pastures and reseeding with endophyte-free (EF) fescue is often recommended to producers. However, EF fescue grass is not as insect and stress tolerant as the EI grass or as tolerant to overgrazing by livestock (Ball *et al.*, 1991). Implementation of grazing management practices, which prevent overgrazing of EF pastures, are often required to maintain stands and productivity. Establishment and maintenance of EF fescue pastures may be difficult in areas where fescue is marginally adapted. Additionally, EI fescue sod will eventually dominate EF fescue grass or other grasses to re-establish its presence within a few short years if not completely destroyed during pasture renovation.

Pasture renovation of EI fescue pastures also is potentially environmentally unsound, because large amounts of nonselective herbicides are required to destroy the EI sod. The destroyed fescue contributes to environmental pollution when it decomposes, as excess nitrogen and phosphorus are released into surrounding watersheds. Application of herbicides and replanting pastures with another grass also expends valuable fuel reserves. Pasture renovation with EF fescue is also expensive costing up to \$375 dollars per hectare and pastures cannot be utilized for up to a year (White, 1990). Cost of pasture renovation of all 15 million hectares of EI fescue pastures is estimated to be 5.6 billion dollars and would require 47.3 million liters of herbicide (White, 1990). Many EI fescue pastures are too hilly for tillage and, if tilled, could result in severe soil erosion.

Toxins Associated with Endophyte-Infected Fescue Pastures and Their Effects

The causative agent(s) of fescue toxicosis has(have) not been definitively proven. Ergopeptide alkaloids synthesized by *Acremonium coenophialum* present in fescue plant leaves and seeds are associated with fescue toxicosis (Lyons *et al.*, 1986). Synthesis of ergot alkaloids in EI fescue grass does not require special environmental or host conditions. Several ergot alkaloids identified in *Acremonium*-infected fescue are: chanoclavine, ergosine, ergosinine (Porter *et al.*, 1979), ergotamine (EG), ergovaline (EV), ergocristine, ergocornine (Yates *et al.*, 1985) and lysergic acid amide (Petrosky and Powell, 1991). Ergovaline is thought to be the alkaloid responsible for fescue toxicosis, because it constitutes 84 to 97% of the total concentration of ergopeptide alkaloids in EI fescue samples (Lyons *et al.*, 1986). Ergovaline persists in forages for a long time. Hay harvested from EI fescue pastures has remained toxic after 2 years (Ball *et al.*, 1991).

Production of ergovaline and alkaloids in general is dependent on factors such as temperature, humidity, and level of nitrogen fertilization during the growing season (Lyons *et al.*, 1986). Since ergovaline concentrations can vary from year to year, analysis of forage for ergovaline content is necessary. Analysis of ergovaline in endophyte-infected pastures requires solvent extraction from forage and high performance liquid chromatography (HPLC) analysis.

Consumption of EI fescue grass and hay has been associated with summer slump syndrome, fescue foot, and fat necrosis in cattle, and reproductive dysfunction in cattle and mares (Ball *et al.*, 1991). Summer slump syndrome is the most common syndrome of fescue toxicosis and is responsible for the greatest economic losses in cattle (Ball *et al.*, 1991). Summer slump syndrome is observed in all fescue growing regions of the U.S.A. and the adverse effects occur year round. Clinical signs are most notable during the summer months in the Southern growing regions, hence the term summer slump. Clinical signs of summer slump syndrome in cattle are poor weight gains, reduced weaning weights in calves, heat intolerance, increased respirations and rectal temperatures, decreased conception rates, rough hair coats, failure to shed winter hair, reduced milk production and nervousness (Hemken *et al.*, 1984; Stuedemann and Hoveland, 1988). Serum cholesterol and alkaline phosphatase (ALP) concentrations are decreased in cattle grazing EI fescue for long time periods (Lipham *et al.*, 1989; Bond *et al.*, 1984; Stuedemann *et al.*, 1985; Garner and Cornell, 1978). Summer slump syndrome also decreases forage intake in cattle. Cattle maintained in EI fescue pastures spend 20% less time grazing (Bond *et al.*, 1984) and consume 24 to 44% less daily dry matter than cattle maintained in EF fescue pastures (Stuedemann *et al.*, 1989). Growing steers and heifers are most sensitive to fescue toxicosis with daily gains reduced by 45 g for each 10 percent of fungus infection (Ball *et al.*, 1991).

Serum prolactin concentrations are often decreased in cattle grazing EI tall fescue (Thompson *et al.*, 1987). Prolactin is important in lactation and immunocompetence.

Hypoprolactemia decreases humoral (Spangelo *et al.*, 1987, Nagy *et al.*, 1983), CMI (Bernton *et al.*, 1988; Nagy and Berczi, 1981), and nonspecific immunity (Fu *et al.*, 1992). Secretion of prolactin from the lactotrophs of the anterior pituitary is under the inhibitory control of the catecholamine, dopamine. Dopamine is secreted into the hypophyseal vessels of the anterior pituitary by tuberoinfundibular dopaminergic neurons. Dopamine inhibits prolactin secretion by interacting with D2-dopamine receptors located on the lactotrophs of the anterior pituitary (Tepperman and Teppermann, 1987). Ergot alkaloids also are dopamine agonists and inhibit prolactin secretion by interacting with D2 receptors located on the lactotrophs of the anterior pituitary (Strickland *et al.*, 1994).

Historical Perspective of Vaccination against Plant Toxins

Nutritional management of livestock is often complicated by plant or fungal toxin contamination of forages and feeds intended for livestock consumption. This contamination may interfere with the nutritional benefits to animals by decreasing feed consumption, producing toxicosis, and by rendering the feed unsuitable for livestock consumption. Producers often use fungicides and herbicides to reduce feed or forage contamination, which may negatively impact the environment. Immunization of livestock to protect against adverse health effects of plant and fungal toxins present in

contaminated forages may allow livestock to benefit nutritionally. Immunization of livestock against plant and fungal toxins also may decrease the need for chemical treatment of affected forages.

Immunization for the protection of animals against plant and fungal toxins has rarely been reported in the literature. Parenteral vaccines that induced specific IgG antibodies against plant or fungal toxins resulted in various degrees of animal protection. Degree of protection against genistein (Cox, 1985), lantana toxin produced by *Lantana camara* (Stewart *et al.*, 1988), and lupinosis caused by mycotoxins produced by *Phomopsis leptostromiformis* (Payne *et al.*, 1993) is related to levels of specific serum IgG titers. Mice vaccinated against the toxic effects of sporidesmin, a toxin produced by the fungus *Pithomyces chartarum*, are protected (Jonas and Erasmunson, 1979). However, protection in mice against sporidesmin is related to the carrier and not to serum antibody levels (IgG and IgM). In the sporidesmin study, antibody titers were assayed by complement fixation, which measures only IgG and IgM as these immunoglobulins can fix complement. Immunoglobulin A (IgA) may have been induced by some of the protein carriers that the sporidesmin derivative was conjugated to. Thus, production of IgA may have imparted protection in the mice against sporidesmin, but this isotype was not measured.

Immunization against fungal and plant toxins in some cases exacerbates clinical signs of disease. Swine vaccinated against zearalenone, a toxin produced by *Fusarium graminearum*, experience greater morbidity to the estrogenic effects when challenged

with the mycotoxin (MacDonald *et al.*, 1990). Ewes vaccinated with protein conjugates of zearalenone also exhibit decreases in reproductive performance (Smith *et al.*, 1992). Despite the protection demonstrated in mice immunized against sporidesmin, ewes vaccinated against sporidesmin develop worse clinical signs than nonimmunized ewes (Fairclough *et al.*, 1984). Rats vaccinated and then challenged with senecionine also exhibit worsening of clinical signs (Culvenor, 1978).

Concepts in Oral Vaccination

In all reported cases of vaccination against plant and fungal toxins, the route of vaccination was parenteral, which induced predominantly an IgG response. As a result, plant and fungal toxins were still absorbed from the gastrointestinal tract into systemic circulation where interaction with target sites/receptors occurred. If IgG antibody responses were not sufficient to neutralize all absorbed toxins or if the toxins had a very high affinity for receptors, then perhaps over time enough toxins escaped the IgG immune response to produce toxicosis. Therefore, neutralization of plant and fungal toxins at the site of absorption (on mucosal surfaces) with IgA antibodies induced by local immunization (oral vaccine) may be beneficial. However, ability of oral vaccination to induce IgA and protect against mycotoxicosis in animals has not to our knowledge previously been investigated.

In the concept of the common mucosal immune system, lymphoid cells from the bone marrow populate the gut-associated lymphoid tissue (GALT), commonly known as the Peyer's patches, and the bronchus-associated lymphoid tissue (BALT). In GALT, environmental antigens enter Peyer's patches through pinocytotic and phagocytic M cells and interact with resident accessory, T and B-cells. Then, antigen sensitized B-cells and lymphoblasts committed to the IgA isotype leave Peyer's patches and enter the regional lymph nodes, lymph, and systemic circulation. These committed and sensitized B-cells populate various exocrine glands and mucosal-associated tissues, where terminal differentiation into IgA secreting plasma cells occurs. Analogous events are thought to occur in BALT (Mestecky, 1987). The GALT is an integral part of the immune system in most animals including cattle (Parsons *et al.*, 1989; Liebler *et al.*, 1988).

Concentrations of specific secretory IgA (sIgA) in secretions at mucosal surfaces are influenced by the route of vaccine administration. Parenteral immunization usually does not stimulate sIgA immune responses at mucosal surfaces because of the compartmentalization of systemic and secretory immune systems. Oral vaccination can produce high amounts of antigen-specific IgA. Two separate pools exist for IgA. Mucosal antibodies of the sIgA isotype are predominately derived from local synthesis and not from circulation. Serum IgA is predominately monomeric and is produced in the bone marrow.

Secretory IgA is the major immunoglobulin class found in exocrine secretions because it functions in the host defense of mucosal surfaces. Daily output of sIgA

exceeds all other immunoglobulins combined (Holmgren *et al.*, 1992). The mucosal surface is the largest area of an organism in contact with the environment. Mucosal surfaces are also the major route of entry for pathogens. Secretory IgA prevents toxin absorption by neutralization of toxins at mucosal surfaces, by prevention of adherence to mucosal surfaces, by reducing charge and hydrophobicity, and by agglutination (Russell and Mestecky, 1988).

Specific Objectives

Research to develop a vaccine against fescue toxicosis was initiated to address the problem of EI fescue pastures and lessen the economic and environmental impact.

Specific objectives were divided into :

Part I

1. Evaluate the ability of cattle grazing EI tall fescue to mount humoral immune responses to vaccination, despite hypoprolactemia.
2. Investigate the effects of consumption of EI tall fescue forage on lymphocyte proliferative responses to mitogens in mice and cattle and characterize cytokine production *in vitro*.

Part II

1. Conjugate ergotamine to various protein carriers via the Mannich reaction.
Assess the magnitude and duration of anti-ergotamine titers induced by various protein carrier-ergotamine conjugates.
2. Assess magnitude and duration of antibody response in mice vaccinated with carrier-ergotamine conjugate encapsulated in microspheres.
3. Evaluate the ability of antibodies induced by carrier-ergotamine conjugates administered orally and parenterally to protect against fescue toxicosis in mice.

Chapter 3: Humoral Immune Responses of Cattle Maintained on Fescue Pastures

INTRODUCTION

Hypoprolactemia in Fescue Toxicosis May Affect the Immune System

Serum prolactin concentrations are often decreased in cattle that graze EI tall fescue (*Festuca arundinacea* Schreb.) (Thompson *et al.*, 1987) and may interfere with ability of cattle to mount an immune response to vaccination. Prolactin, a polypeptide pituitary hormone produced in the anterior pituitary that is associated with lactation, also is important in the normal maintenance of the immune system. Ergot alkaloids are potent inhibitors of prolactin secretion via dopamine D2 receptors. Field reports suggest that cattle grazing EI tall fescue may not respond to vaccination as well as those grazing EF pastures (Sprowls, 1987). Research in rats supports this anecdotal evidence. Rats have a lower titer to vaccination with sheep red blood cells (SRBC) when fed diets of EI fescue seed (Dew *et al.*, 1990). Experimentally, decreases in serum prolactin concentrations in mice with the dopaminergic agonist, bromocriptine, reduce antibody responses to SRBC (Spangelo *et al.*, 1987). Impaired immune responses to SRBC vaccination in

hypophysectomized rats are reversed by subcutaneous administration of exogenous prolactin (Nagy *et al.*, 1983).

Cattle with hypoprolactemia that are maintained in EI fescue pastures may also have impaired CMI, which predisposes them to infection. Cell mediated immune responses require prolactin for production of macrophage activating factors (MAF) by T-cells. Hypoprolactemia impairs CMI by decreasing production by T lymphocytes of a major MAF, gamma interferon (IFN- γ) (Bernton *et al.*, 1988). Hypoprolactemia also decreases lymphocyte proliferation responses to mitogens (Bernton *et al.*, 1988). Lymphocyte proliferation responses to mitogens are decreased in rodents fed an EI fescue seed diet (Dew, 1989). Prolactin induces interleukin-2 (IL-2) receptors on lymphocytes (Mukherjee *et al.*, 1990). These IL-2 receptors are pivotal to immune responses mediated by T-cells. Additionally, hypophysectomized rodents are unresponsive to dinitrochlorobenzene-induced contact sensitivity, which is reversed by exogenous prolactin administration (Nagy and Berczi, 1981).

Non-specific immune mechanisms also may be impaired by fescue toxicosis. Phagocytosis and free radical production by alveolar macrophages and neutrophils are non-specific immune mechanisms important in the defense of the lungs. Superoxide anion secretion by neutrophils, which is important in the killing of pathogens, is dependent on prolactin (Fu *et al.*, 1992). Hydrogen peroxide release, MHC II expression and phagocytic activity of monocytes were decreased in cattle with fescue toxicosis (Saker, 1995).

Anecdotal observations suggest that cattle shipped from EI fescue pastures to feedlots experience a higher incidence of respiratory disease during the first few weeks (Sprowls, 1987). Causative agents of Bovine Respiratory Disease Complex (BRDC) include *Pasteurella* bacteria, infectious bovine rhinotracheitis (IBR), parainfluenza type 3 (PI3), and bovine virus diarrhea (BVD) viruses.

Research indicates that hypoprolactemia may interfere with immune responses. However, research in vaccinated cattle fed EI fescue diets are not in agreement with anecdotal reports or research in rats fed EI fescue diets. Steer calves vaccinated against IBR, PI3, and BVD viruses when fed EI fescue diet had similar humoral immune responses to calves fed EF fescue diet (Dew, 1989). In fact, anti-viral titers of steers fed EI fescue diet tended to be higher in general. However, calves fed EI fescue diet exhibited only mild signs of fescue toxicosis as indicated by body temperatures, weight gains, and serum ALP concentrations. Neither EV concentrations in treatment diets, nor serum prolactin concentrations were measured and correlated to anti-viral titers.

Assessment of the ability of cattle to mount humoral immune responses when grazing EI tall fescue is important to optimize vaccination programs, because reductions of prolactin concentrations may attenuate selected immune responses. If cattle that graze EI tall fescue have impaired immune responses, then cattle could be strategically vaccinated before consuming EI fescue forage.

Therefore, the objectives of this research were: 1) to assess the magnitude of humoral immune responses in cattle that graze EI versus EF tall fescue when vaccinated

with T-cell dependent antigens; and 2) to correlate humoral immune responses with serum prolactin concentrations.

MATERIALS AND METHODS

Animals and Experimental Design

The study site was the Southwest Virginia Agriculture Research and Extension Center at Glade Spring. Glade Spring is located in the Ridge and Valley region of Southwestern Virginia at an approximate latitude of 36° 41 min 13 sec north, longitude of 82° 02 min 01 sec west, and an altitude of 2070 feet.

In 1994, a total of 24 Angus and Angus crossbred steers, nine to ten months of age, were blocked by weight (average BW \pm SEM; 271 \pm 5.1 kg) and randomly allocated into two groups of 12 steers each. Steers of both groups were further allocated into two replicates of six to graze in two EI and two EF fescue pastures. Cattle were supplemented with standardized trace mineral salt (TMS) block (Morton TMS Block). Each pasture was 1.5 ha and the stocking rate was one steer per 0.25 ha. Pastures were fertilized and limed annually based on soil test recommendations. Soils at Glade Spring

are primarily Frederick (sil loam mixed, mesic, Typic Paleudult) and Hagerstown (fine, mixed, mesic, Typic Hapludalf) on well-drained slopes of 15 to 25%.

Pastures of 'Kentucky-31' tall fescue (EI and EF) were established in 1986. Pastures were managed as grazed forage, with the exception of 1993 and 1994, when spring growth was harvested as hay. Tall fescue forage was mowed to a height of 3 inches at the beginning of June, 1994. Desirable stands of tall fescue grass were maintained with negligible contamination of broadleaf weeds and undesirable grasses in pastures as determined by visual inspection. Tall fescue grass in all pastures was tested for the presence of *Acremonium coenophialum* (Morgan-Jones and Gams, 1982; Fescue Toxicity Diagnostic Center, Auburn Univ., Auburn, Al). One group grazed EI tall fescue (EI group) that was about 82 % endophyte-infected. The other group grazed EF tall fescue (EF group) that was about 2 % endophyte-infected. Ergovaline concentrations of the pastures were quantified by HPLC analysis (Hill *et al.*, 1993) at the initiation and termination of the study. Steers were acclimated on their respective pastures for 1 month prior to the beginning of the study on June 16, 1994.

HPLC Analysis of Fescue Forage Samples

Sample Collection

Grab samples of tall fescue were randomly collected by walking the pastures in a W pattern on June 16, July 7, and July 21, 1994. Individual grab samples of harvested plant tissue from each pasture were stored at -20 °C. Samples were freeze-dried and ground in a Stein mill prior to extraction for HPLC analysis.

Extraction of Fescue for Ergovaline Analysis

Tall fescue grass was extracted using the procedure of Rottinghaus *et al.* (1991). One gram of ground grass from each lot was extracted with 40 mL chloroform and 0.01 M NaOH (9:1 v/v) for 12 hours at 4 °C. Also 200 µL EG (4 µg/mL) in methanol was added as an internal standard. The supernatant was filtered through filter paper (Whatman 1PS) to remove residual water. A solid-phase clean-up column was made from TLC plates containing an organic binder (Analtech silica gel HL). Silica gel was scraped from TLC plates and ground with a mortar and pestle. Silica gel HL solid-phase chromatography columns were prepared by placing a 13 mm fritted disk in the bottom of a 6 mL disposable syringe barrel followed by 0.5 g of silica gel HL. After the silica gel layer, a 13 mm disc was placed followed by 1 g of sodium sulfate and another 13 mm disc to complete the column. The solid-phase column was prewashed with 2 mL of chloroform and 20 mL of fescue filtrate was added to the column under vacuum.

Pigments were eluted from the column with 5 mL of acetone and chloroform (75:25 v/v). Residual acetone and chloroform was removed with 1 mL of ethyl ether and the column was allowed to dry. A 0.25 micron filter was attached to the syringe bottom of the solid-phase column, then 4 mL of methanol was added. The methanol was eluted by slowly advancing the plunger manufactured for the syringe. The eluted methanol was evaporated to dryness at 45 °C with nitrogen gas and reconstituted with 1 mL of methanol for HPLC analysis.

High Performance Liquid Chromatography Analysis

HPLC analysis was done according to published methods (Rottinghaus *et al.* (1991). Working standard solutions of EV and EG from 50 to 1000 ng/mL were prepared in methanol and stored at -20 °C. Analyses was performed using a liquid chromatograph equipped with a fluorescence spectrophotometer (excitation wavelength: 250 nm ; emission wavelength: 420 nm) and a 7.5-cm x 4.2-mm C18 3- μ m column. The mobile phase was 34% acetonitrile in a 200 mg/L aqueous solution of ammonium carbonate at a flow rate of 1.5 mL/min. Fluorescence responses to EV and EG were recorded as peak height with a reporting integrator.

Ergotamine and Ergovaline Recovery

Ergotamine recovery was determined from the internal standard of each EI tall fescue sample analyzed for EV. Ergotamine recovery was determined by adding 200 μ L

of methanol containing 4 µg/mL of EG as an internal standard. Ergovaline recovery was determined by adding 100 µl of methanol containing 5 µg/mL EV to 1 g samples of freeze-dried EF tall fescue in 40 mL of chloroform followed by isolation with solid-phase chromatography columns of silica gel as previously described.

Vaccination and Blood Sampling Protocol

After one month acclimation on pastures, all steers were injected intramuscularly (IM) with 1 mL each of two different antigen mixtures on June 16, 1994. One antigen mixture contained 800 µg/mL of lysozyme in physiological saline without adjuvant. The second mixture consisted of 250 µg/mL of concanavalin A (Con A) in physiological saline with 10% SRBC emulsified in Freund's incomplete adjuvant (FIA). To prepare the second antigen mixture, fresh SRBC (7.5 mL) were washed three times in sterile saline and mixed with 42.5 mL of sterile saline containing 18.75 mg of Con A and 25 mL of FIA.

Steers were revaccinated 21 d after the first injection on July 7, 1994 with 1 mL each of the same antigen preparations. Whole blood was collected by jugular venipuncture 14 d post-revaccination on July 21, 1994. Sera collected on d 0, 21 and 35 were frozen at -20 °C for later determinations of antibody levels (titers), serum alkaline phosphatase (ALP), cholesterol, prolactin, growth hormone (GH), and insulin-like

growth factor-I (IGF-I) concentrations. Whole blood was also collected in heparinized tubes for white blood cell (WBC) counts and differentials on d 21 and 35. Prolactin (Wallner *et al.*, 1983), GH, and IGF-I concentrations were determined by radio-immunoassay (RIA) in the Endocrinology Laboratory at Virginia Polytechnic Institute and State University. Total WBC count and differential, serum ALP and cholesterol concentrations were measured by the Clinical Pathology Laboratory at the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM). Serum IgG titers against Con A and lysozyme were determined by ELISA and antibodies to SRBC were determined by hemagglutination. Indices measured to confirm fescue toxicosis in steers that grazed EI tall fescue were serum prolactin, ALP, and cholesterol concentrations, weight gains, and rectal temperatures. Rectal temperatures and body weights were measured on days that whole blood was collected.

Determination of Serum Hemagglutination Titers

The SRBC were obtained from the same sheep that supplied blood for vaccination of the steers. Whole blood was collected via jugular venipuncture into a sterile bottle that contained EDTA. The SRBC were washed twice with phosphate-buffered saline (PBS) and 100 μ L of SRBC was resuspended in 10 mL PBS for a final concentration of 1%. Fifty μ L of PBS was added to each well of a 96 well V bottom

microtiter plate and 100 μL of each serum sample (heat inactivated at 56 $^{\circ}\text{C}$ for 30 min.) was added to wells in duplicate. Two fold serial dilutions were carried out and 50 μL of 1% SRBC in PBS solution was added to each well. Plates were incubated for 4 h at room temperature and visually scored for agglutination. A negative control was included in each plate and titers were expressed as the reciprocal of the highest serum dilution of SRBC agglutination for each steer.

Determination of Anti-Concanavalin A and Lysozyme Antibody Titers

Wells of high binding immunoplates (Nunc Maxisorp[®]) were coated overnight at 4 $^{\circ}\text{C}$ with 5 μg per well of Con A or lysozyme diluted in 50 μL of 0.1 M sodium carbonate-bicarbonate buffer (pH 9.6). Plates were washed five times with PBS + 0.02% Tween 80 (PBST). Wells were blocked for 60 min at 37 $^{\circ}\text{C}$ with 100 μL of 0.5% (w/v) gelatin in PBST. Plates again were washed five times with PBST. Next, serially diluted aliquots (50 μL) of sera were added to each well and plates were incubated for 30 min at 37 $^{\circ}\text{C}$. Unbound antibody was removed by washing five times with PBST. Fifty μL of rabbit anti-bovine IgG (diluted 1/600 in 0.5% (w/v) gelatin in PBST) conjugated to horseradish peroxidase (HRPO) was added to each well. Plates were incubated for 30 min at 37 $^{\circ}\text{C}$ and washed five times with PBST. Bound peroxidase was determined by adding 100 μL per well of o-phenylenediamine (Sigma) and hydrogen peroxide

substrate. Plates were incubated at room temperature for 30 min and the reaction was stopped with 25 μ L per well of 8 N sulfuric acid. Absorbance was read at 490 nm with a multiscan reader (Titertek[®], Molecular Devices Corp.). The titer of each serum was the maximum dilution where the absorbance was greater than the mean plus 3-fold the standard deviation (SD) of triplicates of the same dilution of prevaccination sera.

STATISTICAL ANALYSIS

Data were tested for normality and homogeneity of variance. Data were analyzed by two-tailed Student's t-test (Statstix[®], Analytical Software). Titers were transformed to Log₂ values for statistical evaluation. Probability values < 0.05 were considered significant.

RESULTS

Mean concentration of EV in EI fescue pastures was 280 ppb. Ergovaline was not detected in EF fescue pastures. Recovery of EG and EV internal standards in HPLC analysis ranged from 95 to 100 %. On d 0, after one month of acclimation, ADG (Table

3:1) were lower ($P < 0.05$) in cattle that grazed EI tall fescue. Thereafter, ADG were not different ($P > 0.05$) between the two groups; however, body weight differences were maintained. Rectal temperatures (Table 3:1) were increased ($P < 0.05$) and serum ALP, cholesterol, (Table 3:2) and prolactin (Table 3:3) concentrations were decreased ($P < 0.05$) in the EI group throughout the 35 d trial period. Basal GH concentrations tended to be increased in both groups (Table 3:3) as compared to reference ranges used by the Endocrinology laboratory. However, concentrations of GH were lower ($P < 0.05$) in the EI group on d 0 and 35, but were similar ($P > 0.05$) to EF group on d 21 of the study. Insulin-like growth factor-I concentrations (Table 3:3) were similar ($P > 0.05$) in the EI and EF groups throughout the 35 d trial period and were within normal reference ranges of the Endocrinology laboratory.

Total WBC counts (Table 3:4) were similar ($P > 0.05$) between the EI and EF groups on d 21 and d 35. However, the total WBC counts increased ($P < 0.05$) in the EI group from d 21 to d 35, whereas WBC levels were not different over time in the EF group ($P > 0.05$). Monocyte numbers (Table 3:4) and percentages of monocytes were not different ($P > 0.05$) between the EI and EF groups on the days measured. On d 21, neutrophil numbers were similar ($P > 0.05$) between the groups; however, neutrophil numbers were higher in the EI group on d 35 ($P < 0.05$) compared to the EF group (Table 3:4). Over time in the EI group, numbers of neutrophils ($P < 0.05$) increased from d 21 to 35. A neutrophilic left shift was not present in either group. Although number of lymphocytes in the EI group increased ($P = 0.05$) from d 21 to 35, lymphocyte

numbers and percentages were similar ($P > 0.05$) between the EI and EF groups on the days measured (Table 3:4).

Primary immune responses (Table 3:5) to Con A tended to be higher ($P = .09$) in the EI group. Primary immune responses (Table 3:5) to SRBC were higher ($P < 0.05$) for the EI group. Secondary immune responses to Con A and SRBC were higher ($P < 0.05$) for the EI group (Table 3:5). Titers to lysozyme were not induced in either group indicating an adjuvant was needed to elicit an immune response at the dose used.

Table 3:1. Body Temperature and ADG of Cattle that Grazed EI versus EF Tall Fescue

Item	Day of study		
	0	21	35
Rectal temperature, °C			
EI group	40.8 ± 0.30 *	40.5 ± 0.28 *	40.5 ± 0.11 *
EF group	40.0 ± 0.2	39.8 ± 0.19	39.7 ± 0.22
Item	Day of study		
	-30 to 0	0 to 21	21 to 35
Daily gains, kg/steer ^a			
EI group	0.27 ± 0.1 *	0.27 ± 0.1	0.77 ± 0.1
EF group	1.05 ± 0.1	0.27 ± 0.1	0.73 ± 0.1

^aWeights for gain calculation are not cumulative and measurements made on d 0, 21, 35 on pasture correspond to one month acclimation period, d 0 to 21, and d 21 to 35, respectively.

Each treatment group consisted of n = 12.

Values represent group mean ± standard error.

* Difference (P < 0.05) between treatments within a column.

Table 3:2. Serum Alkaline Phosphatase and Cholesterol Concentrations of Cattle that Grazed EI versus EF Tall Fescue

Item	Day of study	
	0	35
Alkaline phosphatase, (U/L)		
EI group	59.6 ± 4.3 *	53.8 ± 5.0 *
EF group	80.3 ± 8.0	79.6 ± 5.2
Cholesterol, (mg/dL)		
EI group	73.7 ± 4.8 *	74.2 ± 4.6 *
EF group	119.8 ± 7.1	120 ± 8.2

* Difference ($P < 0.05$) between treatments within a column.

Each treatment group consisted of $n = 12$.

Values represent group mean \pm standard error.

Table 3:3. Serum Concentrations of Prolactin, Growth Hormone (GH) and Insulin Growth Factor-I (IGF-I) of Cattle that Grazed EI versus EF Tall Fescue

Item	Day of study ^a		
	0	21	35
Prolactin, ng/mL^b			
EI group	5.5 ± 3.0 *	8.6 ± 3.6 *	9.1 ± 3.5 *
EF group	113.2 ± 21.2	102.8 ± 17.1	121.9 ± 21.9
GH, ng/mL^c			
EI group	12.3 ± .89 *	17.6 ± 2.6	16.3 ± 2.0 *
EF group	20.3 ± 2.3	21.9 ± 5.1	30.2 ± 5.2
IGF-I, ng/mL^d			
EI group	253.5 ± 21.2	201.7 ± 15.4	252 ± 21.1
EF group	290.1 ± 26.4	239.3 ± 31.5	283 ± 30.6

^a Values represent group mean ± SEM.

^b For prolactin determinations, each treatment group consisted of n = 12 on days 0, 21, and 35.

^{cd} For GH and IGF-I determinations, each treatment group consisted of n = 12 on day 0. On day 21, EI treatment group consisted of n = 10 and EF treatment group consisted of n = 9. On day 35, EI treatment group consisted of n = 12 and EF treatment group consisted of n = 11.

* Difference (P < 0.05) between treatments within a column.

Table 3:4. Total WBC Counts and WBC Differentials of Cattle that Grazed EI versus EF Tall Fescue

Item	Day of study	
	21	35
Total WBC count ^a		
EI group	8.4 ± 0.4	10.6 ± 0.8
EF group	9.4 ± 0.8	9.4 ± 0.5
Monocyte numbers ^b		
EI group	0.078 ± 0.03	0.123 ± 0.6
EF group	0.213 ± .01	0.126 ± 0.6
Neutrophil numbers ^c		
EI group	2.7 ± 0.5	3.8 ± 0.3 *
EF group	2.9 ± 0.5	2.9 ± 0.2
Lymphocyte numbers ^d		
EI group	4.7 ± 0.4	6.4 ± 0.5
EF group	5.5 ± 0.4	5.8 ± 0.5

^a For total WBC counts, each treatment group consisted of n = 12.

^{bcd} For WBC differentials, each treatment group consisted of n = 4 on d 21 and n = 12 on d 35. Laboratory constraints were responsible for the n = 4 per treatment group on d 21.

Values represent group mean ± SEM.

Values expressed as 1×10^3 cells/ μ L.

* Difference (P < 0.05) between treatments within a column.

Table 3:5. Humoral Immune Responses of Cattle Vaccinated with Con A and SRBC that Grazed EI versus EF Tall Fescue

Treatment ^c	Primary immune response ^a (Log ₂ titer)		Secondary immune response ^b (Log ₂ titer)	
	Con A ^d	SRBC	Con A	SRBC
EI fescue pasture	5.8 ± 1.1	5.6 ± 0.6 *	9.2 ± 0.3 *	6.0 ± 0.7 *
EF fescue pasture	3.3 ± 0.8	3.1 ± 0.9	7.4 ± 0.7	3.2 ± 0.7

^a Measured on d 21 of study.

^b Measured on d 35 of study, revaccination on d 21.

^c Each treatment group consisted of n = 12.

^d P = 0.09.

Each antibody unit of titer represents a twofold serial dilution of serum, beginning with a 1:64 dilution at a Log₂ titer = 1.

Values represent group mean ± SEM.

* Difference (P < 0.05) between treatments within a column.

DISCUSSION

Fescue Toxicosis Did Not Influence the Leukogram of Cattle

Elevated rectal temperatures and lower serum ALP, cholesterol, prolactin concentrations, and decreased ADG measured in cattle maintained on EI fescue pasture indicated clinical fescue toxicosis.

Total WBC counts and differential indices were within normal laboratory reference ranges for both treatment groups. Thus, leukograms of cattle that grazed EI tall fescue were not negatively influenced by the end of the study period. Acute epinephrine release induced by stress and chronic inflammation increase numbers of neutrophils and lymphocytes (Duncan and Prasse, 1986). Acute epinephrine release from stress associated with blood collection (i.e. rounding up the cattle and restraining procedures) or heat stress may explain the increased neutrophil numbers on day 35 in the EI group. Cattle with fescue toxicosis have increased body temperatures and are more sensitive to heat stress.

Since cattle grazing EI fescue may have a predisposition to BRDC, measurement of plasma fibrinogen may be a better indicator of the presence of subclinical inflammatory disease than a leukogram. Measurement of plasma fibrinogen in the cattle of this study may have been useful, because neutrophil numbers were within reference

ranges. Early cases of BRDC causes fibrinous, nonpurulent inflammation in cattle that elicits little or no neutrophil response. An increase in plasma fibrinogen may be the only laboratory sign of early cases of inflammatory disease (Duncan and Prasse, 1986). In cattle, the neutrophilic response is poor and often does not correlated with severity of disease (Bender, 1995).

Although most cattle in this study did not have obvious clinical signs of respiratory disease, measurement of plasma fibrinogen in cattle grazing EI tall fescue in future experiments may be a valuable indicator of subclinical disease, especially after shipment to feedlots.

Growth Hormone and ADG

A deficiency of GH or protein-calorie malnutrition retards growth. Hyposecretion of GH by the anterior pituitary gland decreases circulating concentrations of both GH and IGF-I. Protein-calorie malnutrition increases GH, but decreases IGF-I concentrations. However, in this study, GH concentrations tended to be increased and IGF-I concentrations were within normal reference ranges for both EI and EF groups. Although GH concentrations were decreased about 50% in EI group on d 0 and 35, GH and IGF-I concentrations overall were not suggestive of GH hyposecretion or protein-

calorie malnutrition, and thus probably were not the cause of low ADG of cattle in this study.

In this research, basal concentrations of GH tended to be increased in both groups as compared to reference ranges of the Endocrinology laboratory. Stress associated with working cattle does not influence GH release by the anterior pituitary gland as it does in rodents (decrease GH release) or primate species (increase GH release) (Malvin, 1993). However, increased GH concentrations would be expected in cattle that graze EI fescue, because dopamine stimulates GH release (Malvin, 1993) and ergot alkaloids are dopamine agonists. Basal concentrations of GH have been increased in cattle that grazed EI fescue, but this is an infrequent finding in cattle with fescue toxicosis. Concentrations of GH stimulated by thyrotropin releasing hormone (TRH) are similar in cattle grazing EI or EF tall fescue (Thompson *et al.*, 1987).

The tendency for increased GH concentrations in both groups may have been a result of age and gender of the cattle. Young animals have higher GH concentrations than mature animals, and steers have higher GH concentrations than heifers (Malvin, 1993). Decreased GH concentrations and normal IGF-I concentrations in the EI group on d 0 and 35 may be because of differences in pulsatile release of GH between the groups. Release of GH into the blood is episodic, therefore extreme fluctuations of plasma levels occur throughout the day. Release of GH among individual animals is not usually synchronized (Malvin, 1993). Similar concentrations of IGF-I further supports the idea that the differences in GH release on d 0 and 35 in the EI group were caused by

differences in pulsatile release of GH. If decreases in GH concentrations were from hyposecretion by the anterior pituitary gland, then concentrations of IGF-I would have also been decreased in the EI group, because IGF-I secretion is dependent on GH.

Likewise, increases in GH in cattle grazing EI fescue as observed by Thompson *et al* (1987) may have been because of differences in pulsatile release of GH, because GH concentrations after TRH stimulation were not different between cattle grazed on either EI or EF fescue pastures.

The low ADG in both groups may be related to environmental stresses. High ambient temperatures may have caused the low ADG in both groups on d 21 and d 35 of the study. Cattle tend to graze and gain less during hot weather and high humidity (Bond *et al.*, 1984). The lower ADG in the EI group on d 0 of the study, after one month of acclimation to respective pastures, could have been caused by increased susceptibility to heat stress. Additionally, seed heads were present on fescue plants on pastures during the acclimation period. Ergovaline concentrations are higher in seed heads than in fescue forage (Rottinghaus *et al.*, 1991). Therefore, cattle may have been exposed to higher concentrations of ergovaline, which could have lowered ADG.

Growth Hormone and Insulin-Like Growth Factor May Have Restored Immune Function of Cattle with Fescue Toxicosis

Increased humoral responses to vaccination in the EI group indicated that despite hypoprolactemia, titer responses were mounted. Titer results in this study are in agreement with results of anti-viral titers in calves housed in temperature-controlled rooms that were fed EI fescue diet (Dew, 1989). Perhaps the ability of cattle with hypoprolactemia to mount humoral immune responses when grazed on EI fescue is related to GH concentrations and balance of T_H cells.

Both prolactin and GH are involved in the maintenance of humoral immunocompetence. Growth hormone is similar in structure to prolactin (Hiestand and Mekler, 1986) and may be able to bind prolactin receptors to exert prolactin-like actions (Bernton *et al.*, 1988). Prolactin receptors are located on lymphocytes (Russell *et al.*, 1984). Prolactin bound to high affinity receptors located on lymphocytes induces activity of the enzyme, ornithine decarboxylase (Klimpel *et al.*, 1979). Induction of ornithine decarboxylase is one of the earliest detectable cellular events in lymphocyte activation (Klimpel *et al.*, 1979). When binding of prolactin to lymphocyte receptors is blocked, GH induces ornithine decarboxylase (Russell *et al.*, 1984).

Release of GH from the anterior pituitary is episodic with extreme fluctuations in circulating concentrations. Because GH concentrations fluctuate so radically, GH stimulates release of IGF-I to regulate cellular growth and other processes in a steady

state manner (Malvin, 1993). Insulin-like growth factor-I, formally called somatomedin-C, is produced predominately by the liver. Growth hormone regulates most of the synthesis and secretion of IGF-I, which is the major mediator of action for GH. The IGF-I may function as a hormone to be released into the blood for endocrine action and as a regulatory molecule in the area of cellular release (Malvin, 1993).

Growth hormone and IGF-I may restore humoral, CMI, and nonspecific immune responses that are impaired by hypoprolactemia. Growth hormone restores antibody responses to SRBC vaccination (Spangelo *et al.*, 1987) and restores selected CMI responses (Berczi *et al.*, 1983) in hypophysectomized rats. Immunoreactive GH produced by lymphocytes stimulates lymphocyte proliferation *in vitro* (Weigent *et al.*, 1991). Growth hormone and IGF-I may restore nonspecific immune response by priming neutrophils (PMN) (Fu *et al.*, 1991) and macrophages (Edwards *et al.*, 1988) to secrete superoxide anion for an enhanced respiratory burst. Growth hormone, but not IGF-I, also primes monocytes for enhanced H₂O₂ production (Warwick-Davies *et al.*, 1995).

Concentrations of GH and IGF-I were not adversely affected by fescue toxicosis and concentrations may have been adequate to act at prolactin receptors. Although, ability of GH or IGF-I to interact with bovine prolactin receptors has not been demonstrated, GH and IGF-I may restore humoral immune responses in hypoprolactemic cattle that graze EI fescue. However, these indices would not explain the increased humoral immune responses to vaccination in the cattle that grazed EI fescue.

Fescue Toxicosis May Stimulate T Helper 2 Subset to Augment Humoral Immune Responses

In this study, not only did cattle with fescue toxicosis mount humoral immune responses, but significantly higher humoral immune responses, despite hypoprolactemia. Fescue toxicosis may significantly increase humoral immune responses by altering the balance of T_H cell activities. T-cell dependent antigens require both T-cells and B-cells for generation of a humoral immune response. T helper cells or CD4⁺ cells can be separated into two major subsets, T_{H1} and T_{H2}, based on the cytokine profile secreted when the cells are activated (Mosmann and Coffman, 1989).

Lymphokines secreted by T_{H1} cells are IFN- γ , IL-2, IL-3, and tumor necrosis factor (Mosmann and Coffman, 1987; Mosmann and Coffman, 1989). The T_{H1} cells mediate delayed type hypersensitivity responses and activate macrophages (Mosmann and Coffman, 1987; Mosmann and Coffman, 1989). Functionally, T_{H1} cells in general activate cell mediated immunity. The T_{H2} cells produce IL-4, IL-5, IL-6 and IL-10 and provide help for B-cell differentiation and antibody production (Mosmann and Coffman, 1987; Mosmann and Coffman, 1989). The T_{H2} cells mediate hapten-carrier helper activity, and augment IgG1 and IgE production (Lise and Audibert, 1989).

Differentiation of T_{H0} cells into cells of either T_{H1} or T_{H2} phenotype after activation is not fully understood, but is thought to depend on several factors. These factors include type, amounts and sequences of cytokines secreted, type of APC involved

in processing of the antigen for presentation to T-cells, and the antigenic load.

Production of IL-10 by B-cells may trigger the switch from T_H1 to T_H2 regulated immune responses (Taylor-Robinson and Phillips, 1994).

Relative numbers of T_H cells are similar in cattle grazing EI fescue and those grazing EF fescue (Dew, 1989). However, T_H2 cells may dominate functionally, because secretion of selected cytokines by T_H1 cells may be inhibited in fescue toxicosis. Hypoprolactemia decreases IFN- γ secretion by T_H1 cells in mice (Bernton *et al.*, 1988) to decrease CMI. Gamma interferon is important in preventing viral replication within cells, enhancing the killing ability of macrophages, augmenting expression of class II proteins, and down regulating the activity of T_H2 cells. Decreases in INF- γ production are associated with normal or increased humoral immune responses (Candore *et al.*, 1993). Additionally, the T_H2 -derived cytokine, interleukin-10, may inhibit T_H1 cell function to decrease CMI and increase humoral immune responses (Powrie *et al.*, 1993).

Decreases in INF- γ production by T_H1 cells and increases in IL-4 production by T_H2 cells often increase specific IgG production. Production of IL-4, IL-5 and antigen specific IgG1 is increased by T_H2 cells in mice with the inability to secrete INF- γ (Graham *et al.*, 1993). Transgenic mice expressing IL-4 have increases in serum levels of IgG1 and antibody immune responses against specific antigens (Burstein *et al.*, 1991). Additionally, IL-4 synergizes with other mediators, such as prostaglandin E2, to significantly increase IgG1 production up to 26-fold (Roper, *et al.*, 1990).

Increased body temperature also may augment immune responses. Cattle that graze EI tall fescue pastures often have increased body temperatures as seen in this study. A plausible mechanism for increased body temperature and augmented humoral immune responses in cattle with fescue toxicosis may be through IL-1 production. Interleukin-1 is an endogenous pyrogen that increases body temperature, and increases humoral immune responses against SRBC vaccination *in vivo* (Reed *et al.*, 1989). Interleukin-1 produced by stimulated macrophages and monocytes (Bernton *et al.*, 1987) increases proliferation of antigen stimulated lymphocytes and lymphokine production (Dinarelli, 1984). Perhaps in fescue toxicosis, an endophyte component absorbed systemically induces production of cytokines. Many fungal carbohydrates are immunostimulants (Wagner and Proksch, 1985).

Interleukin-1 also is a messenger for the interaction of the immune system with the neuroendocrine system. Interleukin-1 produced peripherally by macrophages may act directly on anterior pituitary cells to modulate hormone secretion *in vivo*. Interleukin-1 stimulates *in vitro* release of adrenocorticotrophic hormone, GH, and thyroid stimulating hormone, but decreases prolactin release (Bernton *et al.*, 1987). However, chronic inflammation with sustained IL-1 levels may ultimately deplete pituitary hormones (Bernton *et al.*, 1987).

Additionally, fescue toxicosis may prime B-cells for activation to increase humoral immune responses indirectly by decreasing ALP concentrations. Effects of ALP and signal transduction on the surface Ig receptor of B-cells is a growing research area.

Alkaline phosphatase isoenzymes are in every tissue and exhibit high activity in liver, bone, intestine, kidney, placenta, and leukocytes (Duncan and Prasse, 1986).

Alkaline phosphatase isoenzymes are essentially plasma membrane enzymes that function in the hydrolysis of simple phosphomonoesters, phosphate transfer, and phospho-hydrolysis of phosphoproteins (Metaye *et al.*, 1989). Phosphorylation or dephosphorylation of proteins is important in regulating protein activities and may couple extracellular stimuli to intracellular events.

Alkaline phosphatase isoenzymes are involved in cellular regulation by controlling concentrations of the protein, phosphotyrosine, which is important in the mitogenic response. Alkaline phosphatase isoenzymes have a selectivity for phosphotyrosine residues in proteins. Tyrosine phosphorylation plays an active role in signal transduction and B-cell activation. Phosphorylation of tyrosine is an initial event of cell multiplication induced by growth factors. Mammalian ALP dephosphorylates proteins containing phosphotyrosine residues (Swarup *et al.*, 1981). Thus inhibition of ALP may increase tyrosine phosphorylation to enhance signal transduction and B-cell activation. B-cells are affected more by ALP inhibitors than T-cells, since ALP activity is more critical in B-cells than T-cells (Metaye *et al.*, 1989). Additionally, ALP may modulate the activity of ornithine decarboxylase (Metaye *et al.*, 1989).

CONCLUSIONS

Cattle with overt fescue toxicosis mounted humoral immune responses against two antigens, despite hypoprolactemia. Growth hormone and a predominance of T_H2 cells with concomitant IL-4 production induced by fescue toxicosis could explain the augmentation of humoral immune responses in cattle maintained on EI tall fescue pastures. Suppression of T_H1 cell functions by a decrease in $INF-\gamma$ production may permit T_H2 cells to stimulate synthesis of higher levels of IgG by B-cells. This stimulation could be increased by decreased concentrations of ALP, which enhances signal transduction in B cells to increase titers. Decreased concentrations of serum prolactin may also negatively affect nonspecific immune responses that may predispose cattle to respiratory infections. Therefore, increased morbidity from BRDC in cattle fed EI fescue may be a result of the inability of nonspecific defense mechanisms in the lungs to kill invading pathogens and not failure of cattle to respond to vaccination.

Chapter 4: Lymphocyte Blastogenesis and Cytokine

Production

INTRODUCTION

Cattle and mice fed EI fescue diets differ in lymphocyte proliferative responses to mitogen stimulation. Lymphocyte proliferation to mitogens is suppressed in rodents fed EI fescue seed diet, whereas normal or increased responses are observed in cattle (Dew, 1989). In light of these discrepancies in lymphocyte proliferation between species and the higher humoral immune response observed in vaccinated cattle that grazed EI fescue, research was initiated to study lymphocyte proliferative responses and cytokine production in mice fed fescue. Also, the effect of sera from cattle fed EI or EF fescue diets on bovine lymphocyte proliferative responses to mitogens was studied.

Both EG and EV are alpha 2-adrenergic and dopamine agonists. The base structures of both EV and EG are structurally similar to serotonin, tryptophan, and melatonin. Therefore, EV and EG may interact with alpha adrenergic, dopaminergic, and serotonergic receptor sites. To affect lymphocyte proliferation of T-cells or B-cells, ergot alkaloids may directly bind surface membrane receptors located on immune cells or induce/inhibit production of soluble mediators. Ergovaline and EG are alpha 2-adrenergic agonists that inhibit cAMP. Alpha 2-adrenergic receptors have been

demonstrated on cell surfaces of human lymphocytes (Titinchi and Clark, 1984), thus ergot alkaloids may bind lymphocytes directly to affect the immune responses.

Fescue toxicosis also may induce production of autocrine or paracrine factors to affect lymphocyte proliferation and immune responses. Cytokines and prostaglandins of the E (PGE₂) series are soluble factors that fescue toxicosis could induce to mediate effects on immune responses.

Prostaglandins produced by macrophages/monocytes, follicular dendritic cells, and fibroblasts generally suppress immune responses (Roper, *et al.*, 1990). Many immune stimuli promote PGE₂ secretion, such as IL-1, tumor necrosis factor, lipopolysaccharide (LPS), complement activation, endotoxins, and binding of IgG, IgA, and IgE to Fc receptor (Roper *et al.*, 1990).

Prostaglandin E₂ induces similar effects as fescue toxicosis on MHC II expression, hydrogen peroxide (H₂O₂) release, and phagocytic activity in cattle, and antibody production in mice. Surface expression of class II MHC antigens and transcription of class II MHC genes (Figueiredo *et al.*, 1990) and MHC II up regulation in activated cells (Roper and Phipps, 1992) is inhibited by PGE₂. Additionally, oxygen radical production by LPS activated macrophages (Metzger *et al.*, 1981) and oxidative burst in bovine PMN cells are suppressed by PGE₂ (Phillips *et al.*, 1987). Prostaglandin E₂ inhibits macrophage phagocytosis (Fernandez *et al.*, 1982) and also increases *in vivo* the induction of specific T suppressor cells (Kozlov *et al.*, 1990). Prostaglandin E₂ crosses the blood-brain barrier to increase body temperature (Moltz, 1993).

Similar effects induced by PGE₂ are observed in cattle with fescue toxicosis on MHC II expression, H₂O₂ release, phagocytic activity, and antibody production.

Decreases in H₂O₂ release, MHC II expression, and phagocytic activity of monocytes in cattle that grazed EI fescue forage have been observed (Saker, 1995). Numbers of T suppressor cells are increased in spleens of mice fed EI diet of tall fescue seed (Dew, 1989). Cattle grazing EI tall fescue also have increased body temperatures.

The objectives of this research were to: 1) assess proliferative responses of splenic lymphocytes from mice fed EI or EF fescue diets to mitogens; 2) assess proliferative responses of peripheral blood lymphocytes (PBL) from a normal, donor cow when stimulated with mitogens in the presence of sera from cattle grazing EI or EF fescue forage; 3) assess production of INF- γ , IL-2, IL-4 by splenocytes from mice fed EI or EF fescue diets; 4) determine effects of inhibition of prostaglandin production by indomethacin on lymphocyte proliferative responses of splenocytes from mice fed EI or EF fescue diets; 5) determine effects of yohimbine, an alpha adrenergic antagonist, on proliferation of splenic lymphocytes from mice fed EI or EF fescue diets; and 6) assess ability of anti-EV antibodies to reverse any effects observed *in vitro* on proliferation of splenic lymphocytes from mice fed EI or EF fescue diets.

MATERIALS AND METHODS

Lymphocyte Transformation Assay (LTA)

Recovery of Peripheral Blood Lymphocytes from Donor Cow

Peripheral blood lymphocytes (PBL) from a donor cow were used to assess the effect of sera from steers that grazed EI versus EF tall fescue on lymphocyte proliferation to mitogen stimulation. Blood was aseptically collected from a donor cow via tail vein venipuncture into heparinized tubes. Peripheral blood lymphocytes were isolated with Ficoll gradient separation. Heparinized whole blood was mixed (1:2 ,v/v) with sterile PBS. In a sterile 15 mL tube, 6 mL of diluted blood was layered over 3 mL of Ficoll's gradient (sp. 1.086) and centrifuged at 4 °C for 15 min at 800 x g. The layer containing the lymphocyte fraction was collected with a sterile pipet and washed in 10 mL of RPMI media for 10 min at 400 x g. Red blood cells were lysed with AKA lysis buffer (0.1 M NH_4Cl , 1 g NaHCO_3 , 0.1 mM EDTA in 1 L at pH 7.2 to 7.4) and lymphocytes were washed twice in 10 mL RPMI for 5 min at 400 x g. Lymphocytes were resuspended in 5 mL of complete RPMI (cRPMI) [10% heat inactivated fetal bovine serum, 1mM Hepes, 2 mM L-glutamine, 5 U/dL penicillin, 5 U/dL streptomycin and nonessential amino acids]. The number of viable lymphocytes was determined by trypan blue exclusion and counted using a hemocytometer. Additional cRPMI was added to the cell suspension to achieve a final cell concentration of 5×10^6 cells per mL.

Determination of Optimal Mitogen Concentrations for Use in Bovine LTA

Round bottom wells of a 96-well, tissue culture plate were seeded with 5×10^5 viable cells per well in 200 μL media. The T-cell mitogens, Con A (ICN Corp.) and phytohemagglutinin (PHA) (Sigma), and the B-cell mitogen, *Brucella abortus* 2308 LPS, were added to triplicate wells. Standard curves to establish the optimal concentrations of Con A and brucella LPS were determined by adding 0.1, 1.0, 10, and 20 μg of each mitogen per well in triplicate. A standard curve to establish the optimal concentration for PHA was determined by adding 0.2, 2.0, 10, and 20 μg per well in triplicate. Plates were incubated for 72 h in a tissue culture incubator at 37 °C with an atmosphere of 5% CO_2 and 95% air. Cell proliferation in response to each mitogen was determined by the addition of 1.0 μCi of [methyl- ^3H] thymidine to each well. After an 18 h pulse period, cells were harvested onto glass fiber strips using a cell harvester. Radioactivity was counted in a liquid scintillation counter and reported as counts per minute (CPM).

Effect of Sera from Steers that Grazed EI or EF Tall Fescue

Peripheral blood lymphocytes were collected from the donor cow as previously described. Twelve sera samples were randomly chosen from the 24 steers previously bled for titer determinations that grazed EI or EF tall fescue for 63 days. Serum from 12 steers was diluted in sterile PBS and passed through a 0.25 micron filter to remove bacterial contaminants. Filter sterilized serum was added to triplicate wells containing

optimal mitogen concentrations of Con A (1.0 µg per well) and PHA (2.0 µg per well) for an overall concentration of 5 % serum per well.

Spleen Cell Recovery from Mice Fed EI or EF Fescue Seed Diets

Eight, female mice of the National Cancer Research (NCR) strain were allocated into two treatment groups and fed either EI 'Kentucky 31' or EF 'Forager' varieties of tall fescue seed/chow diets (50:50, w/w) for 6 wk. The EI and EF fescue seed varieties were obtained from Southern States Coop. The EI and EF fescue diets were not analysed for ergovaline concentrations or for nutrient content. Splenic lymphocytes from mice were used to assess lymphocyte proliferative responses to mitogens and cytokine production. Spleens of mice were aseptically removed and placed individually into a small sterile petri dish containing 10 mL of RPMI and a sterile screen. The spleen was gently moved over the screen using the plunger of a 5 cc sterile syringe to disperse the cells. Cells were washed 3 times with 10 mL RPMI at 400 x g for 6 min per wash. Red blood cells were lysed with 10 mL of AKA lysis solution. Cells were washed again in 10 mL of RPMI and then resuspended into 10 mL of cRPMI media.

The number of viable lymphocytes was determined by trypan blue exclusion and counted using a hemocytometer. Additional cRPMI was added to the cell suspension to achieve a final cell concentration of 5×10^6 cells per mL.

Triplicate wells seeded with 5×10^5 lymphocytes per well in 200 µL media from mice fed EI or EF fescue diets for 6 wk were stimulated with Con A (1 µg per well), *E.*

coli LPS (1 µg per well), and PHA (2 µg per well). Wells seeded with 5×10^5 lymphocytes per well in 200 µL media from mice fed EI or EF fescue diets for 6 wk were stimulated with mitogens in the presence of yohimbine (16 nM per well; 6 replicates), indomethacin (1×10^{-7} M per well; 6 replicates), and affinity purified monoclonal antibodies against EV (0.022, 0.22, 2.2, 22.0 µg per well).

Plates were incubated for 48 h in a tissue culture incubator at 37 °C with an atmosphere of 5% CO₂ and 95% air. Cell proliferation in response to each mitogen was determined by the addition of 1.0 µCi of [methyl-3H] thymidine to each well. After an 18 h pulse period, cells were harvested onto glass fiber strips using a cell harvester. Radioactivity was counted in a liquid scintillation counter and reported as CPM.

Cytokine Production by Splenic Lymphocytes *in Vitro* from Mice Fed EI or EF Fescue Seed Diets

Sample Collection

The mitogens, Con A (1.0 µg per well), *E. coli* LPS (1.0 µg per well) and PHA (2.0 µg per well), were added to round bottom wells of a 96-well, tissue culture plate. Wells were seeded with 5×10^5 viable cells per well in 200 µL media from mice fed EI or EF fescue diets for 6 wk. Plates were incubated for 48 h in a tissue culture incubator at 37 °C with an atmosphere of 5% CO₂ and 95% air. Cell supernatant was collected

from each well, centrifuged for 5 min at 400 x g to remove cells, and frozen at -70 °C until analyzed for cytokine content.

Assay Procedure

Interleukin-4, IL-2, and INF- γ were measured by ELISA (MinikitTM by Endogen Cambridge, MA). Wells of high binding immunoplates (Nunc Maxisorp[®]) were coated overnight at 25 °C with 100 μ L per well of anti-mouse, coating antibody specific for either IL-4, IL-2, or INF- γ in PBS at pH 7.4. Plates were emptied and blotted dry. Then plates were blocked for 1 h at 25 °C with 200 μ L per well of assay buffer (PBS with 2 % BSA fraction V and 0.01 % thimerosal, pH 7.2 to 7.4). Plates were washed 5 times in wash buffer (50 mM Tris, 0.2% Tween-20, pH 7.0 to 7.5). Fifty microliters of samples and standards were added in duplicate to wells containing 50 μ L assay buffer and incubated for 12 to 18 h at 25 °C. Standard curves for the cytokines ranged from 20 to 1300 pg per mL for IL-2, from 0.4 to 52.2 ng per mL for INF- γ , and from 7.6 to 488.4 pg per mL for IL-4. Plates were washed 5 times in wash buffer and 100 μ L per well of either anti-mouse IL-2, IL-4, or INF- γ biotinylated detecting antibody was added. After 1 h of incubation at 25 °C, plates were washed 5 times in wash buffer. Then 100 μ L per well of 1:5000 horseradish peroxidase conjugated streptavidin (Zymed Corp) was added for 30 min at 25 °C. Plates were washed 5 times and 100 μ L per well of tetramethylbenzidine (TMB) substrate (Dako Corp. #S1600) was added. Plates were

developed in the dark for 30 min at 25 °C. The reaction was stopped with 100 µL per well of 0.18 M sulfuric acid and absorbance at 450 nm was measured. Standard curves were constructed and equation of the line was determined by linear regression. The derived equation was used to calculate cytokine concentrations in samples assayed.

STATISTICAL ANALYSIS

Randomized complete-block design (SAS®, SAS Institute, Cary, NC) was used to determine significant differences at P value of 0.05 between lymphocyte proliferative responses to Con A, LPS, and PHA mitogens in mice fed EI or EF fescue diets. Mice were blocked by pairs to account for interassay variation and diets tested against pair*diet interaction. Mice were blocked by pairs of mice fed either EI or EF fescue diets that were tested on different days.

Paired T test (two tailed) was used to determine significant differences at P value of 0.05 between lymphocyte proliferative responses to Con A and PHA of sera from cattle that grazed EI or EF tall fescue (Microsoft Excel, Microsoft Corp, USA).

Student's T test (two tailed) was used to determine significant differences at a P value of 0.05 for wells with and without indomethacin and yohimbine for each mitogen (Microsoft Excel, Microsoft Corp, USA).

One way analysis of variance was used to test for significance at a P value of 0.05 between the means of the effect of purified monoclonal antibodies on mitogen responses. Bonferroni pairwise comparisons were used to determine differences among various MAB treatments for both EI and EF groups. Stepwise regression was used to determine if linear or quadratic relationships existed between lymphocyte proliferation and increasing MAB concentrations *in vitro* for both EI and EF groups (Statstix®, Analytical Software).

Wilcoxon signed rank test was used to determine significant differences at a P value of 0.05 for IL-2, IL-4 and INF- γ production by splenocytes from mice fed EI or EF fescue diets (Statstix®, Analytical Software).

RESULTS

Bovine LTA

The optimal concentration of Con A and PHA to stimulate proliferation of bovine PBL was 1 μg per well and 2 μg per well, respectively (Figure 4:1). Brucella LPS failed to stimulate cells at concentrations added.

Sera from steers that grazed both EI and EF tall fescue increased proliferation (P < 0.05) of PBL stimulated with Con A greater than Con A without sera. However, sera

from steers that grazed EI tall fescue stimulated proliferation of PBL in response to Con A greater ($P < 0.05$) than sera from steers that had grazed EF tall fescue (Figure 4:2). In contrast, sera from steers that grazed either EI or EF tall fescue decreased proliferation ($P < 0.05$) responses to PHA (Figure 4:3). Proliferation responses to PHA were not different ($P > 0.05$) between sera from steers that grazed either EI or EF tall fescue.

Murine LTA and Cytokine Production

The standard curve for IL-2 was linear ($r^2 = 0.995$) and the equation of the line was $y = 0.001x + 0.056$. The standard curve for IL-4 was linear ($r^2 = 0.986$) and the equation of the line was $y = 0.001x + 0.101$. The standard curve for INF- γ was linear ($r^2 = 0.986$) and the equation of the line was $y = 0.027x + 0.022$.

Body weights, spleen weights, spleen weights as a percentage of body weights, and total numbers of splenic cells were similar ($P > 0.05$) in mice fed EI or EF fescue diets (Table 4:1). Proliferative responses (Table 4:2) of splenic lymphocytes from mice fed EI or EF fescue diets were similar ($P > 0.05$) for all mitogens. Although not significant, lymphocyte proliferation to Con A was decreased 38% in EI group. Interleukin-4 (Figure 4:4) production (986.5 pg/mL) stimulated with Con A in splenic

lymphocytes from mice fed EI fescue seed diet tended to be increased ($P = 0.1$) compared to mice fed EF diet (409 pg/mL). Interleukin-4 (Figure 4:4) production was similar ($P > 0.05$) between groups for LPS and PHA. Gamma interferon production (Figure 4:5) stimulated with Con A, LPS, and PHA in splenic lymphocytes from mice fed EI fescue seed diet was similar ($P > 0.05$) to mice fed EF diets. However, $\text{INF-}\gamma$ production stimulated by all mitogens in splenic lymphocytes from mice fed EI fescue seed was 35% greater than $\text{INF-}\gamma$ production in splenic lymphocytes from mice fed EF fescue seed diets. Interleukin-2 production (Figure 4:6) stimulated by LPS in splenic lymphocytes from mice fed EI fescue seed diets tended to be lower ($P = 0.18$) as compared to IL-2 produced in splenic lymphocytes from mice fed EF fescue diets. Interleukin-2 production (Figure 4:6) stimulated by PHA in splenic lymphocytes were similar ($P > 0.05$) in both groups. Interleukin-2 production (Figure 4:6) stimulated by Con A in splenic lymphocytes from mice of both groups exceeded the standard curve and, thus differences in IL-2 production between EI and EF groups could not be determined.

Yohimbine increased lymphocyte proliferative responses ($P < 0.05$) to all mitogens in mice fed EI fescue seed diets (Figure 4:7). However, yohimbine did not increase proliferative responses ($P > 0.05$) of splenic lymphocytes from mice fed EF fescue seed diet to Con A, LPS, and PHA.

Indomethacin (Figure 4:8) did not affect ($P > 0.05$) proliferative responses of lymphocytes from mice fed EI diet to PHA, LPS and Con A. Indomethacin did decrease proliferative responses of lymphocytes from mice fed EF diet to Con A, and LPS ($P < 0.05$), but not PHA ($P > 0.05$). Indomethacin (1×10^{-7} M per well) or monoclonal antibodies against EV (MAB) alone did not stimulate proliferation of lymphocytes from mice fed EI or EF diets.

Higher concentrations of MAB (22.0 and 2.2 μg per well) tended to increase proliferation of splenic lymphocytes in response to Con A from mice fed EI fescue diet (Figure 4:9). Lower concentrations of MAB (0.22 and 0.022 μg per well) tended to decrease proliferation. Proliferation was higher for 22.0 μg per well of MAB than for 0.02 μg per well ($P < 0.05$) in the presence of Con A (Figure 4:9). In the EI group, proliferation of splenocytes stimulated with Con A ($P = 0.19$, $r^2 = .65$) or LPS ($P = 0.11$, $r^2 = 0.80$) in the presence of 0.022, 0.22, 2.2, and 22 μg per well of MAB tended to be linear. However, proliferation of splenocytes stimulated with Con A ($P = 0.33$, $r^2 = 0.46$) or LPS ($P = 0.83$, $r^2 = 0.03$) in the presence of 0.022 to 22 μg per well of MAB was not linear in the EF group.

Addition of MAB at any concentration to splenic lymphocytes from mice fed EF fescue diets did not affect proliferative responses ($P > 0.05$) to Con A (Figure 4:9). Proliferative responses of splenic lymphocytes to LPS from mice fed EI or EF fescue diet were similar ($P > 0.05$) after addition of MAB at any concentration (Figure 4:10).

Table 4:1. Body Weights, Spleen Weights, and Total Spleen Cells in Mice Fed EI or EF Diets

	EI Group	EF group	P value
Body weight (g)	29.03± 0.78	29.22 ± 0.56	0.85
Spleen weight (g)	0.125 ± 0.014	0.124 ± 0.012	0.94
Spleen weight as % body weight	0.432 ± 0.046	0.426 ± 0.038	0.93
Spleen cell # (1 X 10⁷)	9.49 ± 1.04	9.15 ± 0.80	0.80

Values reported as mean ± SEM.

Each treatment group n = 4.

Diets consisted of 50% rodent chow and 50% fescue seed.

Mice were fed treatment diets for 6 wk ad libitum.

Table 4:2. Responses of Splenocytes to Mitogens from Mice Fed EI or EF Diets

Mitogen	EI group		EF group	
Con A ^a	38694	± 5288	62141	± 8077
LPS	22148	± 4702	16673	± 1416
PHA	6692	± 1191	9892	± 2982

^aP = 0.19

Values reported as mean CPM ± SEM.

Each treatment group n = 4 with 12 observations per treatment group for LPS, 9 observations per treatment group for Con A, and 12 and 11 observations in EI and EF groups, respectively, for PHA.

Mitogen concentrations were 1 µg per well Con A, 1 µg per well *E. coli* LPS, 2 µg per well PHA added to triplicate wells and incubated for 48 h.

Diets consisted of 50% rodent chow and 50% fescue seed.

Mice were fed treatment diets for 6 wk ad libitum.

No differences (P > 0.05) in splenocyte responses to mitogens between treatment groups were observed.

CPM in control wells without mitogens were 1648 for EI group and 1476 for the EF group.

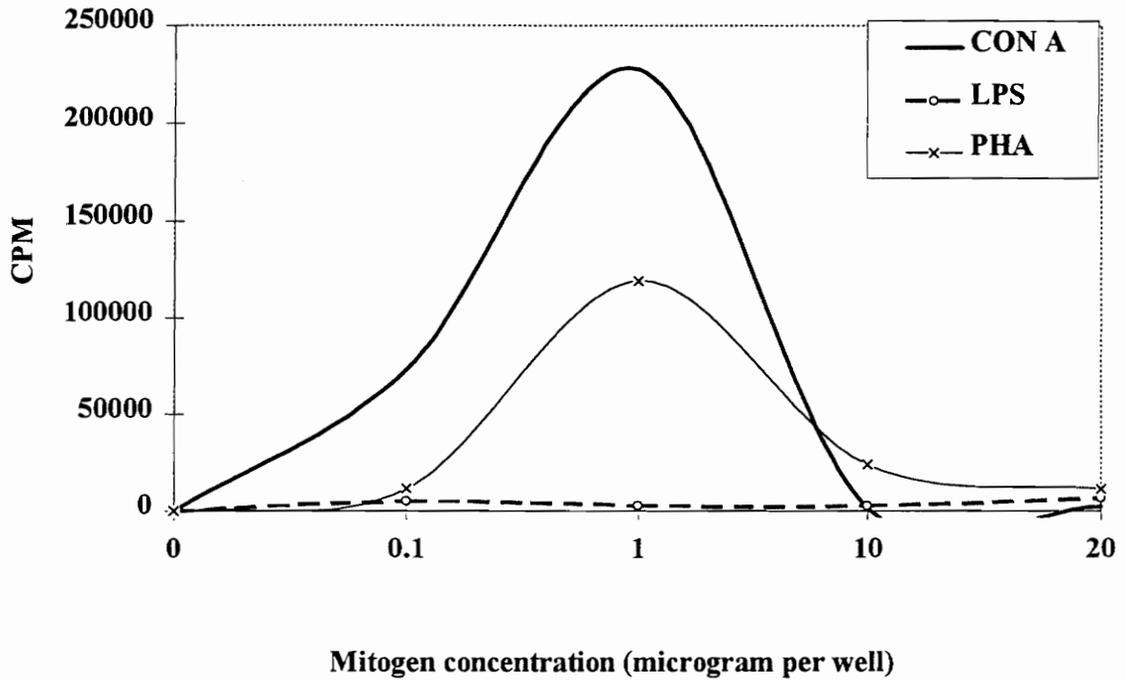


Figure 4:1. Standard curve showing optimal proliferative responses of bovine lymphocytes stimulated with Con A, LPS, and PHA mitogens. Concentrations of Con A and LPS were 0.1, 1.0, 10, and 20 micrograms per well. Concentrations of PHA were 0.2, 2.0, 10, and 20 micrograms per well. Values represent mean CPM for triplicate wells of each mitogen concentration tested. The average CPM of unstimulated control wells was 4195.

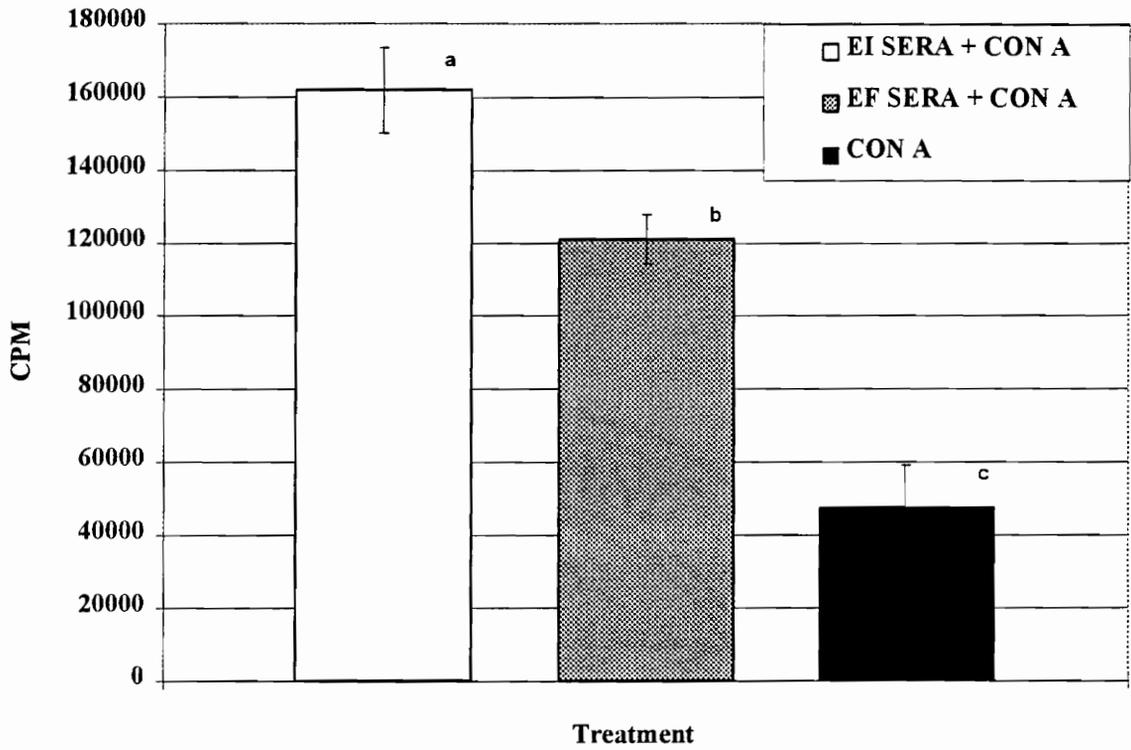


Figure 4:2. Effect of sera from steers that grazed either EI or EF tall fescue on proliferation of peripheral blood lymphocytes from a normal, donor cow to 1 μ g per well of Con A. Values represent mean \pm SEM of each treatment group (n = 6) expressed as counts per minute. Unlike superscripts indicate significant differences at P < 0.05. The average CPM of unstimulated control wells was 2089.

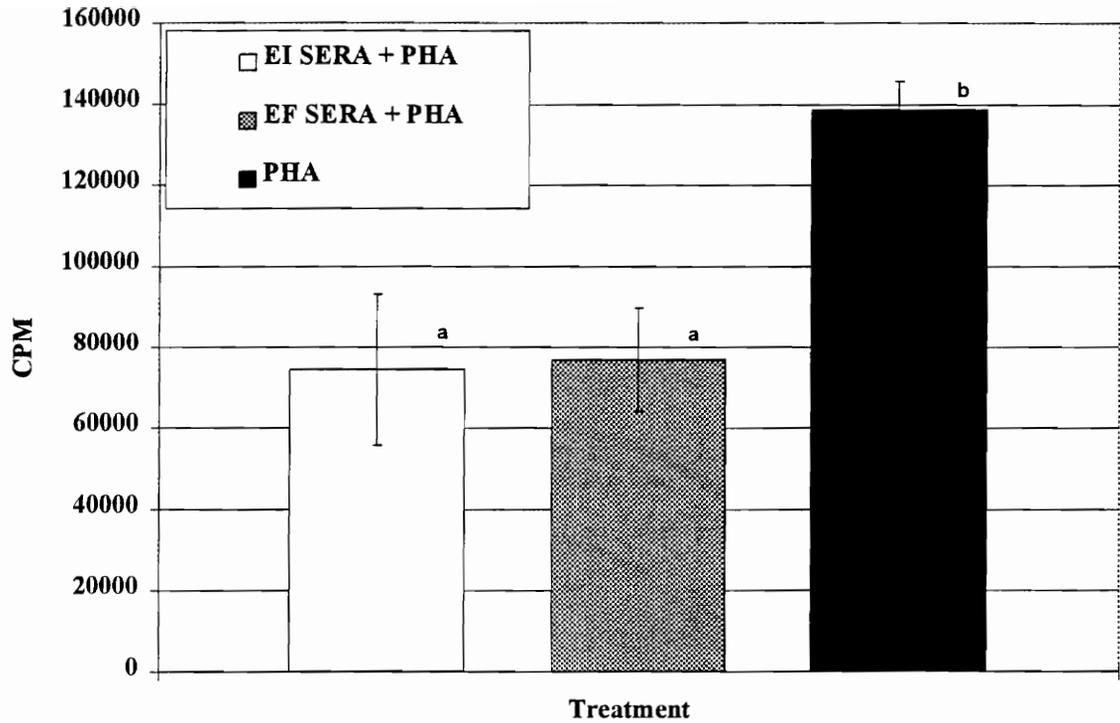


Figure 4:3. Effect of sera from steers that grazed either EI or EF tall fescue on proliferation of peripheral blood lymphocytes from a normal, donor cow to 2 μ g per well of PHA. Values represent mean \pm SEM of each treatment group (n = 6) expressed as counts per minute. Unlike superscripts indicate significant differences at $P < 0.05$. The average CPM of unstimulated control wells was 2089.

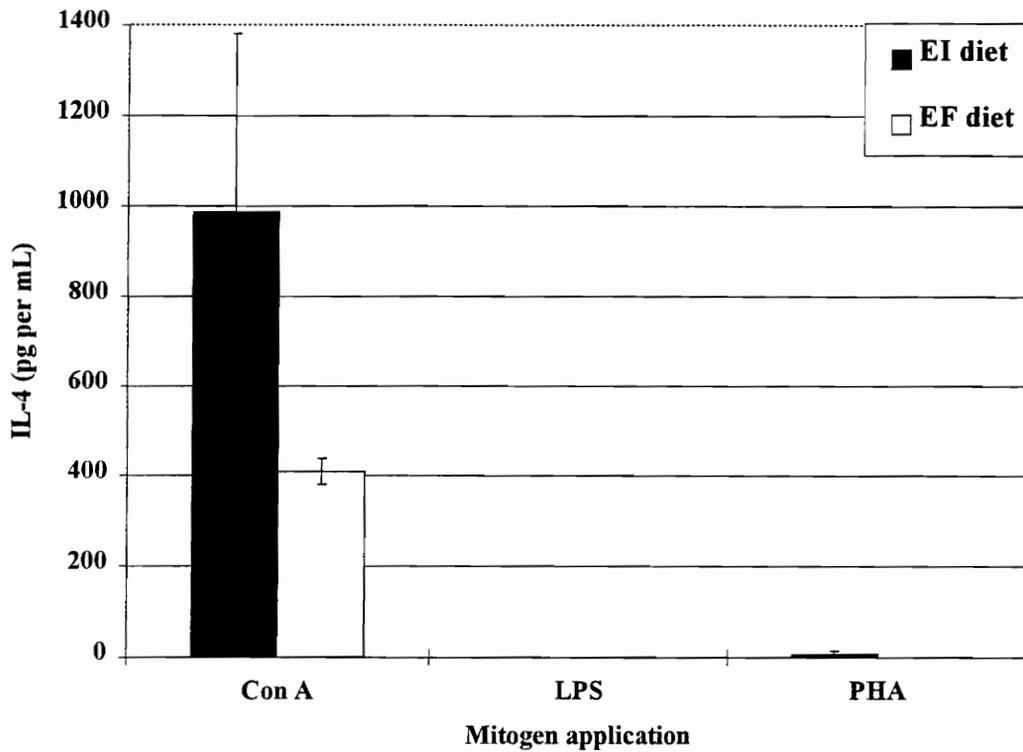


Figure 4:4. Production of IL-4 (pg per mL) *in vitro* by splenocytes from mice fed EI or EF fescue diets for 6 wk. Splenocytes were stimulated with 1 μ g per well of Con A and LPS and 2 μ g per well PHA. Values represent mean \pm SEM of IL-4 production for each dietary group (n = 4) as determined by ELISA. No significant differences at $P < 0.05$ were detected between dietary groups for each mitogen.

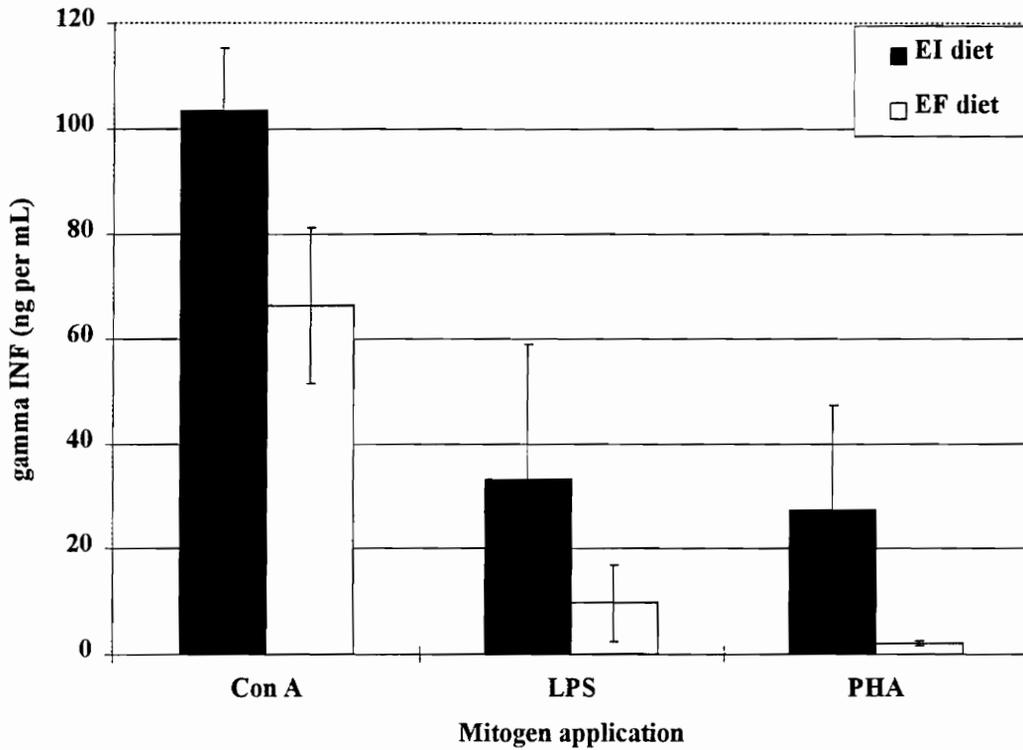


Figure 4:5. Production of INF- γ (ng per mL) *in vitro* by splenocytes from mice fed EI or EF fescue diets for 6 wk. Splenocytes were stimulated with 1 μ g per well of Con A and LPS and 2 μ g per well PHA. Values represent mean \pm SEM of INF- γ production for each group (n = 4) as determined by ELISA. No significant differences at P < 0.05 were detected between dietary groups for each mitogen.

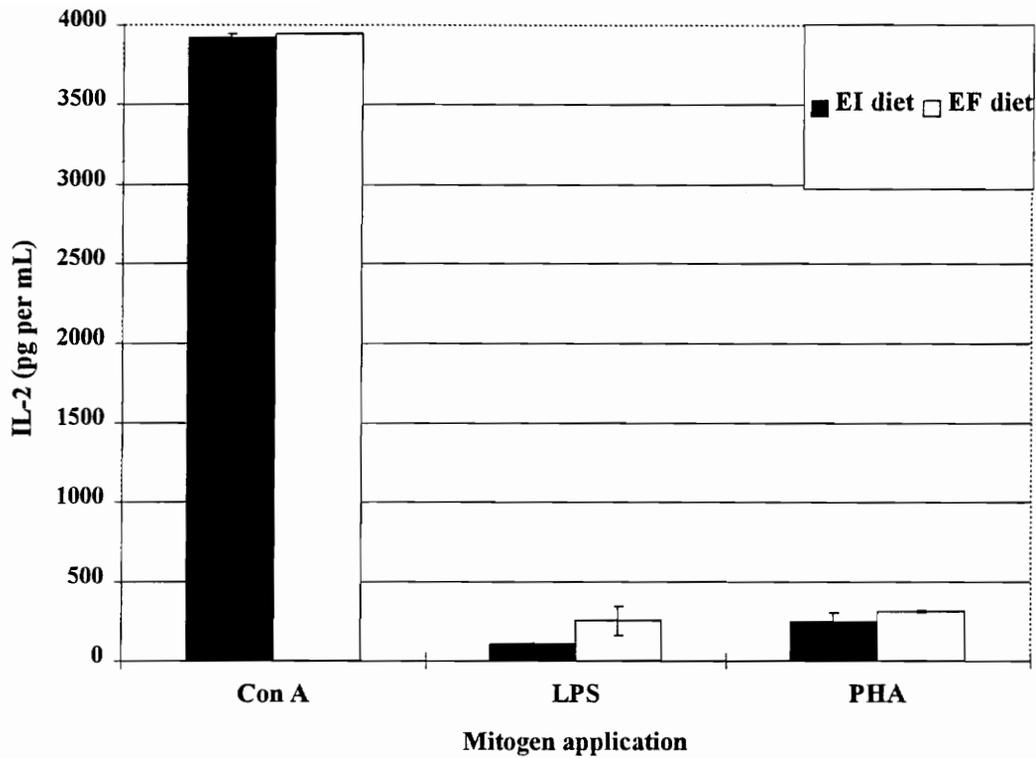


Figure 4:6. Production of IL-2 (pg per mL) *in vitro* by splenocytes from mice fed EI or EF fescue diets for 6 wk. Splenocytes were stimulated with 1 μ g per well of Con A and LPS and 2 μ g per well PHA. Values represent mean \pm SEM of IL-2 production for each group (n = 3) as determined by ELISA. No significant differences at P < 0.05 were detected between dietary groups for each mitogen.

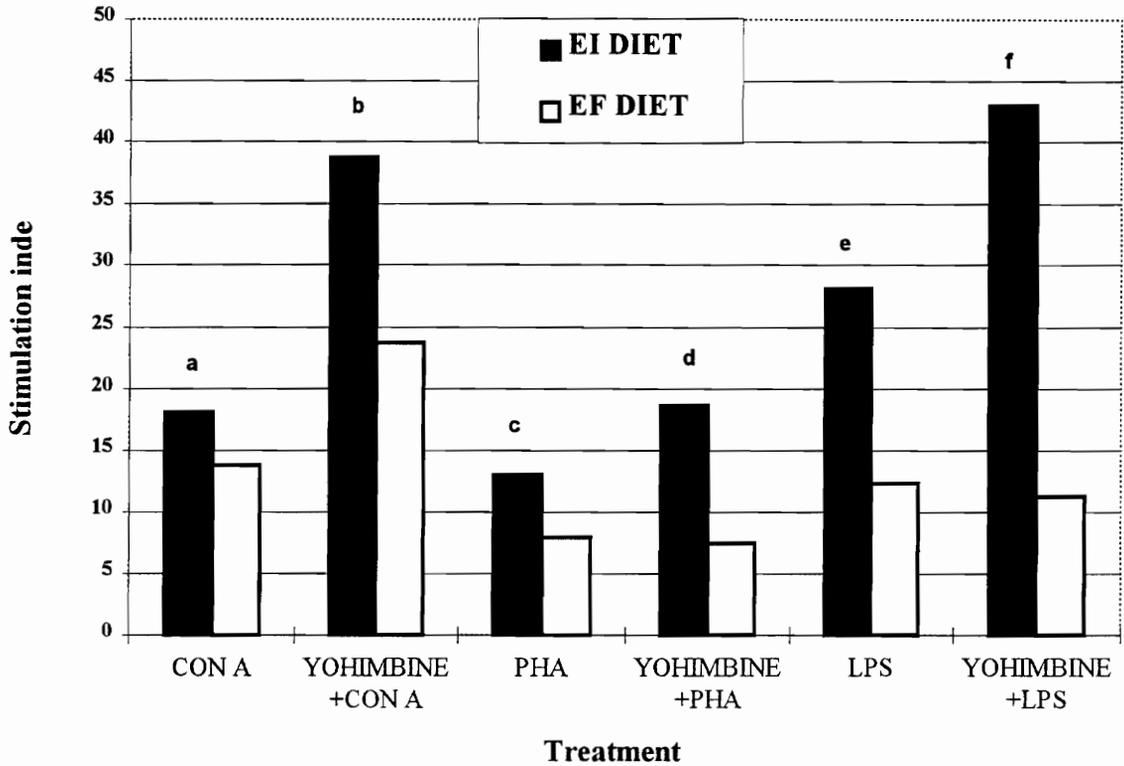


Figure 4:7. Effects of yohimbine on mitogen responses of splenic lymphocytes from mice fed EI or EF fescue diets for 6 wk. Splenocytes were stimulated with 1 μ g per well of Con A and LPS and 2 μ g per well PHA in the presence of 16 nM of yohimbine for each treatment diet. Values represent lymphocyte proliferation for each group (n = 1) expressed as stimulation index [stimulation index = CPM of treatment/CPM negative control]. Unlike superscripts within diets for each mitogen indicate significant differences at P < 0.05. The average CPM of unstimulated control wells for EI or EF groups were 1091 and 1231, respectively.

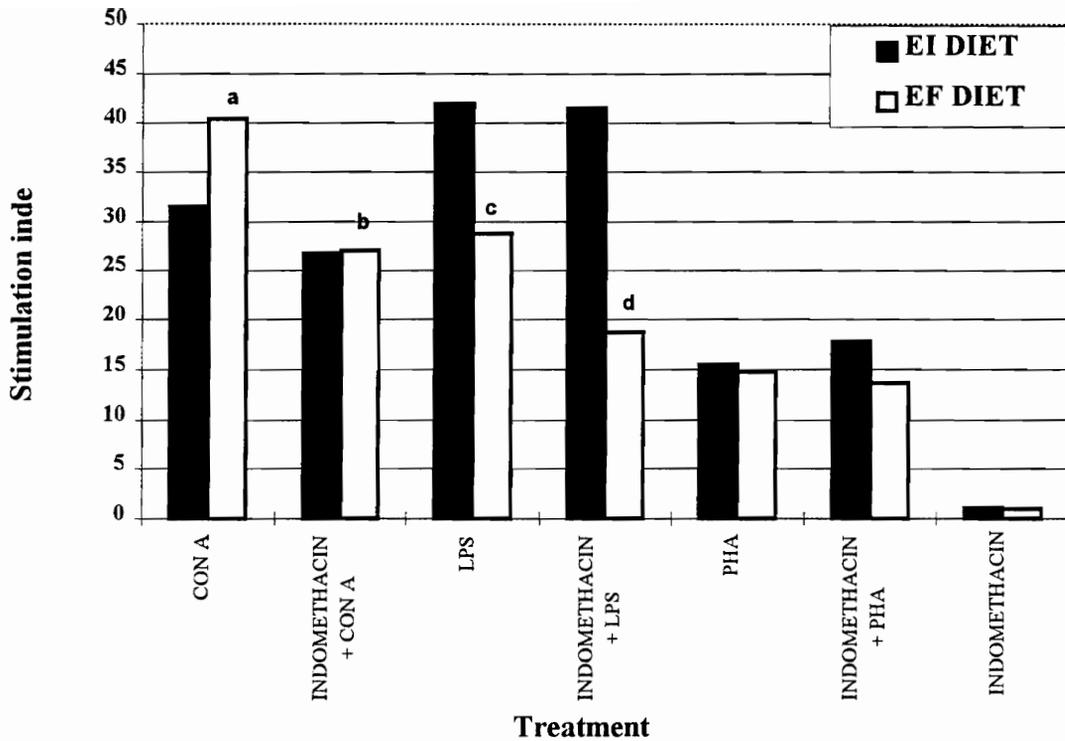


Figure 4:8. Effects of indomethacin on mitogen responses of splenic lymphocytes from mice fed EI or EF fescue diets for 6 wk. Splenocytes were stimulated with 1 μ g per well of Con A and LPS and 2 μ g per well PHA in the presence of 1×10^{-7} M indomethacin for each treatment diet. Values represent lymphocyte proliferation for each group ($n = 1$) expressed as stimulation index [stimulation index = CPM of treatment/CPM negative control]. Unlike superscripts within diets for each mitogen indicate significant differences at $P < 0.05$. The average CPM of unstimulated control wells for EI or EF groups were 1000 and 716, respectively.

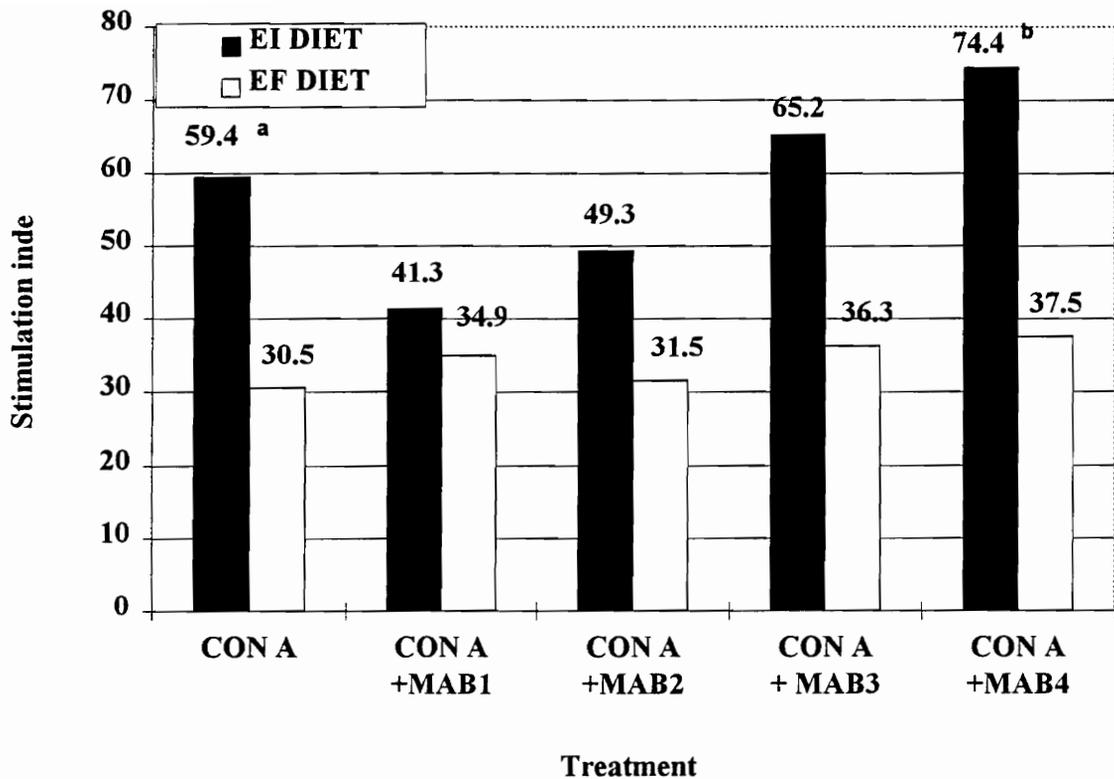


Figure 4:9. Effects of monoclonal IgG antibodies (MAB) against ergovaline on proliferation of lymphocytes to Con A from mice fed EI or EF fescue diets. Mice were fed fescue treatment diets for 6 wk. Splenocytes were stimulated with 1 μ g per well of Con A in the presence of various MAB concentrations for each treatment diet. Concentrations of MAB were MAB1 = 0.022, MAB2 = 0.22, MAB3 = 2.2, and MAB4 = 22.0 μ g per well. Values represent lymphocyte proliferation for each group (n = 1) expressed as stimulation index [stimulation index = CPM of treatment/CPM negative control]. Unlike superscripts within diets indicate significant differences at $P < 0.05$. There tended to be a linear relationship in the EI group ($P = 0.19$, $r^2 = 0.65$) between degree of splenocyte proliferation and MAB concentration, but a linear effect was not present in the EF group ($P = 0.33$, $r^2 = 0.46$). The average CPM of unstimulated control wells for EI or EF groups were 905 and 3274, respectively.

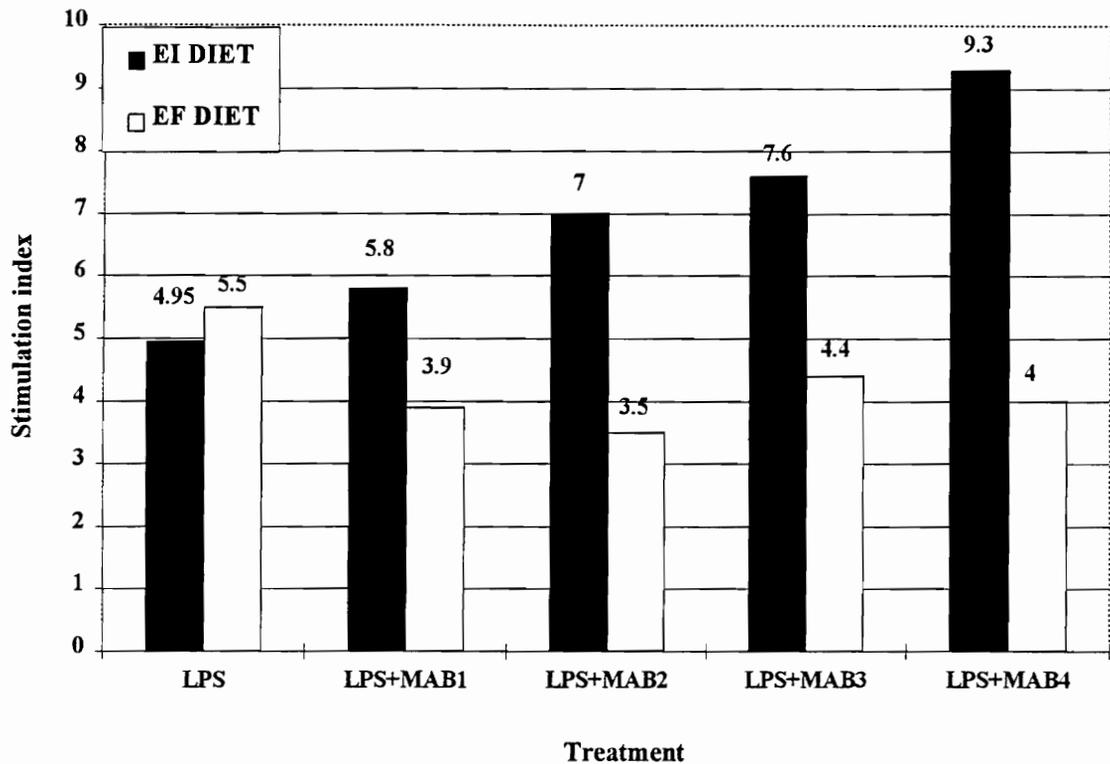


Figure 4:10. Effects of monoclonal IgG antibodies (MAB) directed against ergovaline on proliferation of lymphocytes from mice fed EI or EF fescue diets. Mice were fed fescue treatment diets for 6 wk. Splenocytes were stimulated with 1 μ g per well of LPS in the presence of various MAB concentrations for each treatment diet. Concentrations of MAB were MAB1 = 0.022, MAB2 = 0.22, MAB3 = 2.2, and MAB4 = 22.0 μ g per well. Values represent lymphocyte proliferation for each group (n = 1) expressed as stimulation index [stimulation index = CPM of treatment/CPM negative control]. No significant differences ($P < 0.05$) within diets among various treatments were present. The average CPM of unstimulated control wells for EI or EF groups were 905 and 3274, respectively. There tended to be a linear relationship in the EI group ($P = 0.11$, $r^2 = 0.80$) between degree of splenocyte proliferation and MAB concentration, but a linear effect was not present in the EF group ($P = 0.83$, $r^2 = 0.03$).

DISCUSSION

Proliferative responses of splenic lymphocytes from mice fed EI seed to mitogens and of PBL stimulated with Con A in the presence of sera from cattle that grazed EI fescue forage were not impaired. In fact, proliferative responses of donor PBL stimulated with Con A were increased when sera from cattle fed EI fescue diet were added to wells in the LTA as compared to when sera from cattle fed EF fescue diet were added. Dew (1989) reported increased proliferative responses of PBL from cattle that grazed EI tall fescue to mitogens. Similar proliferation of lymphocytes to mitogen stimulation in mice fed EI or EF diets in this study is not in agreement with previously reported work (Dew *et al.*, 1990). However, in the previous study, decreases in lymphocyte proliferation were observed in mice fed EI diet of fescue seed versus 100% rodent chow diet. Therefore, dietary deficiencies in previous studies may have impacted results.

Although lymphocyte proliferation responses to Con A were statistically similar between the treatment groups, lymphocyte proliferation was decreased 38% in the EI group and INF- γ and IL-4 concentrations tended to be increased in supernatant of cultured splenic lymphocytes stimulated with Con A. The presence of increased concentrations of both INF- γ and IL-4 simultaneously is unusual because these cytokines possess an antagonistic relationship. However, the presence of endogenous IL-4 *in vitro*

stimulates INF- γ production in IL-4 transgenic mice (Noble and Kemeny, 1995).

Perhaps fescue toxicosis induces the secretion of IL-4 from T_H0 cells that stimulate INF- γ secretion. Additionally, numbers of natural killer cells are increased in cattle grazing EI tall fescue (Dew, 1989). Natural killer cells are activated by Con A *in vitro* (Shen *et al.*, 1994) and produce large amounts of INF- γ (Hunter *et al.*, 1995). Therefore, mice fed EI fescue seed diets may have had increased numbers of natural killer cells that were stimulated by Con A *in vitro* to produce large amounts of INF- γ . Previously, hypoprolactemia in mice was shown to decrease INF- γ production (Bernton *et al.*, 1988). However, prolactin concentrations were not determined in the mice used in the LTA and cytokine studies, therefore INF- γ secretion as related to prolactin concentrations in this study is unknown.

Interleukin-4 is produced early during lymphocyte proliferation and is often called B-cell growth factor. Interleukin-4 is produced again during chronic stimulation where it induces T_H2 cells. Increased concentrations of IL-4 in the EI group may indicate that the T-cells may differentiate into T_H2 subset of CD4⁺ cells. The T_H2 cells produce IL-4, IL-5 and IL-6, mediate hapten-carrier helper activity, and augment IgG1 and IgE (Lise and Audibert, 1989).

Production of IL-2 tended to be decreased in LPS stimulated splenocytes from mice in the EI group and may be a result of decreases in prolactin that occur in fescue toxicosis. Although prolactin was not analyzed in this study, decreases of prolactin have

been pivotal in expression of IL-2 receptors (Mukherjee *et al.*, 1990). Additionally, production of PGE2 inhibits IL-2 production (Kozlov *et al.*, 1990).

Indomethacin Treatment Did Not Affect Lymphocyte Proliferation in the EI Group

Production of prostaglandins (PG) by monocytes suppresses proliferation of lymphocytes (Aune, 1987). Addition of indomethacin to suppress PG production *in vitro* usually increases lymphocyte proliferation (Inamizu *et al.*, 1985). Contrary to what was expected, lymphocyte proliferation decreased with inhibition of PG production by indomethacin in the EF group. However, inhibition of PG with indomethacin did not decrease or increase proliferation in the EI group. Perhaps the concentration of indomethacin was insufficient to inhibit PG production if activated cells were producing large amounts of PG. Additionally, indomethacin may not have affected proliferation in the EI group, if cells were not stimulated to produce PG. The enzyme cyclooxygenase is inhibited by indomethacin. Inhibition of cyclooxygenase inhibits production of thromboxanes and other prostaglandins, besides PGE2, which could affect lymphocyte proliferation.

Lymphocyte Proliferation is Increased by Alpha Adrenergic Antagonist in EI Treated Mice

Agents that increase intracellular cAMP levels, such as cholera toxin, decrease lymphocyte proliferation and agents that inhibit cAMP increase proliferation (Tizard, 1987). Therefore, EV binding to alpha adrenergic receptors on lymphocytes should stimulate lymphocyte proliferation by cAMP inhibition. Furthermore, blocking alpha adrenergic receptors by competitive inhibition with an alpha adrenergic antagonist, such as yohimbine, should lower the cAMP-induced proliferation.

In this study, addition of yohimbine did not affect lymphocyte proliferation in the EF group. However, contrary to what was expected, yohimbine increased proliferative responses in the EI group. Yohimbine is selective for alpha 2 receptors and binds receptors rapidly. Apparent saturation of alpha receptor sites occur at yohimbine concentrations of 14 to 16 nM (Titinchi and Clark, 1984). Perhaps yohimbine displaced EV from alpha adrenergic receptors to increase EV concentrations at some other receptor that stimulates lymphocyte proliferation. Although not considered in this study, effects of EG or EV on dopaminergic and serotonergic receptor sites may influence immune responses.

The interaction of ergot alkaloids with the guanine nucleotide regulatory protein (G protein) is another mechanism by which EG or EV may influence immune responses. The guanine nucleotide regulatory protein links receptors on cell surfaces to

phospholipase C for regulation of cell processes. The sIg receptors of B-cells are coupled to phospholipase C (Snow, 1991) by way of pertussis toxin-sensitive G protein. Binding of ligands to G protein can activate either the stimulatory or inhibitory subunit of the G protein. Therefore, activation of G receptor can have inhibitory or stimulatory effects on cell processes. Ergovaline may bind receptors and stimulate cells through G proteins located on cell surfaces. The A and B rings of the lysergic base structure of EV are structurally similar to guanidine. Metabolic biotransformation of EV may yield intact A and B ring structures that may interact with the G protein because of its similarity to guanidine. Immunomodulating drugs that are analogues of bromolevamisole and guanidine derivatives (levamisole, imidazole, theophylline and aminoguanidine) also inhibit ALP (Metaye *et al.*, 1988) via the inhibition of adenylate cyclase (Metaye *et al.*, 1992) and enhance mitogen-induced lymphocyte proliferation (Metaye *et al.*, 1989). Levamisole also increases INF- γ production in individuals with impaired T-cell responses (Tizard, 1987). Although hypoprolactemia may interfere with T-cell function in fescue toxicosis, perhaps EV exerts similar effects as levamisole and thereby stimulates immune function.

Anti-EV Antibodies Induce a Biphasic Response on Lymphocyte Proliferation

Low amounts of purified, MAB against EV tended to decrease lymphocyte proliferation in the EI group. Neutralization of EV by anti-EV antibodies may prevent interaction with alpha adrenergic receptors. The antibody neutralization would prevent inhibition of cAMP and stimulation of lymphocyte proliferation. Therefore, lymphocyte proliferation would be decreased in the EI group by MAB.

Addition of high amounts of purified MAB against EV increased proliferation in EI group. These results demonstrate a biphasic response of anti-EV antibodies on proliferation of lymphocytes from mice fed EI diet and not from mice fed EF fescue diet. The cause of this biphasic response of lymphocyte proliferation to various concentrations of MAB is unknown. Replication of these results is needed to further define the effects of specific antibodies on lymphocyte proliferation.

CONCLUSIONS

Fescue toxicosis did not negatively influence *in vitro* immune responses. Proliferation of splenic lymphocytes from mice fed either EI or EF fescue diets was similar. However, T-cell proliferation to Con A tended to be decreased in mice fed EI

fescue diet. On the other hand, sera from steers that grazed EI fescue stimulated T-cell proliferation of PBL from a normal, donor cow to Con A greater than sera from cattle that grazed on EF fescue pastures. These results agree with previous research in steers (Dew, 1989). Apparently, species (murine versus bovine) or dietary (seed versus grass) differences may explain these results.

Production of IL-2, IL-4, and IFN- γ by splenocytes from mice fed EI or EF fescue diets were similar. However, IL-4 and INF- γ tended to be increased in splenocytes stimulated with Con A from mice fed EI fescue diets. The production of IL-4 in the EI group may favor the dominance of TH₂ subset, which may explain the increased humoral immune responses observed in vaccinated steers.

Low concentrations of purified, anti-EV antibodies decreased proliferation of lymphocytes from mice fed EI fescue diet to Con A. This suggested that presence of antibodies may impair T-cell proliferation.

Inhibition of PG production by indomethacin did not affect proliferation of lymphocytes from mice fed the EI fescue diet. Yohimbine increased proliferation of lymphocytes from mice fed the EI fescue diet. Proliferative effects observed with indomethacin and yohimbine may not be a treatment effect, but rather a mouse effect because of the small number of samples in the study. Therefore, studies replicating these results are needed.

Chapter 5: Preparation of Protein-Ergotamine Conjugates and Vaccination of Mice and Steers

INTRODUCTION

Ergotamine (EG), an ergopeptide alkaloid, was conjugated to protein carriers by the Mannich reaction. The protein-EG conjugates were used in this research to investigate the development of an oral or parenteral vaccine to protect against fescue toxicosis. Protein carriers assessed were subunit B of cholera toxin (CTB), whole cholera toxin (CT), Con A, and bovine serum albumin (BSA). The magnitude and duration of humoral and mucosal immune responses induced by parenteral and oral administration of these protein-EG conjugates were evaluated initially in mice. Parenteral administration of CTB-EG and Con A-EG conjugates were evaluated in cattle. Duration and magnitude of specific immune responses against protein-EG conjugates given orally or parenterally were important to determine the frequency of vaccine administration and the best route of vaccine administration.

Vaccine Considerations

Ergotamine was the model antigen used in the development of a vaccine against fescue toxicosis in this study because of its availability and structural similarities to ergovaline (EV). Ergotamine is inexpensive and available commercially. Ergovaline is not commercially available and its synthesis is complicated with many chemical steps (Smith, 1993). Ergotamine and EV are both ergopeptine alkaloids, which are structurally similar. Structurally, EG differs from EV only at the terminal amino acid residue of the cyclol tripeptide at the C 8 position of the ergoline ring. The terminal amino acid of the cyclol tripeptide in EG is phenylalanine, whereas in EV the terminal amino acid is valine (Shelby and Kelley, 1991).

Both EV and EG appear to have a similar mechanism of action in the hypothalamus. Levodopa and homovanillic acid are decreased in the median eminence of steers fed diets that contain EI fescue seed or EF fescue seed treated with EG tartrate for 28 days (Marple *et al.*, 1988). Ergotamine and EV also have similar physiological effects, since both alkaloids decrease serum prolactin and cause vasoconstriction of blood vessels (McCollough *et al.*, 1994). At the molecular level, both EG and EV inhibit cyclic adenosine monophosphate (cAMP), which may be the basis of their mechanism of action (Kerley *et al.*, 1994).

Ergotamine must be conjugated to a protein carrier for immunogenicity because of its low molecular weight of 531 daltons. An indole nitrogen in the structures of EG

and EV enables conjugation to protein carriers containing lysine residues via the Mannich reaction. Conjugation of ergot peptide alkaloids to carrier proteins via the indole nitrogen directs antibody specificity to the tricyclic peptide moiety of the alkaloid (Berde and Schild, 1978a).

The Mannich reaction is often used to conjugate small neurotransmitter ligands and indolealkylamines with carrier proteins utilizing formaldehyde as a chemical linker as described by Ranadive *et al.*, (1967). The Mannich reaction was previously used to prepare protein-conjugates of serotonin-BSA or serotonin-human gamma globulin (Flurkey *et al.*, 1985), lysergic acid diethylamide-BSA (Castro *et al.*, 1973) lysergic acid diethylamide-human serum albumin (Taunton-Rigby *et al.*, 1973), and N-maleyl-dopamine-BSA (Miwa *et al.*, 1977). These conjugates prepared by the Mannich reaction were used to induce polyclonal and monoclonal antibodies.

Specific immune responses against EV, EG, and lysergol were induced by parenteral administration of the respective alkaloids conjugated to protein carriers. Polyclonal antibodies against EG are elicited in rabbits with BSA-EG conjugates made via the Mannich reaction for use in a competitive inhibition ELISA for the detection of EG in grain samples (Shelby and Kelley, 1990). The BSA-EV conjugates induce anti-EV antibodies of the IgG isotype in mice (Kelley and Shelby, 1990). A murine hybridoma that produces monoclonal antibodies specifically against the peptide moiety of EV has been developed using BSA as the protein carrier (Kelley and Shelby, 1990). The monoclonal anti-EV antibodies produced by this hybridoma cross react strongly with

EG. A murine monoclonal antibody specific to the lysergic moiety of ergot alkaloids has also been produced (Hill *et al.*, 1994). Cattle actively immunized with a lysergol-protein conjugate develop short-lived antibody titers against the lysergic moiety of ergot alkaloids (Hill *et al.*, 1994).

Several protein carriers for EG were studied by this research in the development of a conjugate vaccine against fescue toxicosis. Immunogenicity of a hapten can be modified by the carrier protein used. Cholera toxin and CTB were protein carriers conjugated to EG for use in oral and parenteral vaccines against fescue toxicosis. Cholera toxin contains a similar number of lysine groups on a molar basis as BSA, which supports its use as a carrier for EG (Azcona-Olivera *et al.*, 1992). Cholera toxin is a protein produced by *Vibrio cholera*, which consists of two subunits. The two subunits are a 28,000 KDa A subunit and a 57,500 KDa B subunit. The non-toxic, pentameric B subunit binds the GM1 ganglioside receptor, which is located on virtually all membrane surfaces of nucleated cells. The A subunit is responsible for the toxicity of cholera toxin. The A subunit is inserted into cell membranes of intestinal epithelial to activate adenylate cyclase enzyme, which results in the copious fluid secretion seen in clinical cholera (Elson, 1989). Extraction of the nontoxic subunit B from the holotoxin removes the toxic effects of subunit A to produce a protein that retains adjuvant/carrier properties.

Both CTB and CT function as carrier proteins, because of their large molecular size, and as adjuvants. The adjuvanticity of CTB and CT is only observed when co-administered with an antigen via the same route. The CTB binds to lymphoid cells and

activates immunocompetent cells in the mucosal-associated lymphoid tissues (Tamura *et al.*, 1989b) to augment immune responses. The main advantages of CTB as an adjuvant are that low concentrations of CTB and hapten are needed for immunization and the immunity produced is long lasting (Russell and Wu, 1991). The CTB induces long-lived specific IgA antibodies and specific IgG antibodies when co-administered orally or intranasally with unrelated antigens (Czerkinsky *et al.*, 1989). Oral immunization with CT in microgram amounts induces both specific sIgA and specific plasma IgG responses originating in GALT (Elson and Ealding, 1984a). A prolonged memory response to CTB has been observed at mucosal surfaces (Russell and Wu, 1991). Cholera toxin given orally does not produce oral tolerance, which is a desirable property in an oral immunogen (Elson and Ealding, 1984b). The oral adjuvant effect of CTB has been demonstrated in mice (McKenzie and Halsey, 1984; Chen and Quinnan, 1989) and it is thought that a similar oral adjuvant effect would occur in other animals.

Long-lived, specific IgG antibodies are also induced when CTB is co-administered parenterally with antigens (Hirabayashi *et al.*, 1990). A prolonged anti-EG titer is important, since dietary EV present in EI fescue forage will not induce an anamnestic immune response because of the low molecular weight of EV. Cholera toxin B is a component of a recently developed and licensed oral cholera vaccine and is also a part of a vaccine in clinical testing against enterotoxigenic *E.coli* diarrhea (Lebens, *et al.* 1993).

Concanavalin A was also examined as a potential carrier for the hapten, EG. The use of carrier proteins that are mitogens *in vitro* may significantly increase the immunogenicity of conjugate vaccines and may also potentially avoid epitopic suppression. Concanavalin A, a glycoprotein extract from the jack bean, binds to mannose residues on the surface of T lymphocytes and induces the cells to proliferate (Liener *et al.*, 1986). Concanavalin A has not previously been used as a carrier protein for small haptens in vaccination. It was expected that conjugation of Con A to EG via the Mannich reaction would result in anti-EG antibody production because of its large molecular size and mitogenic effects on T-cells.

Poly-L-lysine (PLL) and lysozyme also were examined as possible protein carriers for EG. Conjugation of protein carriers to EG by the Mannich reaction depends on the presence of lysine residues in proteins. Therefore, PLL with its many lysine residues was chosen as a potential carrier for EG for use as a coating antigen in ELISA. Lysozyme also was chosen as a potential carrier for EG, because it would be antigenic based on its degree of complexity, molecular weight, and foreignness.

Route of vaccine administration may also be an important determining factor of protection against fescue toxicosis. Parenteral vaccination induces systemic IgM and IgG isotypes, but rarely serum or sIgA isotypes. Oral vaccination can produce high concentrations of antigen-specific sIgA at mucosal sites. Secretory IgA is the major immunoglobulin class found in exocrine secretions, since it functions in the host defense of mucosal surfaces. Daily output of sIgA exceeds all other immunoglobulins combined

(Holmgren *et al.*, 1992). Lymphoid tissues associated with mucosal membranes contain more immunocytes, including B and T lymphocytes and plasma cells, than any other tissue in the body. Moreover, intestines are the richest lymphoid tissue present in the body (Mestecky, 1987). The IgA responses originating at one mucosal site disseminate to other mucosal associated lymphoid tissues by migration of sensitized B and T-cells, hence the basis for oral and intranasal immunization strategies. Neutralization of EV at the site of absorption (i.e. mucosal surfaces) with specific antibodies induced by oral vaccination may be beneficial in the protection against fescue toxicosis.

MATERIALS AND METHODS

Preparation of Protein-Ergotamine Conjugates by the Mannich Reaction

Ergotamine (tartrate salt: Sigma Chemical Co) was conjugated to BSA, Con A, CT, lysozyme, and CTB for vaccination. Ergotamine also was conjugated to poly-L-lysine (PLL) and ovalbumin for use as solid phase antigens in the indirect ELISA. Proteins were linked to EG using the Mannich reaction by the method previously described (Kelley and Shelby, 1990). Conjugates were evaluated on 250 μm thin-layer chromatographic (TLC) plates of silica gel developed in chloroform/methanol (9:1, v/v) solvent system and observed under a long wave lamp. Protein content of each protein-

EG conjugate was determined by bicinchoninic acid assay (BCA). The relative alkaloid content of each conjugate was determined by titration in the ELISA.

Preparation of CT-EG and CTB-EG Conjugates

Two mg of CTB or CT was dissolved in 100 μ L distilled water and 200 μ L of 3 M sodium acetate. While the protein solution was stirred, 400 μ L of 37% formaldehyde was added dropwise. The solution was stirred for 5 minutes. One mg of EG dissolved in 250 μ L of methanol:water (1:1, v/v) was added to the CTB solution and the mixture was stirred for 5.5 h at room temperature in the dark. The reaction mixture was diluted to a final volume of 2 mL with distilled water and dialyzed against distilled water for 4 days with water changes every 12 h.

Preparation of BSA-EG, Lysozyme-EG, and Ovalbumin-EG Conjugates

Twenty mg of protein was dissolved in 100 μ L of distilled water and 200 μ L of 3 M sodium acetate. While the protein solution was stirred, 400 μ L of 37% formaldehyde was added dropwise. The solution was stirred for 5 minutes. Two mg of EG dissolved in 300 μ L of methanol and 100 μ L of distilled water was added to each of the respective protein solutions and the mixtures were stirred for 5.5 hours at room temperature in the dark. The reaction mixtures were diluted to a final volume of 6 mL with distilled water and dialyzed against distilled water for 4 days with frequent water changes every 12 h.

Preparation of Con A-EG and Poly-L-Lysine-EG Conjugates

Starting volumes of reactants were varied to determine optimal reactant conditions that produced a protein-EG conjugate with high protein and alkaloid content. Twenty mg of Con A or PLL was dissolved in either 1 fold, 2 fold, 3 fold, or 4 fold the volumes of distilled water (100 μL) and 3 M sodium acetate (200 μL). While the protein solution was stirred, either 1 fold, 2 fold, 3 fold, or 4 fold the volume of 37% formaldehyde (400 μL) was added dropwise. The solution was stirred for 5 minutes. Two mg of EG dissolved in 300 μL of methanol and 100 μL of distilled water was added to each of the respective protein solutions and the mixtures were stirred for 5.5 hours at room temperature in the dark. The reaction mixtures were diluted to a final volume of 6 mL with distilled water and dialyzed against distilled water for 4 days with water changes every 12 h.

Evaluation of Protein-Ergotamine Conjugates

Thin Layer Chromatography Analysis

Five μL of each protein-EG conjugate and EG standard (1 $\mu\text{g}/\mu\text{L}$) was spotted on 5 X 20 cm, 250 μm TLC plates and the spotted samples dried. The TLC plate was developed in chloroform/methanol (9:1 v/v). Each protein-EG conjugate appeared as a fluorescent spot at the origin, whereas free EG migrated when observed under a long

wave lamp. Unconjugated protein controls remained at the origin and did not fluoresce when observed under a long wave lamp.

BCA Protein Analysis

Protein concentration of each protein-EG conjugate was determined by BCA analysis. A set of protein standards was prepared with BSA (2 mg/mL) ranging from 7.8 µg to 2000 µg per mL. Two mL of working reagent (BCA™ Protein Assay, Pierce) was added to 100 µL of protein-EG conjugate, protein standards, and a water reference in borosilicate tubes. Tubes were incubated at 37 °C for 30 min, then cooled to room temperature. Absorbance of standards, protein-EG conjugate, and water reference was measured on a double beam spectrophotometer (Gilford) at 562 nm. A standard curve was constructed by plotting absorbance versus protein concentration. The equation of the line for the standard curve was determined by linear regression. Protein content of the conjugate was determined from the derived equation.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Protein-EG conjugates were analyzed by 10% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis according to the method of Lammeli (1970). Protein-EG conjugates were separated on both mini-gel and standard sized gels. Protocols for both gel sizes were similar. Briefly, protein-EG conjugates and unconjugated protein controls were boiled for 5 min in equal volumes of sample buffer (0.0625 M Tris, pH

6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromo-phenol blue).

Samples then were loaded into appropriate lanes of the stacking gel (0.125 M Tris-HCl, pH 6.8). Resolving gel (0.375 M Tris-HCl, pH 8.8) contained a final concentration of 12.5% or 15% acrylamide-bisacrylamide. A mixture of molecular weight markers (BioRad Laboratories, CA) ranging from 8,600 to 207,000 daltons was also loaded into lanes of the stacking gel to estimate molecular weights of separated proteins in the protein-EG conjugates. Gels were electrophoresed at constant amperage (30 mA/gel) for approximately 1 h in reservoir buffer (0.025 M Tromethamine, 0.19 M glycine, pH 8.3). Gels were stained overnight in 0.2% Coomassie blue stain. Gels were destained to the desired level with 10% glacial acetic acid, 40% methanol, and 50% distilled water.

Determination of Relative EG Content of Protein-EG Conjugates by Titration in

ELISA

Wells of high binding immunoplates (Nunc Maxisorp®) were coated overnight at 4 °C with 50 µL of each protein-EG conjugate (5 µg per well) diluted in 0.1 M sodium carbonate-bicarbonate buffer (pH 9.6). Plates were washed five times with phosphate-buffered saline (PBS) containing 0.02% Tween-80 (PBST). Wells were blocked for 60 min at 37 °C with 100 µL of 1.0% BSA (w/v) in PBST and washed five times with PBST. Next, serially diluted aliquots (50 µL) of murine monoclonal antibody specific for EV were added to each well and plates were incubated for 30 min at 37 °C. Unbound antibody was removed by washing five times with PBST. Fifty µL of goat anti-mouse

IgG (diluted 1/600 in 1.0% BSA in PBST) conjugated to HRPO was added to each well. Plates were incubated for 30 min at 37 °C and washed five times with PBST. Bound peroxidase was determined by adding 100 µL per well of o-phenylenediamine (Sigma) and H₂O₂ substrate. Plates were incubated at room temperature for 30 min and the reaction was stopped with 25 µL per well of 8 N sulfuric acid. Absorbance at 490 nm was read by using a multiscan reader (Titertek[®], Molecular Devices Corp.). The titer of each protein-EG conjugate was the maximum dilution where the absorbance was greater than the mean plus 3-fold the standard deviation of wells containing no monoclonal antibody.

Biological Activity of CT-EG and CTB-EG Conjugates after the Mannich Reaction

Wells of high binding immunoplates (Nunc Maxisorp[®]) were coated overnight at 4 °C with 50 µL of GM1 ganglioside (2.5 µg per well) in 0.1 M sodium carbonate-bicarbonate buffer (pH 9.6). Plates were washed five times with PBST. Wells were blocked for 60 min at 37 °C with 100 µL of 1.0% BSA (w/v) in PBST and then washed five times with PBST. Next, either 5 µg of CT, 6 µg of CTB and of CTB-EG conjugate, or 10 µg of CT-EG conjugate were added per well and incubated for 1 h. Then 50 µL of control serum or 50 µL of anti-CTB IgG serum diluted 1:50 was added to each well and incubated for 30 min at 37 °C. Plates were washed five times with PBST and then 50 µL of goat anti-mouse IgG (diluted 1/600 in 1.0% BSA in PBST) conjugated to HRPO was added to each well. Plates were incubated for 30 min at 37 °C and washed five times

with PBST. Bound peroxidase was determined by adding 100 μL per well of o-phenylenediamine (Sigma) and H_2O_2 substrate. Plates were incubated at room temperature for 30 min and the reaction was stopped with 25 μL per well of 8 N sulfuric acid. The absorbance at 490 nm was read by using a multiscan reader (Titertek[®], Molecular Devices Corp.).

Biological Activity of Con A-EG and Con A Conjugates after the Mannich Reaction

The ability of Con A to stimulate lymphocyte proliferation *in vitro* in the lymphocyte transformation assay after conjugation to EG was assessed. Concanavalin A was reacted without EG and with EG in the Mannich reaction (Appendix I). Conjugates were evaluated by TLC and BCA analysis. Then 1 μg and 10 μg per well of Con A, Con A-EG conjugate, and Con A reacted in the Mannich reaction without EG were added to triplicate wells containing 4×10^5 spleen cells in 200 μL of complete RPMI media. Plates were incubated for 48 h in a tissue culture incubator at 37 °C and an atmosphere of 5% CO_2 and 95% air. Cell proliferation in response to each mitogen was determined by the addition of 1.0 μCi of [methyl-3H] thymidine to each well. After an 18 h pulse period, cells were harvested onto glass fiber strips using a cell harvester. Radioactivity was counted in a liquid scintillation counter and reported as CPM.

Safety of CT-EG Conjugate Oral Vaccine

Twenty female Balb/c mice (23 g average BW) were blocked by weight and randomly assigned to two treatment groups of 10 mice each. One group received CT-EG conjugate and the other received CT alone by oral gavage. Cholera toxin was conjugated to EG by the Mannich reaction as described previously and evaluated for protein content by BCA analysis. Thin layer chromatography was used to confirm conjugation of CT to EG, and biological activity of CT-EG conjugate was evaluated by ELISA. Cholera toxin without EG was dissolved into PBS.

CT-EG conjugate (0.5 $\mu\text{g}/\mu\text{L}$) or CT in PBS (0.5 $\mu\text{g}/\mu\text{L}$) was administered by oral gavage at doses of 10, 20, 30, or 40 μg . Mice were observed daily for 1 week to record morbidity and mortality in each treatment group.

Determination of Magnitude and Duration of Anti-Ergotamine IgG Titers Induced by Different Protein-Ergotamine Conjugates in Mice and Steers

Parenteral Vaccination of Mice with Protein-Ergotamine Conjugates

Balb/c female mice, six weeks of age, were vaccinated parenterally with different protein-EG conjugates to determine the magnitude and duration of primary and secondary humoral immune responses against EG. Groups, each consisting of six mice, were vaccinated intraperitoneally (IP) with one of the following protein-EG conjugates:

250 µg BSA-EG conjugate + Freund's complete adjuvant (FCA); 250 µg Con A-EG conjugate + FCA; 50 µg CTB-EG conjugate + FCA; and 50 µg CTB-EG conjugate without adjuvant.

Mice were revaccinated IP 15 days later with the following amount of protein-EG conjugates: 100 µg BSA-EG conjugate + Freund's incomplete adjuvant (FIA); 100 µg Con A-EG conjugate + FIA; 50 µg CTB-EG conjugate + FIA; and 50 µg CTB-EG without adjuvant.

To determine the effects of revaccination with protein-EG conjugates on the magnitude and duration of anamnestic humoral immune responses against EG, mice were given a third vaccination IP on d 105. The third vaccination was with the following amounts of protein-EG conjugates emulsified in FIA: 100 µg BSA-EG conjugate; 100 µg Con A-EG conjugate; and 100 µg CTB-EG conjugate.

The group of six mice previously vaccinated twice IP with CTB-EG conjugate without adjuvant were revaccinated IP with 50 µg of CT-EG conjugate without adjuvant on d 105. The ability of CT-EG conjugate to induce anti-EG titers after vaccination with CTB-EG conjugate, thus was determined. Mice anesthetized with halothane were bled every 2 to 4 wk via the retro-orbital sinus. Serum was collected and frozen at -20 °C until titer determinations by ELISA.

Parenteral Vaccination of Cattle with Con A-EG and CTB-EG Conjugates

Three Angus crossbred steers were vaccinated IM with 1 mg of Con A-EG conjugate (Appendix I) emulsified in FIA. Specific titers were measured to determine the ability of Con A-EG conjugate to induce anti-EG antibodies. Cattle were bled by jugular venipuncture prior to vaccination and sera were pooled for use as a prevaccination control in ELISA. Two weeks later, steers were bled by jugular venipuncture to collect sera for determination of primary humoral immune responses against EG. Also, cattle were revaccinated with 0.5 mg Con A-EG conjugate emulsified in FIA. Two weeks later, steers were bled by jugular venipuncture again for determination of secondary humoral immune responses (anti-EG titer) by ELISA.

Two Angus heifers were vaccinated IM with 250 µg CTB-EG without adjuvant to determine if administration of an adjuvant with CTB-EG conjugate was required to induce anti-EG titers in cattle when vaccinated with CTB-EG conjugate. Cattle were bled by jugular venipuncture prior to vaccination and sera were pooled for use as a prevaccination control in ELISA. Two weeks later, heifers were bled and revaccinated with 250 µg CTB-EG without adjuvant. Heifers were bled and then revaccinated 45 d later with 0.5 mg CTB-EG conjugate in FIA. Heifers were bled 11 d later for anti-EG titer determination by ELISA.

Determination of Anti-Ergotamine IgG Antibodies in Mouse Serum by Indirect ELISA
(Appendix VII)

Wells of high binding immunoplates (Nunc Maxisorp®) were coated overnight at 4 °C with 50 µL of ovalbumin-EG (5 µg per well) diluted in 0.1 M sodium carbonate-bicarbonate buffer (pH 9.6). Plates were washed five times with PBST. Wells were blocked for 60 min at 37 °C with 100 µL of 0.3% (w/v) gelatin in PBST and washed five times with PBST. Next, serially diluted aliquots (50 µL) of sera were added to each well and plates were incubated for 30 min at 37 °C. Unbound antibody was removed by washing five times with PBST. Fifty µL of goat anti-mouse IgG (diluted 1/600 in 0.3% (w/v) gelatin in PBST) conjugated to HRPO was added to each well. Plates were incubated for 30 min at 37 °C and washed five times with PBST. Bound peroxidase was determined by adding 100 µL per well of o-phenylenediamine (Sigma) and H₂O₂ substrate. Plates were incubated at room temperature for 30 min and the reaction was stopped with 25 µL per well of 8 N sulfuric acid. Absorbance at 490 nm was read by using a multiscan reader (Titertek®, Molecular Devices Corp.). The titer of each serum was the maximum dilution where the absorbance was greater than the mean plus 3-fold the standard deviation of triplicates of the same dilution of control sera.

Determination of Anti-Ergotamine IgG Antibodies in Cattle Serum by Indirect ELISA

Wells of medium binding immunoplates (Costar®) were coated overnight at 4 °C with 50 µL BSA-EG conjugate (5 µg per well) diluted in 0.1 M sodium carbonate-

bicarbonate buffer (pH 9.6). Plates were washed five times with PBST. Wells were blocked for 60 min at 37 °C with 100 µL of 0.3% (w/v) fish gelatin in PBST and washed five times with PBST. Next, serially diluted aliquots (50 µL) of sera were added to each well and plates were incubated for 30 min at 37 °C. Unbound antibody was removed by washing five times with PBST. Fifty µL of rabbit anti-bovine IgG (diluted 1/600 in 0.3% fish gelatin in PBST) conjugated to HRPO was added to each well. Plates were incubated for 30 min at 37 °C and washed five times with PBST. Bound peroxidase was determined by adding 100 µL per well of o-phenylenediamine (Sigma) and H₂O₂ substrate. Plates were incubated at room temperature for 30 min and the reaction was stopped with 25 µL per well of 8 N sulfuric acid. Absorbance at 490 nm was read by using a multiscan reader (Titertek[®], Molecular Devices Corp.). The titer of each serum was the maximum dilution where the absorbance was greater than the mean plus 3-fold the standard deviation of triplicates of the same dilution of control sera.

Oral Vaccination of Mice with CTB-EG Conjugate

A group of 6 mice was orally vaccinated with 15 µg of CTB-EG conjugate along with 5 µg of unconjugated CT, a mucosal adjuvant. Specific antibody concentrations were measured in feces to determine the ability of CTB-EG conjugate and unconjugated CT, when given orally, to induce mucosal anti-EG antibodies. Mice were boosted 10 days later with the same dose of CTB-EG conjugate and CT. Mice were bled by the retro-orbital sinus under halothane anesthesia every 2 wk to collect sera for determination

of serum IgG titers against EG by ELISA. Fecal pellets were collected from individual mice every 3 to 5 days for 5 wk and frozen at -70 °C until testing for IgA and IgG coproantibodies against EG and CTB by ELISA.

Mice were revaccinated IP 93 days after the initial oral vaccination with 100 µg of CTB-EG in FIA. Specific antibody levels were measured in feces to assess induction of anamnestic immune responses in the gut mucosa by parenteral vaccination in mice previously vaccinated orally with CTB-EG conjugate. Mice were bled retro-orbitally under halothane anesthesia every 2 wk for 5 wk to collect sera for determination of serum IgG titers by ELISA. Fecal pellets were collected from individual mice every 3 to 5 days for 3 wk and frozen at -70 °C until testing for IgA and IgG coproantibodies against EG and CTB by ELISA.

Determination of Anti-Ergotamine IgA and IgG Coproantibodies in Mice Orally Vaccinated with CTB-EG Conjugate

Sample preparation

Mice were temporarily placed in cages without shavings. Fecal pellets were collected after 1 h and frozen at -70 °C until analysis. Samples of 150 mg were weighed and placed into microcentrifuge tubes that contained 1.5 mL PBS. The mixture was incubated at room temperature for 15 minutes. Samples were vortexed, left to settle for 15 min, and then revortexed until all material was in suspension. The suspension was

centrifuged for 10 min at 800 x g. The supernatant was immediately tested in the ELISA for IgG and sIgA immunoglobulins.

Determination of anti-EG coproantibodies by indirect ELISA (Appendix VIII)

Wells of high binding immunoplates (Nunc Maxisorp®) were coated overnight at 4 °C with 50 µL of BSA-EG conjugate (5 µg per well) or of CTB (0.5 µg per well) diluted in 0.1 M sodium carbonate-bicarbonate buffer (pH 9.6). Plates were washed with PBST. Wells were blocked for 3 h at 37 °C with 200 µL of 0.5% gelatin in PBST. Plates were washed with PBST and 100 µL of test sample was added per well and incubated at 37 °C for 2 h. Plates were washed with PBST to remove unbound antibody and 100 µL of goat anti-mouse IgG or IgA conjugated to HRPO (diluted 1/600 in 0.5% gelatin in PBST) was added to each well. Plates were incubated for 2 h at 37 °C. Plates were washed and the bound peroxidase determined by adding 200 µL of o-phenylenediamine (Sigma) and H₂O₂ substrate. Plates were incubated for 30 min at room temperature in darkness. The reaction was stopped with 25 µL of 8 N sulfuric acid. The absorbance at 490 nm was by using a multiscan reader (Titertek®, Molecular Devices Corp.). Mucosal sIgA and IgG were quantally determined by measuring the optical density (OD) at wavelength of 490 nm (DeVos and Dick, 1991).

RESULTS

As previously reported (Shelby and Kelley, 1990), the protein-EG conjugates on TLC plates exhibited intense fluorescence at the origin, whereas free EG fluoresced and migrated. Unconjugated protein controls remained at the origin and did not fluoresce.

All protein-EG conjugates showed bands with higher molecular weights than their respective protein controls (Figures 5:1a & 5:1b) when analyzed on SDS Page gels. The CTB-EG (Lane 3, Figure 5:1a) and CT-EG (Lane 5, Figure 5:1a) conjugates showed faint bands at 207 KDa, because of the low protein content per μL of conjugate. The CTB-EG (Lane 4, Figure 5:1b) did not appear as a band on the gel as it should have, probably because of the low protein content per μL of conjugate. The BSA (Lane 6, Figure 5:1a) and CT controls (Lane 1, Figure 5:1b) did not separate well on their respective gels, because too much protein was added to each sample well. Cross linking of proteins in the protein-EG conjugates was evident by SDS Page analysis (Figures 5:1a & 5:1b). Crosslinking of proteins was especially evident in lanes with Con A-EG conjugate (Lane 7, Figure 5:1b), Con A control (Lane 8, Figure 5:1b), and Con A without EG after the Mannich reaction (Lane 9, Figure 5:1b). Lanes with Con A-EG conjugate and Con A without EG after the Mannich reaction both have bands between 207 and 139 KDa. The bands between 207 and 139 KDa are not present in the lane with Con A control. Unconjugated EG did not appear as a band (Lane 3, Figure 5:1b), because it has only three peptides in its structure.

The Mannich reaction was used to conjugate BSA, Con A, CTB, CT, and ovalbumin to EG. However, the Mannich reaction did not conjugate poly-L-lysine or lysozyme to EG, since no antibody reaction was detected (Table 5:1). The CTB, CT and BSA conjugates apparently bound more EG than Con A or ovalbumin conjugates as determined by titration in ELISA (Table 5:1). The amount of EG conjugated to Con A was optimized by a 3-fold increase in reactant starting volumes (Table 5:2).

The Mannich reaction reduced the biological activity of Con A, CTB, and CT conjugates. The ability of Con A-EG conjugate and Con A after the Mannich reaction to stimulate lymphocyte proliferation in the lymphocyte transformation assay (LTA) was abolished for Con A-EG conjugate and greatly decreased for Con A (Table 5:3). The ability of CTB subunit present in CT-EG and CTB-EG conjugates to bind the GM1 receptor likewise was greatly reduced (Table 5:4).

The CT-EG conjugate was safe when given orally. Oral doses of CT-EG conjugate or CT ranging from 10 to 40 μg per mouse caused rough haircoats, but no diarrhea or death.

The magnitude and duration of primary and secondary humoral immune responses against EG (Figure 5:2) induced by parenteral administration were greatest for the BSA-EG conjugate. Anti-EG IgG titers increased linearly post-vaccination to peak around d 28. After d 28, titers slowly declined by d 105 post-vaccination. The Con A-EG and CTB-EG conjugates administered with adjuvant induced primary and secondary humoral immune responses against EG with similar kinetics to each other. In contrast to

BSA-EG conjugate, anti-EG IgG titers induced by Con A-EG and CTB-EG conjugates did not increase as rapidly after vaccination. Titers were increasing at d 36 post-vaccination and peaked between d 36 and 84 post-vaccination. Titers were similar to pre-vaccination levels by d 105 post-vaccination. The duration of the antibody response elicited by CTB-EG conjugate with adjuvant was not prolonged, but was similar to that induced by a 5-fold larger dose of Con A-EG conjugate. The CTB-EG conjugate without adjuvant induced low anti-EG IgG titers (Figure 5:2).

Revaccination of mice a third time on d 105 (Figure 5:3) with BSA-EG and Con-EG conjugates with adjuvant induced humoral immune responses similar to secondary immune responses. Revaccination with BSA-EG conjugate induced anti-EG titers that peaked earlier with a magnitude and duration similar to the secondary response. Again, the magnitude and duration of anti-EG IgG antibodies induced were greatest for the BSA-EG conjugate. Revaccination a third time with Con A-EG conjugate IP induced a humoral immune response that peaked earlier than the secondary response with a similar magnitude, but with a shortened duration. However, revaccination with CTB-EG in FIA induced lower anti-EG titers than the second vaccination. The humoral immune response induced by a third vaccination with CTB-EG in FIA peaked earlier than the secondary response, and had a shortened magnitude and duration. Magnitude of the anti-EG response induced by third vaccination of CTB-EG in FIA was decreased by 50% and the duration was only measurable for 15 days. In contrast, revaccination with CT-EG conjugate without adjuvant on d 105 to mice twice previously vaccinated with CTB-EG

conjugate without adjuvant induced an anti-EG titer greater in magnitude and duration, than responses induced by Con A-EG or CTB-EG conjugates emulsified in adjuvant.

In steers, Con A-EG conjugate emulsified in FIA failed to induce anti-EG antibodies. The CTB-EG conjugate without adjuvant did not induce anti-EG antibodies after two vaccinations. Anti-CTB antibodies ($\text{Log}_2 = 3$; where each unit of antibody titer represents a twofold serial dilution of serum, beginning with a 1:64 dilution at a Log_2 titer = 1) were detected after revaccination with CTB-EG conjugate. However, anti-EG IgG antibodies ($\text{Log}_2 = 6$) were induced 13 d after a third vaccination with CTB-EG conjugate in FIA.

The CTB-EG conjugate given orally to mice induced both mucosal and systemic anti-EG and anti-CTB antibodies. A systemic titer IgG response was observed from d 10 to 21 post-oral vaccination and decreased by d 30. The systemic titer response to oral vaccination was lower ($\text{Log}_2 = 1$) than the titers observed after the treatments shown in Figure 5:2. The systemic IgG response preceded the appearance of sIgA and IgG anti-EG antibodies at the mucosal level. The mucosal response against EG and carrier (CTB) (Figure 5:4) peaked at d 29 post-vaccination and the duration was short lived. Mucosal immune responses were higher for the carrier, CTB, than for EG. Kinetics of mucosal sIgA and IgG against EG and CTB were similar with sIgA attaining higher peak levels.

Intraperitoneal administration of CTB-EG conjugate in FIA 93 d post-oral vaccination rapidly induced anamnestic immune responses at intestinal surfaces for both IgG and sIgA isotypes against EG and CTB (Figure 5:4). Magnitude of IgA and IgG

anamnestic immune responses at intestinal surfaces was decreased after IP administration of CTB-EG conjugate, but the duration was extended as compared to only oral vaccination. However, magnitude and duration of the systemic IgG titer after IP revaccination was extended as compared to only oral vaccination. The systemic IgG response (Log_2 titer = 3.5) peaked 10 days after IP vaccination and remained elevated (Log_2 titer = 2.75) at 3 wk post vaccination, but declined (Log_2 titer = 0.25) by 5 wk post-vaccination.

Table 5:1. Relative Ergotamine Content of Protein-Ergotamine Conjugates as Determined by ELISA

Protein-EG conjugate ^a	Anti-EG titer ^b
Con A	1:64
BSA	1:512 to 1:2048
CT	1:512
CTB	1:512 to 1:2048
Ovalbumin	1:64
Lysozyme	1:4
Poly-L-lysine	0 to 1:2

^a 5 µg/well of each protein-EG conjugate was the antigen coating in ELISA.

^b Anti-EG titers determined by indirect ELISA using anti-EV monoclonal antibody as the primary antibody.

Table 5:2. Relative Ergotamine Content of Concanavalin A-Ergotamine Conjugates Prepared by Various Reactant Volumes in the Mannich Reaction

Reactant condition ^a	Anti-EG titer ^b	Protein content ($\mu\text{g}/\mu\text{L}$) ^c
1-fold	1:2	0.75
2-fold	1:8	1.63
3-fold	1:64	2.11
4-fold	1:8	1.52

^a Starting volumes of reactants in the Mannich reaction were increased 2, 3, and 4-fold.

^b Anti-EG titers determined by indirect ELISA using anti-EV monoclonal antibody as the primary antibody.

^c Protein content determined by BCA analysis.

Table 5:3. Activity of Con A-EG Conjugate and Con A in the Lymphocyte Proliferation Assay after the Mannich Reaction

	Counts per minute (CPM) \pm SEM
Con A ^a	137,000 \pm 13,990
Con A-EG conjugate	250 \pm 31.5
Con A (rxn)	12,281 \pm 925

^a 1 μ g/well Con A, 10 μ g/well Con A-EG, and 10 μ g/well Con A without EG post Mannich reaction.

CPM for unstimulated control wells was 3790 \pm 556.

Table 5:4. Ability of CTB-EG and CT-EG Conjugates to Bind GM1 Receptor in ELISA

	Fold greater than optical density of negative control
CTB-EG conjugate^a	2.1
CT-EG conjugate	5.6
CT control	12.9
CTB control	13.7

^a 5 µg/well of CT, 6 µg/well of CTB and of CTB-EG conjugate, and 10 µg/well of CT-EG conjugate.

2.5 µg/well GMI coating antigen.

Negative control average optical density ± SEM at 490 nm was 0.105 ± 0.013.

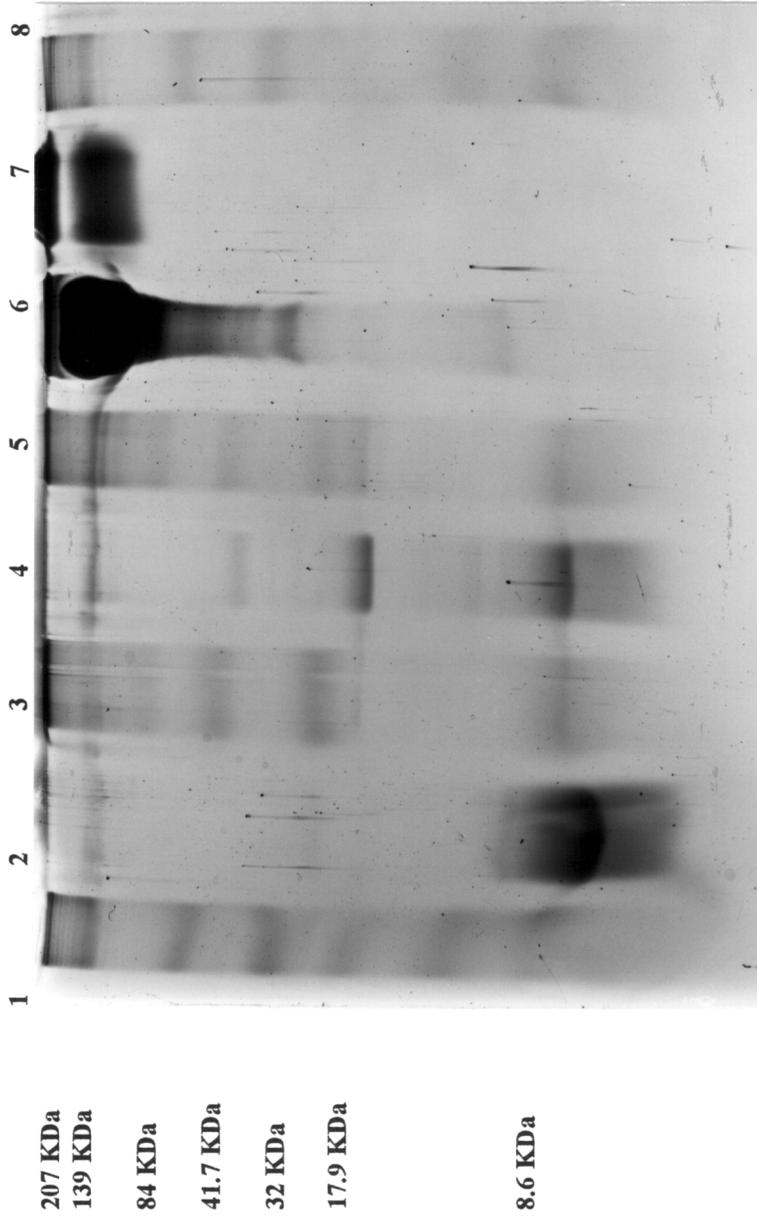


Figure 5:1a. SDS PAGE Of Protein-Ergotamine Conjugates Stained with Coomassie Blue

Key

- Lane 1 Molecular weight marker
- Lane 2 Cholera toxin subunit B control
- Lane 3 CTB-EG conjugate
- Lane 4 Cholera toxin control
- Lane 5 CT-EG conjugate
- Lane 6 BSA control
- Lane 7 BSA-EG conjugate
- Lane 8 Molecular weight marker

* Mini gel with 12.5% PAGE

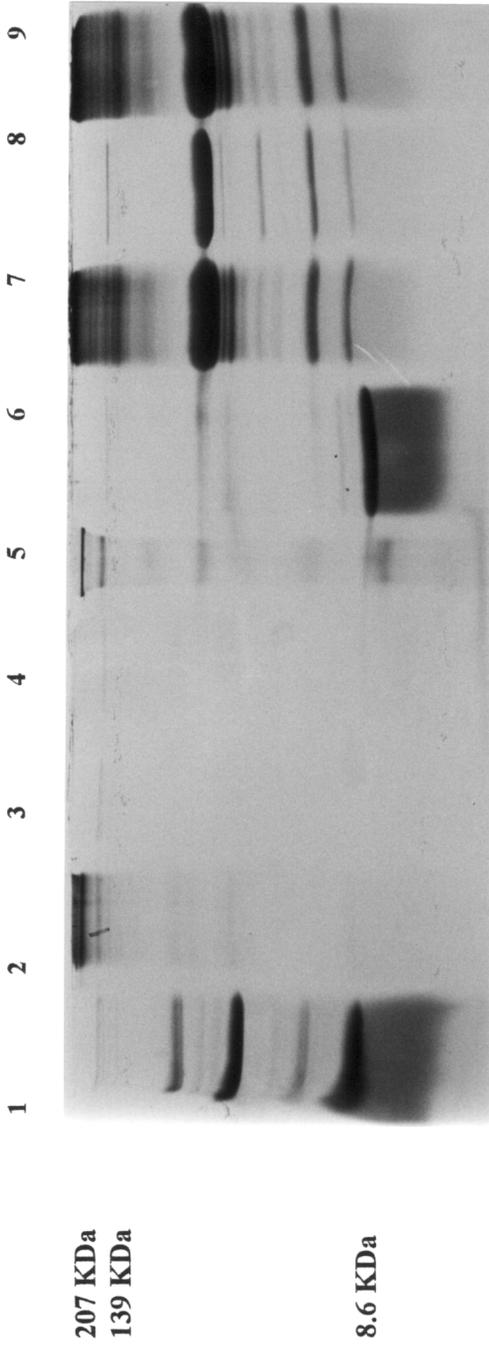
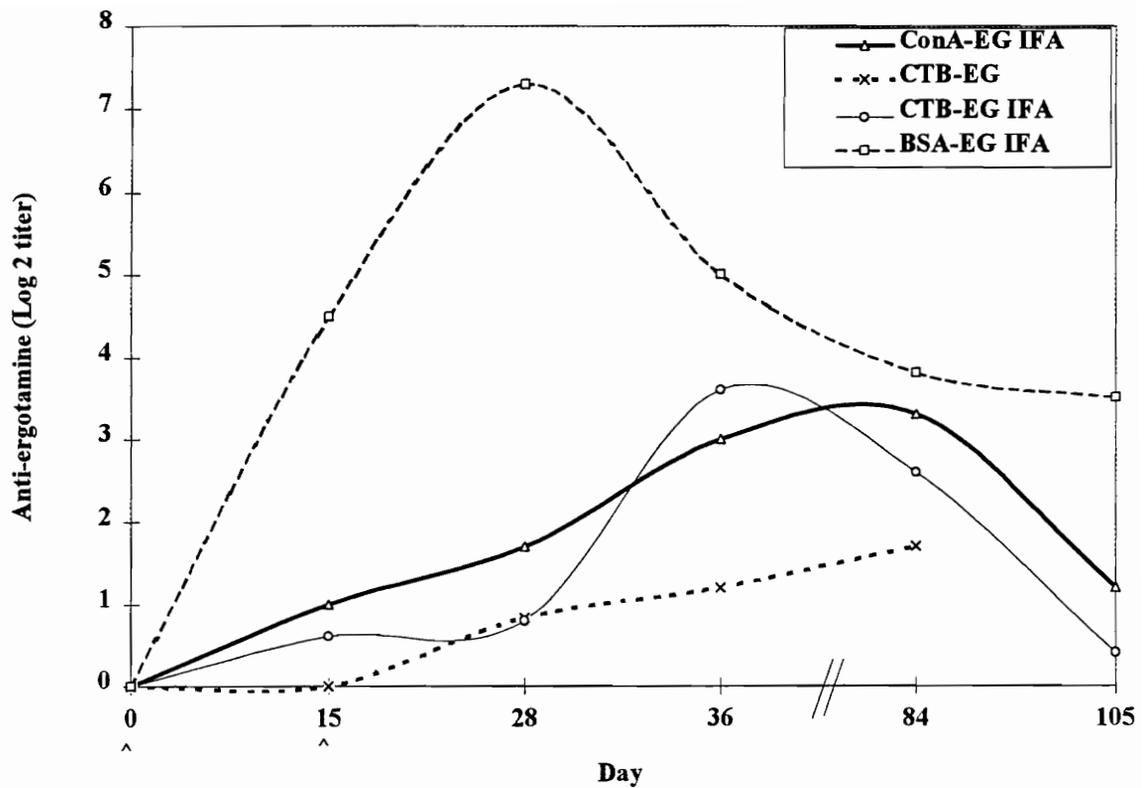


Figure 5:1b. SDS PAGE Of Protein-Ergotamine Conjugates Stained with Coomassie Blue

Key

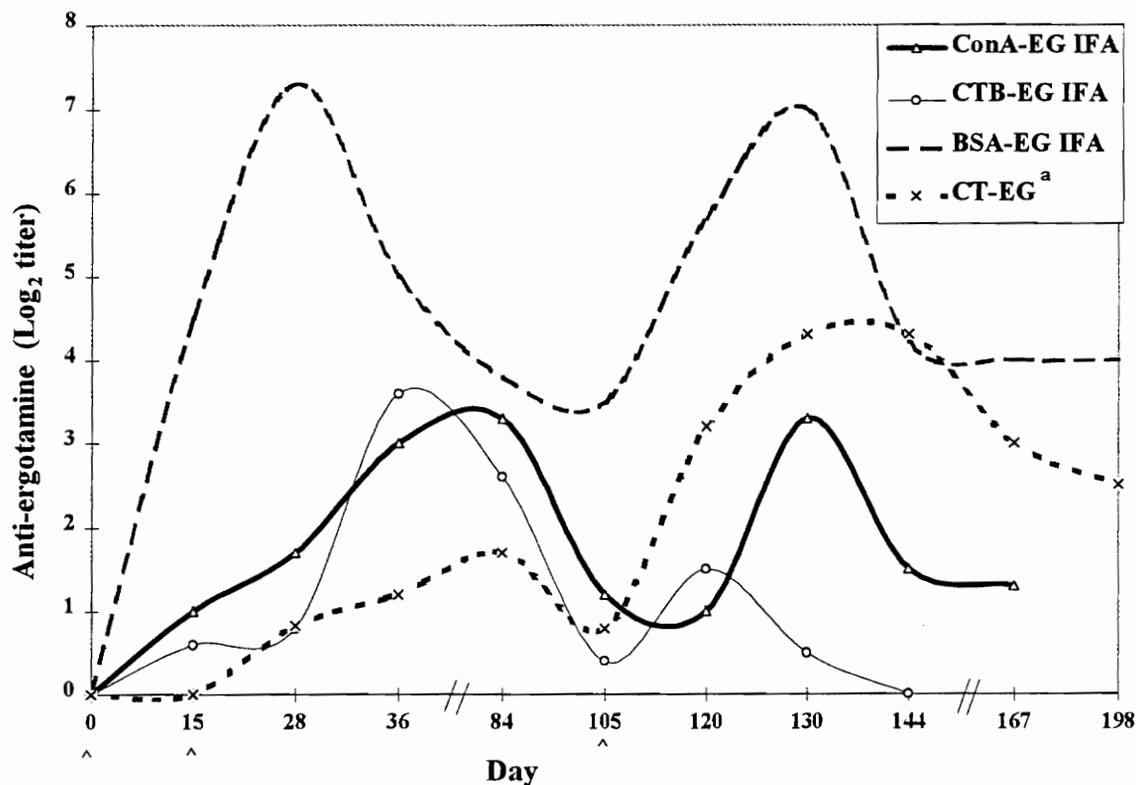
- Lane 1 Cholera toxin control
- Lane 2 CT-EG conjugate
- Lane 3 Ergotamine control
- Lane 4 CTB-EG conjugate
- Lane 5 Molecular weight marker
- Lane 6 Cholera toxin subunit B control
- Lane 7 Con A-EG conjugate
- Lane 8 Con A control
- Lane 9 Con A without EG after Mannich reaction

* Standard gel with 15% PAGE



^ indicates vaccination on d 0 and 15

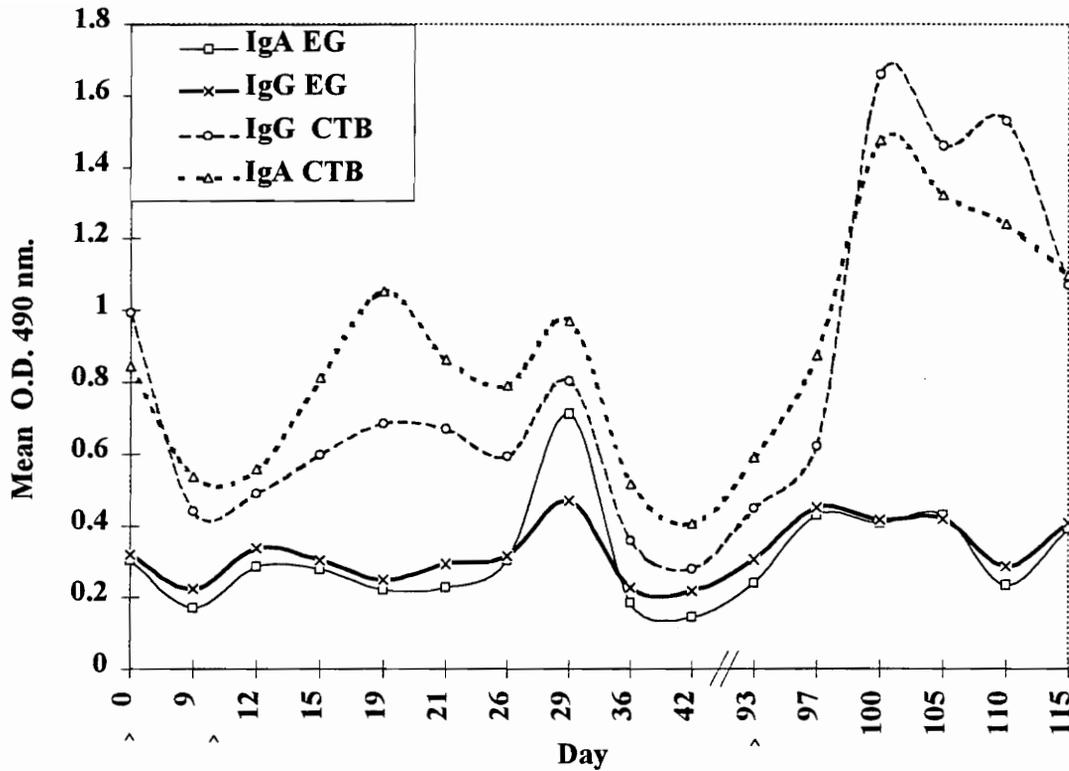
Figure 5:2. Magnitude and duration of primary and secondary humoral immune responses (anti-ergotamine IgG titers) in mice induced by protein-ergotamine conjugates. Each unit of antibody titer represents a two-fold serial dilution of serum, beginning with a 1:64 dilution at a Log_2 titer = 1. Mice in each treatment group ($n = 6$) were vaccinated IP with one of the protein-EG conjugates on d 0 and 15. Anti-EG titers were determined by ELISA.



^ indicates vaccination

Figure 5:3. Effects of third vaccination with protein-ergotamine (EG) conjugates on magnitude and duration of anamnestic humoral immune responses (anti-EG IgG titers) in mice. Each protein-EG conjugate was given IP to mice (n = 6) on d 0 and 15 to induce primary and secondary responses. Mice were revaccinated IP with protein-EG conjugates on d 105. Each unit of antibody titer represents a twofold serial dilution of serum, beginning with a 1:64 dilution at a Log₂ titer = 1.

^a CT-EG conjugate without adjuvant was given IP on d 105 to mice previously vaccinated IP with CTB-EG conjugate without adjuvant.



^ indicates vaccination

Figure 5:4. Mucosal anti-ergotamine IgA and IgG levels induced by CTB-EG conjugate given orally to mice. Mice (n = 6) were orally vaccinated with 15 μ g CTB-EG conjugate and 5 μ g of CT on d 0 and d 10. Mice were revaccinated intraperitoneally on d 93 with CTB-EG conjugate in FIA to induce anamnestic mucosal responses.

DISCUSSION

The Mannich reaction successfully conjugated BSA, Con A, CTB, CT, and ovalbumin to EG. Apparently, these proteins contain an adequate number of lysine residues in the proper orientation for conjugation to the indole nitrogen of EG. However, the Mannich reaction did not conjugate PLL or lysozyme to EG. The formation of homoconjugates of PLL resulted in precipitation of protein that probably interfered with conjugation of PLL to EG. Low lysine residue content and formation of protein homoconjugates also interfered with conjugation of lysozyme to EG.

The EG content of CTB and BSA conjugates was similar and the EG content of ovalbumin and Con A conjugates was similar. The average number of EG residues per molecule of carrier protein was previously determined as 7.7 for BSA and 1.3 for ovalbumin (Shelby and Kelley, 1990).

The Mannich reaction did not completely abolish the ability of CTB in CT-EG and CTB-EG conjugates to bind the GM1 receptor to exert biological effects. However, the ability to bind GM1 receptors was greatly reduced. Decreased ability of CTB subunit of CT-EG and CTB-EG conjugates to bind GM1 receptor may be a result of inactivation of CTB by the formaldehyde chemical linker or conjugated hapten, EG. Either the formaldehyde chemical linker or EG present in CT or CTB-EG conjugates may interfere with binding to GM1 receptor by such mechanisms as steric hindrance.

Competitive inhibition by unconjugated protein present in CT-EG and CTB-EG conjugates may decrease the ability of CTB subunit to bind GM1 receptor.

Unconjugated CT and CTB are present in CT-EG and CTB-EG conjugates, because the efficiency of conjugation of proteins to EG via the Mannich reaction is not 100%. In ELISA, immunoplates coated with GM1 receptor that were reacted with CTB-EG conjugate followed by monoclonal anti-EV antibodies demonstrated the ability of CTB-EG conjugate to bind GM1 receptor. However, binding of GM1 receptor by CTB-EG conjugate was reduced compared to CTB control (data not shown).

Ability of Con A and Con A-EG to act as mitogens in the LTA also was negatively influenced by the Mannich reaction. Perhaps Con A was denatured by the low pH of the Mannich reaction or formaldehyde inactivated the binding sites. Conjugation of EG to Con A decreased the ability of Con A to act as a mitogen in the LTA, since lymphocyte proliferation was not induced when Con A-EG was used as the mitogen in the test. Therefore, Con A conjugated to EG for use in vaccination may not stimulate T-cell proliferation over other non-mitogenic protein carriers because Con A-EG conjugates lack biological activity. Nonetheless, Con A may be a useful protein carrier for haptens because of its large molecular weight and complex structure.

The CT-EG conjugate given orally at high doses did not cause mortality or morbidity in mice. Thus, oral administration of CT-EG conjugate vaccine was safe at doses 4-fold greater than what was required to induce mucosal immune responses.

Overall, the Mannich reaction is useful in vaccine development for haptens, such as indolealkylamines, which are not denatured during the coupling reaction. Advantages of the Mannich reaction are that CTB retained some GM1 binding capacity, conjugates formed are non-toxic, and the procedure is not labor intensive. However, conjugation of proteins to EG via the Mannich reaction has several disadvantages that may decrease immune responses to haptens, such as formation of protein homoconjugates. Additionally, the coupling reaction is not controllable to allow production of conjugates with defined variations in protein to EG ratios.

Parenteral and oral vaccination of mice with protein-EG conjugates prepared by the Mannich reaction induced anti-EG antibodies. Anti-EG titers were induced by parenteral vaccination of mice with BSA-EG, CT-EG, CTB-EG and Con A-EG conjugates. Magnitude and duration of humoral immune responses induced by BSA-EG conjugates were greatest. Magnitude and duration of humoral immune responses induced by Con A-EG and CTB-EG conjugates were similar. Time before anti-EG titers were detectable in mice after vaccination with Con A-EG and CTB-EG conjugates was longer than for BSA-EG conjugate. The CTB-EG conjugate, which may retain some CTB activity, may increase the time for development of titers because CTB inhibits B-cell proliferation and induces isotype differentiation (Woogen *et al.*, 1987). Revaccination with BSA-EG and Con A conjugates in mice did not impair magnitude and duration of humoral immune responses. However, repeated parenteral vaccination of CTB-EG conjugate with adjuvant decreased immune responses against EG. High doses of CTB-

EG conjugate in FIA decreased anti-EG titers and may have induced tolerance.

Tolerance develops in mature individuals when an immune response is not observed against an otherwise immunogenic antigen. Tolerance to a specific antigen may be induced by high or low doses of antigen, antigen exposure during fetal development, deaggregated antigen, and antigen given in the absence of APC. Low doses of antigen induce T-cell tolerance and high doses of antigen induce both T and B-cell tolerance (Tizard, 1987). Nonetheless, both high and low dose tolerance will inhibit antibody production because T-cell help is required for B-cell response to T-cell dependent antigens. The exact mechanisms of tolerance have not been clearly defined. Possible mechanisms for tolerance may include stimulation of T suppressor cells or clonal deletion (Tizard, 1987). The high dose of CTB-EG conjugate with adjuvant may have stimulated T suppressor cells induced by previous immunizations. Ergotamine may have a role in such inductions, since fescue toxicosis increases suppressor T-cells in mice (Dew, 1989). Additionally, presence of unconjugated CTB carrier in the CTB-EG conjugate may have induced epitope suppression of EG to decrease anti-EG titers.

The CTB-EG conjugate without adjuvant did not induce titers in mice or cattle after parenteral administration. Thus, CTB-EG conjugates will require either an adjuvant, or different doses to achieve adequate titers when administered parenterally. However, CTB-EG conjugate without adjuvant induced memory cell populations, because anti-EG titers were induced in mice after third vaccination with CT-EG conjugate without adjuvant and in cattle after revaccination with CTB-EG conjugate

emulsified in FIA. The CT-EG conjugate was more effective at inducing titers than CTB-EG in the absence of adjuvant in mice. Therefore, immune responses to parenteral vaccination may be aided by adding non-toxic doses of CT as an adjuvant to CTB-EG conjugates. The CTB, as a protein carrier for EG, did not prolong titers in this study as reported for other antigens (Hirabayashi *et al.*, 1990). Perhaps the decreased or absence of binding of GM1 receptor by CTB in CTB-EG conjugate interfered with the interaction of CTB with lymphocytes to generate prolonged titers.

Parenteral revaccination of cattle with CTB-EG conjugate emulsified in FIA induced higher anti-EG titers than after vaccination with CTB-EG conjugate alone. Concanavalin A-EG emulsified in FIA did not induce anti-EG titers by d 28 in cattle. Perhaps a longer lag time is required for induction of measurable anti-EG titers after vaccination of cattle with Con A-EG conjugate as demonstrated in mice. Cattle were not bled after d 28, therefore anti-EG titers may have been missed. Consequently, additional vaccination of cattle with CTB-EG and Con A-EG conjugates is needed to further characterize magnitude and duration of induced humoral immune responses.

Oral vaccination of mice with CTB-EG conjugate with CT as an adjuvant induced mucosal IgA and IgG antibodies against both EG and CTB. Determination of specific sIgA in oral vaccination protocols is important because sIgA can provide protection in the absence of demonstrable serum IgG antibodies. Thus, protection may be correlated best with local sIgA secretion rather than circulating antibody. Oral vaccination of mice with CTB-EG conjugate also induced short-lived systemic anti-EG IgG titers.

Intraperitoneal administration of CTB-EG conjugate post-oral vaccination rapidly induced anamnestic immune responses for both IgG and IgA isotypes at intestinal surfaces. Intraperitoneal administration of CTB-EG conjugate post-oral vaccination also induced a systemic IgG immune response with a greater magnitude and duration than the mucosal response. A population of memory cells committed to producing anti-EG antibodies probably was stimulated by the IP vaccination. These results are in agreement with previous research demonstrating that parenteral immunization with an antigen, to which an individual was previously immunized by the oral or intranasal routes, induces sIgA responses at mucosal surfaces (Mestecky, 1987). The effect of repeated oral vaccinations of CTB-EG conjugate on immune responses was not investigated in this research. A theoretical concern about the repeated use of CTB as a carrier for mucosal immunization is the interference by the established, long-lived CTB antibodies present in the mucosal surfaces in generating a secondary immune response to EG. However, Tamura *et al.* (1989a) demonstrated that pre-existing immunity to CTB in mice did not limit the ability of CTB to enhance the protective mucosal sIgA and systemic antibody response to influenza vaccination.

To protect cattle against fescue toxicosis, repeated parenteral vaccination of cattle may be required to induce adequate systemic anti-EG titers. However, repeated parenteral vaccination is too expensive and labor intensive for cattle producers. Also repeated vaccination may induce tolerance to the antigen with some protein carriers as demonstrated in the mice experiments. Cattle repeatedly vaccinated with a protein-

lysergol conjugate had decreasing titers, despite revaccination (Hill *et al.*, 1994).

Suppressor T-cell populations are increased in mice fed a diet of EI tall fescue (Dew, 1989) and may favor development of tolerance. Therefore, traditional parenteral vaccination may not be the optimal route of vaccine administration in the prevention of fescue toxicosis.

Oral vaccination is an infrequently used route of administration, but may be an important route in the protection of cattle against fescue toxicosis. However, oral vaccination to induce mucosal immune responses is more complicated in cattle because of the complex nature of their gastrointestinal tract. The limitations of inducing a mucosal immune response by oral vaccination in cattle may be overcome with microspheres or with intranasal vaccination. Novel immunization protocols developed against fescue toxicosis could involve initial mucosal (oral or intranasal) immunization to induce anti-EG IgA effector and memory cell populations followed by subcutaneous immunization. Intranasal inoculation of mice with influenza and CTB was 10-100 times more effective on a dose basis as oral immunization in producing intestinal anti-CTB and anti-viral IgA antibodies (Hirabayashi *et al.*, 1990) apparently from the migration of lymphocytes sensitized in BALT to GALT. A single intranasal immunization of 1 μ g CTB with influenza vaccine induced a high level of mucosal IgA and serum anti-viral antibodies that protected mice against viral infection for 16 weeks demonstrating a long-lived protective titer (Tamura *et al.*, 1989b). Subcutaneous administration of microsphere-encapsulated vaccine also may prolong mucosal and systemic titers for

months and decrease the frequency of vaccination. Additionally, systemic anti-EG IgG antibodies induced by parenteral administration could neutralize any EV absorbed systemically.

In conclusion, the Mannich reaction was used to conjugate EG to BSA, Con A, ovalbumin, CT and CTB protein carriers. Biological activity of CTB-EG, CT-EG, and Con A-EG conjugates was impaired by the Mannich reaction. Concanavalin A-EG and CTB-EG conjugates injected IP with adjuvant induced systemic IgG antibodies against EG with similar kinetics in mice. The CTB-EG conjugate did not prolong antibody titers as compared to the other carrier-EG conjugates, although smaller doses were needed to elicit titers in mice. The CTB-EG conjugate required an adjuvant to induce anti-EG titers in both mice and cattle. Concanavalin A-EG conjugate did not induce titers in cattle. Oral vaccination of mice with the CTB-EG conjugate induced both mucosal IgA and IgG isotypes, in addition to a systemic IgG response. Intraperitoneal administration of CTB-EG conjugate to mice previously vaccinated orally elicited an anamnestic mucosal response and systemic anti-EG response. This research suggests that an anti-EG antibody response of adequate magnitude and duration will require either frequent vaccine administration or an enhanced vaccine delivery system, such as microspheres, to protect against fescue toxicosis.

Chapter 6: Microspheres as a Vaccine Delivery System in Vaccination against Fescue Toxicosis

INTRODUCTION

The humoral immune response against a specific antigen is antigen driven. The stimulus for lymphocyte proliferation ceases when an antigen is eliminated. If the antigen persists, then the stimulus for lymphocyte proliferation continues and the immune response is prolonged. Thus, encapsulation of protein-EG conjugate into poly(DL-lactide-co-glycolide) microspheres may prolong humoral immune responses against EG by providing continuous release of EG.

Microspheres synthesized from poly(DL-lactide-co-glycolide) (DL-PLG) are biodegradable and biocompatible polyesters. Poly (DL-lactide-co-glycolide) microspheres are in the class of copolymers from which resorbable sutures, resorbable surgical clips, and controlled-release implants are made. These copolymers have a history of safe and effective use and are approved for use in humans. They have an acceptable shelf life without the need for stabilizers or refrigeration. Encapsulation of antigens into microspheres does not require expensive equipment, and is not time or labor intensive. Microspheres have no intrinsic mitogenic activity, but enhance the immune response to an antigen when the antigen is incorporated within the copolymer

matrix (Eldridge *et al.*, 1991). Microspheres, when given parenterally, induce a minimal inflammatory response and biodegrade into the normal metabolic components, lactic and glycolic acid, through a nonenzymatic hydrolysis of ester linkages.

Microspheres as a vaccine delivery system offer many advantages in oral, intranasal, or subcutaneous vaccination protocols. Microspheres act as a vaccine depot to prolong titers and target vaccines/adjuvants to specific sites. Tetanus microencapsulated microspheres administered subcutaneously produce a higher antibody response over a 6 month period than the free antigen (Alonso *et al.*, 1993). Microspheres also protect vaccines against digestion in the gut, amplify the immune response (adjuvant effect), and promote antigen uptake (Russell and Mestecky, 1988). Microspheres as a vaccine vehicle may protect an oral vaccine from degradation by the low pH of the stomach and from proteolytic enzymes and bile salts in the gastrointestinal tract. A lower dose of vaccine may be administered orally or intranasally, because more of the vaccine would reach the target site intact. Microspheres also target the vaccine to the mucosal immune system by stimulating uptake by the M cells of GALT and the lymphoepithelial cells in BALT. Intact antigen delivered to sIgA inductive sites may effectively induce a disseminated sIgA immune response.

Microspheres may be useful in parenteral vaccination protocols against fescue toxicosis in cattle. Microspheres made of different lactide to glycolide ratios allow a single injection to release one or more booster doses at predicted intervals. Subcutaneous administration of protein-EG conjugate encapsulated into optimally formulated

microspheres may provide continuous release of vaccine to induce prolonged anti-EG titers. A subcutaneous depot of protein-EG vaccine would make repeated vaccination unnecessary and could also provide antigen to induce an anamnestic response for sIgA in cattle previously vaccinated orally or intranasally.

Microsphere size, appearance, and release kinetics can be altered by formulating microspheres with different vaccine loading doses and different lactide to glycolide ratios. The rate at which DL-PLG microspheres degrade, which influences antigen release, is dependent on the ratio of lactide to glycolide in the walls of the microsphere (Miller *et al.*, 1977). The rate of vaccine release is important in the development of a microencapsulated conjugate vaccine given by different immunization routes to ensure the availability of the antigen at the appropriate time. Microspheres with fast vaccine release rates are optimum for oral and intranasal immunization, whereas microspheres possessing slow, continuous vaccine release rates are best for subcutaneous immunization.

Characterization of microspheres is important since their size, appearance, and release kinetics can vary depending on polymer formulation and vaccine/polymer ratio. Determination of the amount of vaccine encapsulated in the microspheres is important for administering the appropriate dose and assessing the vaccine effectiveness. Also, low or high dose tolerance can develop to the antigen if an inappropriate dose is given. Assessment of surface appearance by scanning electron microscopy (SEM) is important in the determination of microsphere quality and size.

The objectives of this study were: 1) prepare microsphere-encapsulated BSA-EG conjugate; 2) characterize microsphere size and surface appearance by SEM; and 3) assess duration of secondary humoral immune responses against EG induced by microsphere-encapsulated conjugate of BSA-EG given subcutaneously to mice.

MATERIALS AND METHODS

Microsphere Preparation and Characterization

The Mannich reaction was used to conjugate BSA to EG (Appendix III). Conjugation was confirmed by TLC analysis (Appendix V) and protein content of the conjugate determined by BCA analysis (Appendix VI).

Microspheres were prepared and characterized according to published methods (Eldridge *et al.*, 1991). Nine mg of BSA-EG conjugate was dissolved in 600 μ L of distilled water and emulsified in 4 mL of methylene chloride containing 500 mg of the polymer poly(DL-PLG) with a lactide to glycolide acid ratio of 50/50 (MW 50,000 to 75,000). While the mixture was stirred, the polymer solution was added to 60 mL of 8% (v/v) aqueous solution of polyvinyl alcohol. The mixture was emulsified by sonication at output 4 (50W) for 10 sec with an ultrasonic probe. The solution was stirred

magnetically for 1 h for further emulsification. Solvent was diluted by adding 3.5 L of distilled water. Microspheres were collected by centrifugation, washed three times with double distilled water, and freeze dried to yield a free flowing powder. The size, appearance, and surface appearance of microspheres were examined by SEM.

Microspheres were coated with gold and mounted on double sided tape for SEM examination by the Ultrastructural Laboratory at VMRCVM. The mean diameter and the range of sizes (1-10, 11-20, 21-30, 31-45 microns) were recorded.

Determination of Amount of BSA-EG Conjugate Encapsulated in Microspheres

The amount of BSA-EG conjugate encapsulated per unit weight of microspheres was determined by dissolving 30 mg of microspheres into 200 μ L methylene chloride and 500 μ L distilled water. The amount of protein was determined by micro-BCA™ assay (Pierce Inc.). A set of protein standards was prepared with BSA (2 mg/mL) in a 96 well plate. The standard curve ranged from 0.156 μ g to 20 μ g per 200 μ L. Twenty microliters of microsphere sample were added to each well containing 180 μ L of working reagent. The plate was incubated at 37 °C for 30 min, then cooled to room temperature. Absorbance was measured at 570 nm . A standard curve was constructed by plotting absorbance versus protein concentration. Linear regression was used to

determine the equation of the line. Protein content was determined from the derived equation of the line.

Evaluation of Microsphere Preparations *in Vivo*

Eight female Balb/c mice were randomly allocated to two treatment groups. All mice were vaccinated subcutaneously with 250 μg of BSA-EG conjugate in FCA to induce a primary immune response against EG. Two weeks later, mice were bled from the retro-orbital sinus under halothane anesthesia and revaccinated to induce a secondary immune response against EG. One group was revaccinated subcutaneously with 10 mg of microspheres with encapsulated BSA-EG conjugate that were soaked overnight in 100 μg BSA-EG conjugate in FIA. Microspheres were soaked overnight to allow adsorption of the conjugate to microsphere surfaces. The second group was revaccinated subcutaneously with only 100 μg BSA-EG conjugate in FIA. Mice were bled from the retro-orbital sinus under halothane anesthesia every 3 to 4 wk for 14 wk. The IgG titers against EG in sera were determined by ELISA (Appendix VII).

STATISTICAL ANALYSIS

Student T test (two tailed) was used to determine significant differences at $P < 0.05$ between treatment groups (Microsoft Excel®, Microsoft Corp., USA).

RESULTS

The surface appearance of poly(DL-PLG) microspheres encapsulating BSA-EG conjugate was smooth and spherical (Figure 6:1). Some microspheres contained very small pores on the surface (Figure 6:2). Microspheres ranged from 7.5 microns to 45 microns in size (Figure 6:3). The average microsphere was 22.7 microns in diameter (SD = 9.5, n = 40) (Figure 6:4). Microspheres degraded very rapidly upon SEM examination at 15 KV (Figure 6:5). Microspheres did not deteriorate as fast upon examination at 5 KV. However, the surface detail was not as clear (Figure 6:6) when examined at 5 KV.

Protein content as determined by micro-BCA analysis was 32 μ g BSA-EG conjugate per 10 mg of microspheres. As expected, primary immune responses of the two groups were the same. However, magnitude and duration of secondary immune

responses against EG were also similar between the groups throughout the 105 d study (Table 6:1).

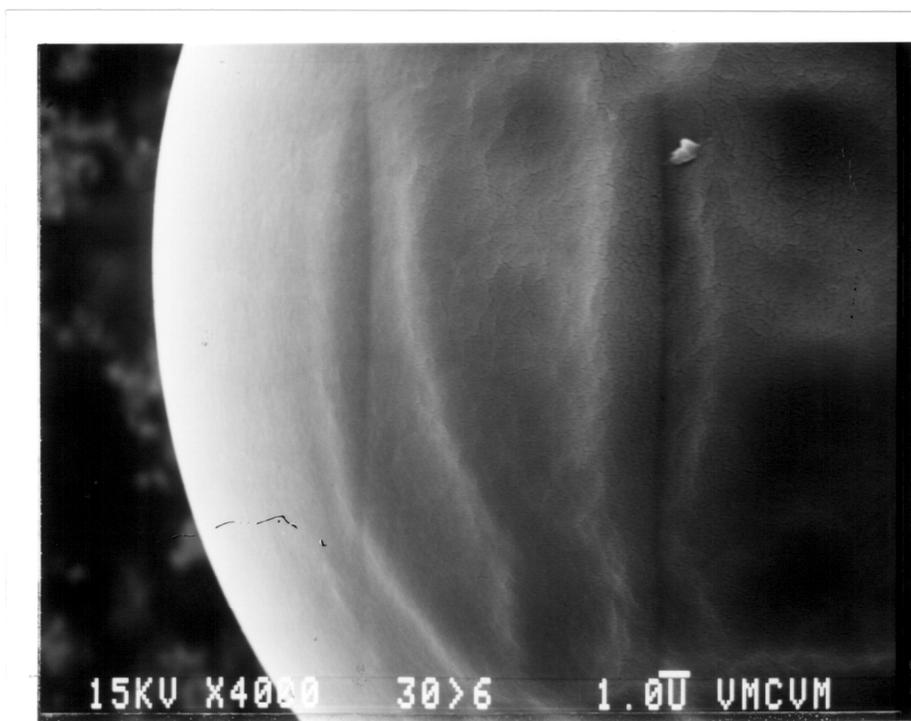


Figure 6:1. Scanning electron micrograph of poly (DL-PLG) microspheres encapsulating BSA-EG conjugate at 15 KV and 4000 X magnification showing the smooth and spherical appearance of the microsphere surface. The microsphere is beginning to deteriorate as a result of the high KV as shown by the formation of ridges on the microsphere surface.

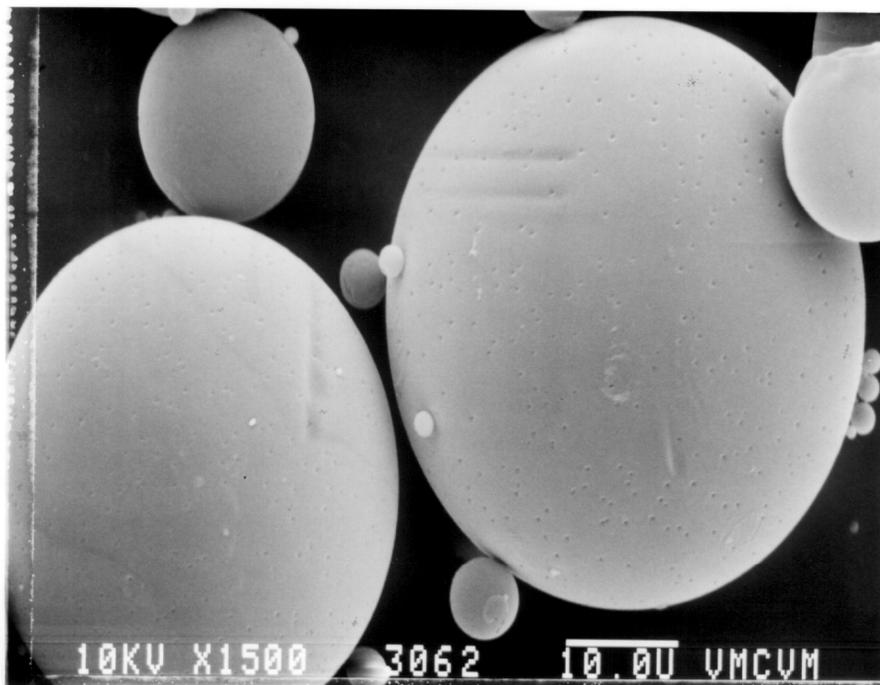


Figure 6:2 Scanning electron micrograph of poly (DL-PLG) microspheres encapsulating BSA-EG conjugate at 10 KV and 1500 X magnification showing the small pores present in the walls of some microspheres.

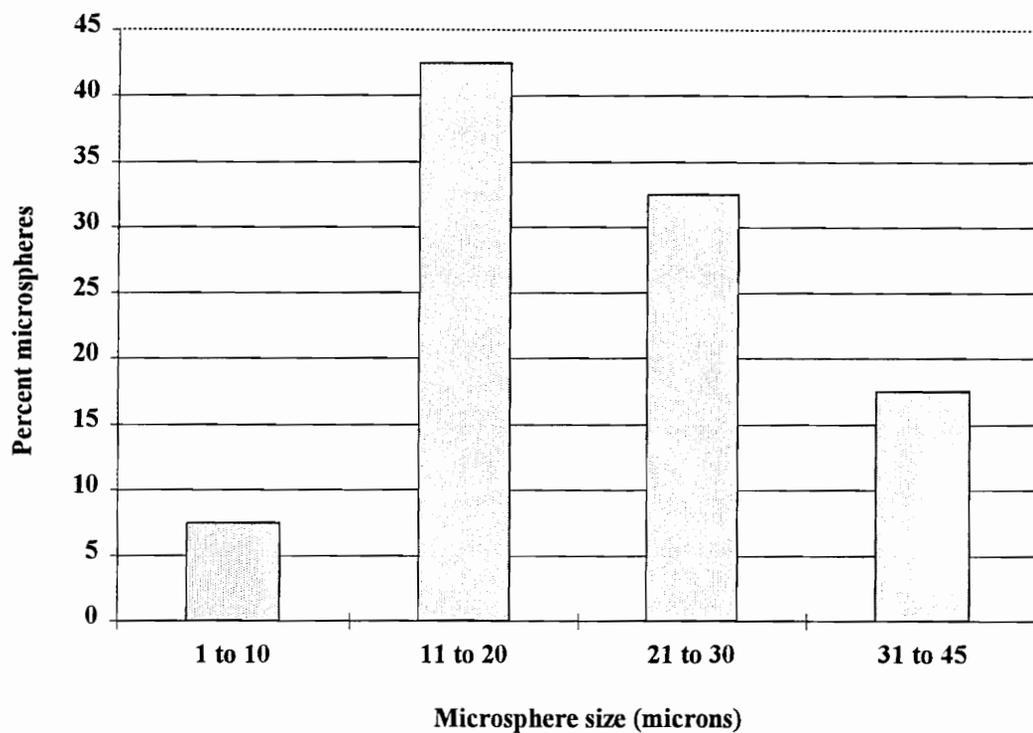


Figure 6:3. Range of sizes for microsphere encapsulated BSA-EG conjugate.

Average size of poly (DL-PLG) microspheres with lactide to glycolide ratio of 50:50 was 22.7 microns in diameter ($n = 40$, $SD = 9.5$).

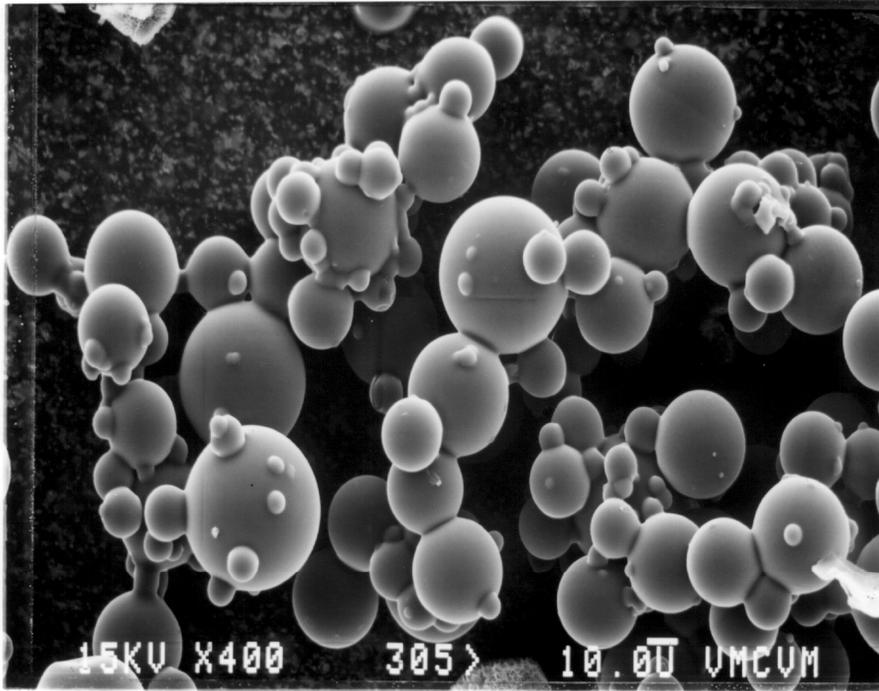


Figure 6:4 Scanning electron micrograph of poly (DL-PLG) microspheres encapsulating BSA-EG conjugate at 15 KV and 400 X magnification showing the range in microsphere sizes. Microsphere sizes ranged from 7.5 to 45 microns. The average microsphere size was 22.7 microns (SD = 9.5, n = 40) in diameter.

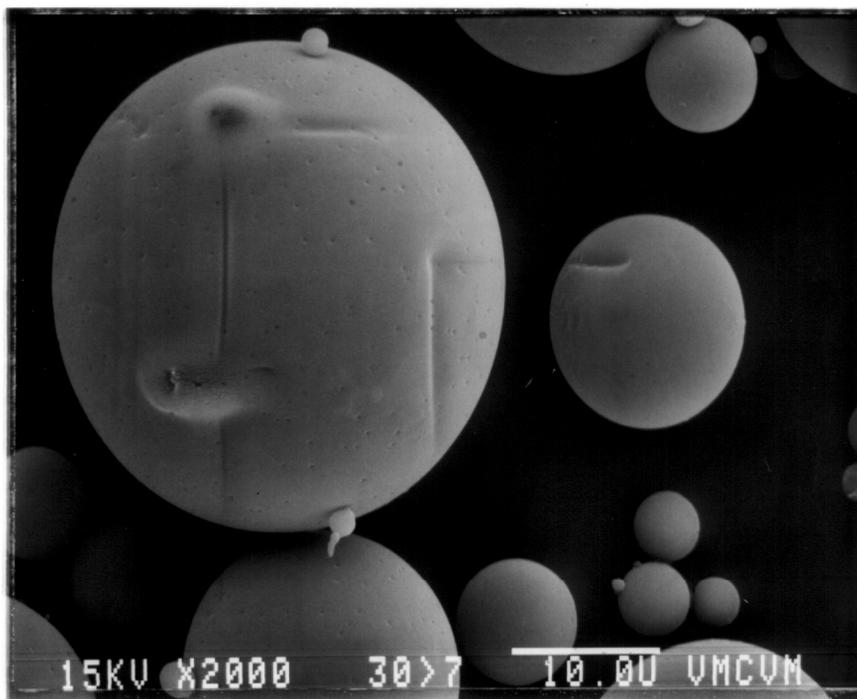


Figure 6:5 Scanning electron micrograph of poly (DL-PLG) microspheres encapsulating BSA-EG conjugate at 15 KV and 2000 X magnification showing formation of fissures and buckling of microsphere surface prior to deterioration.

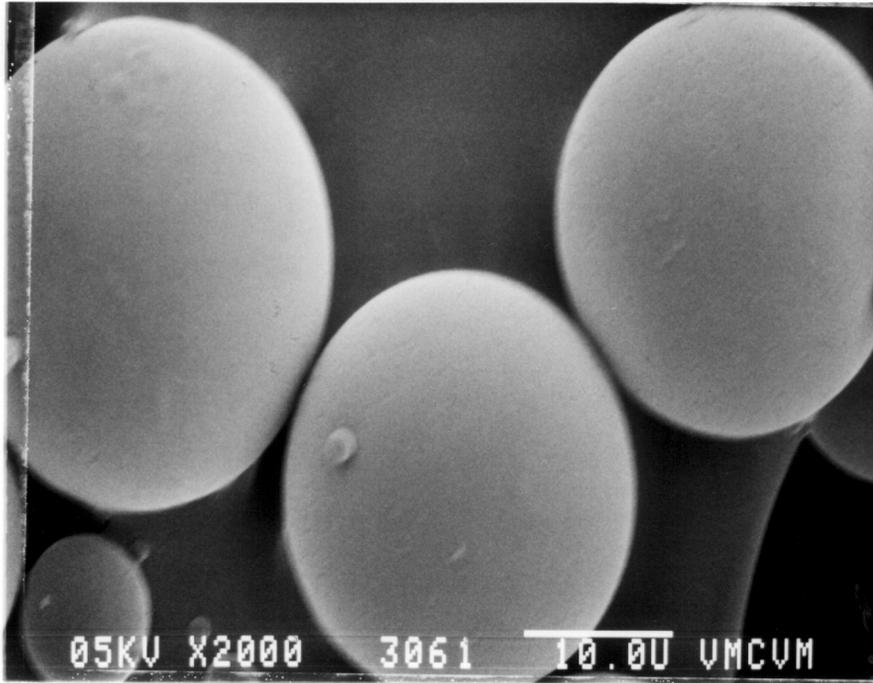


Figure 6:6 Scanning electron micrograph of poly (DL-PLG) microspheres encapsulating BSA-EG conjugate at 5 KV and 2000 X magnification showing the decrease in surface detail.

Table 6:1. Anti-Ergotamine IgG Titers of Mice Vaccinated Subcutaneously with Microencapsulated and Free BSA-Ergotamine Conjugate

Anti-Ergotamine IgG Titer ^a			
Days post-vaccination	Microsphere group ^b	Control group ^c	± SEM
14	4.0	4.0	0.25
28	2.3	2.3	0.25
55	5	5	0.5
75	1.75	2.25	0.6
105	3.75	3.25	0.9

^a Values represent group mean expressed as Log₂ titers where Log₂ = 1 is equivalent to 1:64 titer.

^b Mice revaccinated on d 14 with 10 mg of microspheres with encapsulated BSA-EG conjugate soaked overnight in 100 µg BSA-EG conjugate in FIA.

^c Mice revaccinated on d 14 with 100 µg BSA-EG conjugate in FIA.

^{bc} Each treatment group n = 4.

No differences (P > 0.05) in anti-EG titers were observed between treatment groups.

DISCUSSION

The smooth and spherical appearance of microsphere-encapsulated BSA-EG conjugate indicated that the microspheres were of high quality. Some microspheres contained very small pores on the surface. High quality microspheres with smooth, spherical surfaces exhibit continuous release profiles. Poor quality microspheres (i.e. irregular in shape with porous surfaces) tend to release encapsulated materials rapidly (Beck *et al.*, 1985). The smooth and spherical appearance, small size, and presence of pores in some microspheres with encapsulated BSA-EG conjugate indicated that degradation of microspheres would occur in a biphasic nature. In biphasic degradation of microsphere encapsulated vaccines, the first release phase is the burst effect. The burst effect is the early release of encapsulated material through pores present in the microsphere walls, and of material adsorbed onto the external surface of microspheres. The second phase of release is the actual release of encapsulated material as the microsphere walls are slowly biodegraded producing a continuous release of antigen.

The range of microsphere sizes in BSA-EG microencapsulated conjugate was from 7.5 microns to 45 microns. The average microsphere size was 22.7 microns. Microsphere size is important in the induction of an effective immune response. Microspheres less than 9 microns are optimal for uptake and processing by APCs, such as macrophages, when given subcutaneously (Eldridge *et al.*, 1991), intranasally

(Almeida *et al.*, 1993), and orally (Lin *et al.*, 1991). This effectively loads relatively large amounts of antigen into the phagocytic accessory cells (Eldridge *et al.*, 1991). Macrophages are the predominant APCs in the induction of an effective immune response to microencapsulated vaccines (Alving, 1991). Sizes of microspheres with microencapsulated BSA-EG conjugate were as small as 7.5 microns, thus some microspheres would be phagocytized by macrophages to induce an effective immune response against EG.

Primary immune responses of the two treatment groups were the same, since both groups were initially vaccinated with the same vaccine. However, revaccination with microsphere-encapsulated BSA-EG conjugate given subcutaneously did not increase the magnitude or duration of secondary immune responses to EG.

Alterations in experimental methods may have prolonged duration of secondary humoral immune responses against EG in mice vaccinated with microspheres. Administration of more microencapsulated BSA-EG conjugate may have prolonged titers. The amount of BSA-EG microencapsulated conjugate may have been insufficient to prolong titers, since the 10 mg of microspheres given to mice contained only 32 μg of protein. The amount of BSA-EG conjugate adsorbed onto the microsphere surfaces is unknown, but may have slightly increased the conjugate dose given to each mouse. Additionally, soaking microspheres overnight in conjugate plus adjuvant prior to administration could have altered the structure of microspheres that allowed for faster release kinetics.

Additionally, microspheres formulated with an increased ratio of lactide to glycolide may prolong release of EG and also increase microsphere size. Microspheres formulated with a high lactide to glycolide ratio and low vaccine loading do not exhibit a burst effect and have a slow, continuous release of antigen. These microspheres are best for subcutaneous administration. The higher the ratio of lactide to glycolide, the longer the duration of release (Beck *et al.*, 1985). Also, microspheres larger than 100 microns take longer to degrade and thus may provide a prolonged release of antigen.

Microspheres prepared with a low lactide to glycolide ratio and high vaccine loading exhibit fast vaccine release rates. These microspheres are best for oral and intranasal administration. Perhaps, size and formulation of BSA-EG microencapsulated microspheres in this study were best for oral or intranasal vaccination.

In conclusion, BSA-EG conjugate was encapsulated into poly(DL-PLG) microspheres (ratio 50:50, lactide:glycolide) at a concentration of 32 µg/10 mg of microspheres. The surface appearance was smooth and spherical with some microspheres having small pores on the surface. Microspheres encapsulating BSA-EG conjugate ranged from 7.5 to 45 microns in size with an average size of 22.7 microns in diameter. Secondary humoral immune responses against EG were not prolonged in mice vaccinated with the microspheres. Increasing the amount of BSA-EG microencapsulated conjugate given or increasing the ratio of lactide to glycolide in the microsphere preparation may have prolonged titers against EG in mice.

Chapter 7: Immunization of Mice against Fescue Toxicosis

INTRODUCTION

Immunization for the protection of animals against plant or fungal toxins is rarely reported in the literature. Previous parenteral vaccines that induce specific IgG antibodies against plant or fungal toxins resulted in various degrees of animal protection. Degree of protection against genistein (Cox, 1985), lantana toxin (Stewart *et al.*, 1988), and lupinosis (Payne *et al.*, 1993) is related to levels of specific serum IgG titers. Jonas and Erasmunson (1979) reported that mice vaccinated against the toxic effects of sporidesmin are protected. However, protection in mice against sporidesmin is related to the carrier and not to serum antibody levels (IgG and IgM).

A murine monoclonal antibody specific to the lysergic moiety of ergot alkaloids was infused intravascularly by jugular venipuncture into cattle grazing EI fescue (Hill *et al.*, 1994). Serum prolactin concentrations in the passively immunized cattle increased by 7 ng/mL immediately post-infusion. The increase in prolactin concentrations suggests that ergot alkaloid toxins in the blood have a higher affinity for the specific antibody than for the dopamine D2 receptor, which further supports the feasibility of vaccination as a potential solution to fescue toxicosis. Cattle actively immunized with a lysergol-protein conjugate develop short-lived antibody titers against the lysergic moiety of ergot alkaloids (Hill *et al.*, 1994). As discussed previously, monoclonal and polyclonal IgG

antibodies against EG and EV have been induced *in vivo*. However, the ability of these antibodies specific for EV or EG to confer a protective effect *in vivo* against fescue toxicosis in the murine model has not been investigated.

Parenteral vaccines against plant or fungal toxins are not always protective and in some cases exacerbate toxin-induced toxicosis. For instance, vaccination against zearalenone (MacDonald *et al.*, 1990; Smith *et al.*, 1992), and senecionine (Culvenor, 1978) worsen clinical signs of toxicosis instead of conferring protection to animals. Ewes vaccinated against sporidesmin develop worse clinical signs than nonimmunized ewes, despite the protection demonstrated in mice immunized against sporidesmin (Fairclough *et al.*, 1984).

In our research, both parenteral and oral routes of vaccination were explored in the protection against fescue toxicosis. Parenteral administration is the traditional route of vaccination used in most immunization regimes. Parenteral vaccination induces systemic IgM and IgG isotypes, but rarely serum IgA or secretory IgA (sIgA) isotypes. Parenteral vaccination usually does not stimulate sIgA immune response at mucosal surfaces because of the compartmentalization of systemic and secretory immune systems. Plant toxins are absorbed from the gastrointestinal tract into systemic circulation, where interaction with target sites/receptors may occur. If a specific IgG antibody response is insufficient to neutralize all absorbed toxins or if the plant toxins have a high affinity for nonantibody, toxin receptors, eventually enough toxins may escape the IgG immune response to produce a toxicosis.

Oral vaccination is increasingly utilized in immunization protocols. Oral vaccines are currently used in humans to protect against polio and cholera. Oral vaccines are being investigated for protection against tetanus, *Salmonella typhi*, and *Streptococcus mutans* in humans, and *Pasteurella haemolytica* and *Brucella abortus* in cattle.

Oral vaccination can produce high levels of mucosal antigen-specific sIgA. Neutralization of EV at the site of absorption (i.e. mucosal surfaces) with specific antibodies induced by oral vaccination with CTB-EG conjugate may be beneficial. The primary function of the mucosal immune system is to prevent the attachment and entry of microbial and food antigens. The mucosal surface represents the largest area of an animal in contact with the environment and is the major route of entry for pathogens. Lymphoid tissues associated with mucosal membranes contain more immunocytes, including B and T lymphocytes and plasma cells, than any other tissue in the body. Moreover, intestines are the richest lymphoid tissue present in the body (Mestecky, 1987).

The major immunoglobulin class found in exocrine secretions is sIgA, since it functions in the host defense of mucosal surfaces. Daily output of sIgA (50 to 100 mg/kg body weight per day) exceeds all other immunoglobulins combined (Holmgren *et al.*, 1992). Specific sIgA in secretions can protect in the absence of demonstrable serum antibodies, and often protection is correlated best with local sIgA secretion rather than circulating antibody. The IgA responses originating at one mucosal site disseminate to other mucosal associated lymphoid tissues by migration of sensitized B and T cells,

hence the basis for oral and intranasal immunization strategies. Parenteral immunization rarely induces serum or sIgA isotypes. However, parenteral immunization with an antigen, to which an individual was previously immunized by the oral or intranasal routes, will induce sIgA response at mucosal surfaces (Mestecky, 1987).

The purpose of this study was to generate a systemic and mucosal immune response against EG and to evaluate the protection afforded in mice against fescue toxicosis. The mouse was the animal model used to assess the protective effect of vaccination against fescue toxicosis. Mice fed EI fescue seed exhibit similar clinical responses as observed in cattle such as decreased feed intake, weight gains, serum prolactin, milk production, conception rates and litter sizes (Zavos *et al.*, 1987; Zavos *et al.*, 1988). Therefore, mice may serve as an animal model for fescue toxicosis in cattle. Direct immunization of mucosal surfaces by oral vaccination to protect against mycotoxicosis in animals has not previously been investigated. Passive immunization of mice with monoclonal anti-EV antibodies of the IgG isotype will test the importance of serum IgG in the protection against fescue toxicosis. Weight gains, concentrations of serum ALP, cholesterol and prolactin were the indices used to evaluate the ability of anti-EG antibodies to confer protection.

MATERIALS AND METHODS

Affinity Column Purification of Anti-Ergovaline Monoclonal Antibodies for Passive Immunization of Mice

A hybridoma producing anti-EV IgG1 antibodies was obtained from Dr. Kelley at Auburn University, AL. The hybridoma produces 26 to 40 μg antibodies/mL of culture supernatant (mean 34 $\mu\text{g}/\text{mL}$) with antibody specificity directed against the peptide part of the ergot peptide alkaloid structure and cross reactivity with EG, ergonine, and ergosine (Kelley and Shelby, 1990). This is important since cross reaction with endogenous substances containing an ergoline ring may be undesirable.

The cryopreserved hybridoma was thawed and propagated in complete RPMI 1640 containing supernatant from peritoneal feeder macrophages. Cells were grown in tissue culture flasks and incubated at 37 °C with 5% CO₂. Supernatant was collected from the hybridoma periodically and frozen at -20 °C.

A 5 mL affinity column was used to isolate the monoclonal antibodies from the cell medium. The column used was anti-mouse IgG bound to agarose (Sigma Immunochemicals) with a binding capacity of 0.4 mg IgG/mL of resin.

For purification, 450 mL of hybridoma supernatant was thawed. Fifty mL aliquots of supernatant were slowly added to the affinity column followed by 50 mL of

PBS to wash the column. The wash fraction then was discarded. The monoclonal antibodies were stripped off the column with 20 mL of 0.2 M glycine buffer solution (pH 2.3). The column was washed with 20 mL PBS. The wash fraction was combined with the glycine fraction and pH was adjusted to 7.0 with NaOH. The filtrate was dialysed (12,000-14,000 KDa) against distilled water for 24 h at 4 °C and lyophilized. The IgG concentration per mL of supernatant was determined by BCA protein analysis (Appendix VI). Activity of purified monoclonal antibodies was determined by ELISA.

Diet Preparation

An identical genotype of EI and EF 'Kentucky 31' tall fescue seed (Lot# L68-2-RS-3) was obtained from International Seed Inc. and stored at -20 °C. Levels of EV in the seed were determined by HPLC analysis as previously described (Hill *et al.*, 1993) (Appendix II). Certified ground rodent chow was mixed with equal parts of ground EI and EF fescue seed by weight. The seed:chow diets were analyzed for nutrient content. Dietary intake was limited to 5 to 6 g per mouse daily. Water was provided ad libitum.

Immunization

The Mannich reaction was used to conjugate BSA to EG (Appendix III) and CTB to EG (Appendix IV). Conjugation was confirmed by TLC analysis (Appendix V) and protein content determined by BCA analysis (Appendix VI).

Fifty Balb/c male mice (22.02 ± 0.15 g; average BW \pm SEM), 6 weeks of age, were blocked by weight and randomly allocated from outcome groups into 5 groups of 10 mice each. Ten mice were injected IP with 250 μ g BSA-EG conjugate in FCA and boosted 14 days later with 100 μ g BSA-EG conjugate in FIA. Anti-EG IgG titers were determined on days 14 and 34 post vaccination by ELISA. One day before treatment diets, ten mice were injected IP with 200 μ g of monoclonal IgG antibodies specific for EV that were affinity purified. After 1 wk on treatment diets, the same mice were injected IP with an additional 500 μ g of monoclonal IgG antibodies specific for EV. Another ten mice were orally vaccinated twice with 15 μ g CTB-EG conjugate and 5 μ g CT at 10 day intervals. All vaccinations and revaccinations with protein-EG conjugates occurred before mice were exposed to fescue diets.

Treatment groups (Table 7:0) were as follows: 1) group passively immunized and fed EI fescue seed diet (MAB group); 2) group parenterally immunized with BSA-EG conjugate and fed EI fescue seed diet (BSA-EG group); 3) group orally immunized with CTB-EG conjugate and fed EI fescue seed diet (CTB-EG group); 4) group

nonimmunized and fed EI fescue seed diet (EI group); and 5) group nonimmunized and fed EF fescue seed diet (EF group). Mice were individually housed in shoebox cages of clear polystyrene and exposure to a 14:10 h light:dark cycle. Ambient temperature during the study was 25° C. Ambient temperature was 16° C for 5 days prior to start of the study. Mice were acclimatized to ground rodent chow for 1 week. Titers were determined at the end of the acclimation period prior to the start of the study period. After 13 days on treatment, mice were anesthetized with halothane and blood was collected from the retro-orbital sinus. Sera obtained were analyzed for prolactin, ALP, cholesterol concentrations and anti-EG IgG antibodies (Appendix VII). Serum ALP and cholesterol concentrations were determined by the Clinical Pathology Laboratory at the Virginia-Maryland Regional College of Veterinary Medicine. Feces were collected from all groups prior to the start of the study and at the completion of the study. Fecal supernatants (DeVos and Dick, 1991) were assayed for anti-EG sIgA and IgG by ELISA (Appendix VIII). Mice were weighed on day 0 and day 12 of the trial period.

Determination of Mouse Prolactin by RIA

Prolactin was measured by RIA. Reagents were purchased from R.F. Parlow. Mouse prolactin standard (25 ng per mL in PBS with 1% BSA) was used to construct a standard curve. The standard curve ranged from 0.25 ng to 5 ng prolactin/tube and was

constructed by adding different amounts of mouse prolactin standard to borosilicate tubes containing 1% BSA in PBS for a total volume of 500 μL . Mouse prolactin was iodinated with I^{125} for use as a tracer by the chloramine T reaction (Sinha *et al.*, 1972). Then 40 μL of serum was added to borosilicate tubes containing 460 μL of 1% BSA in PBS. Also 100 μL of rabbit anti-mouse prolactin antibody and 100 μL of tracer (mouse prolactin iodinated with iodine 125) were added to each tube. After incubating for 24 h at 25 $^{\circ}\text{C}$, 100 μL of sheep anti-rabbit prolactin antibody was added to each tube. Tubes were incubated for 3 d at 4 $^{\circ}\text{C}$, after which, 1 mL of double distilled PBS was added per tube. Tubes were centrifuged for 30 min at 1500 x g. The supernatant was decanted and rims of tubes were rinsed with water and the rims dried. The pellet in each tube was then counted for radioactivity of I^{125} . Duplicate tubes of serum samples and standards were assayed.

STATISTICAL ANALYSIS

Differences in weight gain, ALP, cholesterol, and serum prolactin concentrations among the treatment groups were determined to be significant at probability values < 0.05 by analysis of variance. Bonferroni test on pairwise comparisons between the means was used to control type I errors. The following pairwise comparisons were made: parenterally vaccinated group versus EI group, EI versus EF group, orally

vaccinated group versus EF group, EI group versus MAB group, parenterally vaccinated group versus EF group, MAB versus EF group, orally vaccinated group versus EF group, and parenterally vaccinated group versus orally vaccinated group. Spearman rank correlation was used for correlation analysis (SAS®, SAS Institute, Cary, NC).

RESULTS

The anti-EG IgG titers (Log_2 titers =8) were elevated in the BSA-EG immunized mice throughout the feeding trial. The anti-EV titers (Log_2 titers = 3.1) of the passively immunized mice were lower than the anti-EG IgG titers of mice actively immunized with BSA-EG conjugate. The anti-EG IgG titers in mice orally vaccinated with CTB-EG conjugate peaked (Log_2 titers =1) 14 d after the 10 d booster and declined by the start of the diet trial. The sIgA concentrations in the feces of the CTB-EG orally vaccinated group peaked 2 wk before the dietary trial and decreased to background levels by the d 12 of the study. However, sIgA concentrations in the feces of the BSA-EG vaccinated group were increased throughout the 13 day trial period.

Mortality occurred in two treatment groups that reduced the number of mice to a total of 46 after 13 days on the feeding trial. One mouse from the CTB-EG group died after oral vaccination, but before the feeding trial. One mouse from the EF group and two mice from the CTB-EG group died during the feeding trial. Necropsy of a mouse

from the CTB-EG group by a pathologist at VMRCVM showed gross gastrointestinal hemorrhage and normal histological findings of major organs. The intestines were normal, but the lumen contained only large, gram positive bacilli and no gram negative bacteria.

The EV content of the dietary treatments was 1500 ppb for the EI diet and none detected in the EF diet. The respective diets were similar upon nutritional analysis (Table 7:1). Although serum prolactin levels (Table 7:2) were not different ($P > 0.05$) among groups, prolactin levels were positively correlated ($P < 0.05$, $\rho = 0.31$) with titers. Concentrations of ALP (Table 7:2) tended to be decreased in the unvaccinated EI group as compared to the EF group. However, ALP concentrations were decreased ($P < 0.05$) in the actively and passively immunized groups consuming the EI diet versus the EF group. Also, ALP concentrations in the parenterally immunized group were decreased ($P < 0.05$) compared to the unvaccinated EI group. The ALP concentrations of the passively immunized group tended to be decreased when compared with the unvaccinated EI group. The ALP levels were negatively correlated ($P < 0.05$, $\rho = -0.60$) with titers. Cholesterol concentrations (Table 7:2) tended to be decreased in the EI, oral, parenteral and passively immunized groups as compared to the EF group. Cholesterol concentrations tended to be negatively correlated to titers ($P > 0.15$, $\rho = -0.22$). Weight gain (Figure 7:1) was increased ($P < 0.05$) in the parenterally immunized group versus the unvaccinated EI group. Passive immunization and oral

immunization tended to increase weight gain as compared to the EI group. Weight gains were positively correlated ($P < 0.05$, $\rho = 0.37$) to titers.

Table 7:0. Treatment Groups of Mice in Fescue Dietary Trial

Treatment Group ^a	Immunization Route	Fescue seed: chowdiet
BSA-EG conjugate ^b	IP	EI
CTB-EG conjugate	PO	EI
EI	-	EI
MAB ^c	IP	EI
EF	-	EF

^a Each treatment group n = 10; except CTB-EG n = 7; and EF n = 9.

^b For BSA-EG group, anti-EG IgG titers (Log₂ titers = 8).

^c For MAB group, anti-EV titers (Log₂ titers = 3.1).

Table 7:1. Nutrient and Mineral Analysis of EI and EF Treatment Diets

Diet analysis ^a	EI seed : chow diet ^b	EF seed : chow diet ^c
Dry matter, %	89.4	89.4
Crude protein, %	21.2	21.2
Acid detergent fiber, %	11.2	11.3
Neutral detergent fiber, %	28.8	27.3
TDN, %	76	76
Net energy (Mcal/lb.)	.79	.79
Calcium, %	.64	.72
Phosphorus, %	.59	.62
Magnesium, %	.22	.23
Potassium, %	.73	.77
Sodium, %	.18	.17
Iron, ppm	199	221
Zinc, ppm	80	78
Copper, ppm	10	10
Manganese, ppm	68	71
Molybdenum, ppm	1.6	2.3

^a Dry matter basis.

^b EI seed:chow diet contained 1500 ppb EV by HPLC analysis.

^c EF seed:chow diet contained no detectable EV to the level of 50 ppb.

^{bc} Treatment diets consisted of 50: 50 rodent chow and fescue seed.

Table 7:2. Effects of Vaccination on Serum ALP, Prolactin and Cholesterol Concentrations in Mice Fed EI versus EF Fescue Seed

Treatment Group ^a	ALP (U/L)	Cholesterol (mg/dL)	Prolactin (ng/mL)
BSA-EG I.P. ^b	86.0 ± 2.9	90.6 ± 4.1	8.5 ± 0.9
CTB-EG P.O.	106.7 ± 6.7	95.0 ± 3.7	7.4 ± 1.3
EI	104.4 ± 3.0	91.5 ± 4.3	6.5 ± 1.1
MAB ^c	96.3 ± 2.4	89.9 ± 3.2	8.2 ± 1.3
EF	114.9 ± 3.5	102.6 ± 3.2	7.3 ± 1.8

^aEach treatment group n = 10; except CTB-EG n = 7; and EF n = 9.

Values represent group mean ± SEM.

^b For BSA-EG group, anti-EG IgG titers (Log₂ titers = 8).

^c For MAB group, anti-EV titers (Log₂ titers = 3.1).

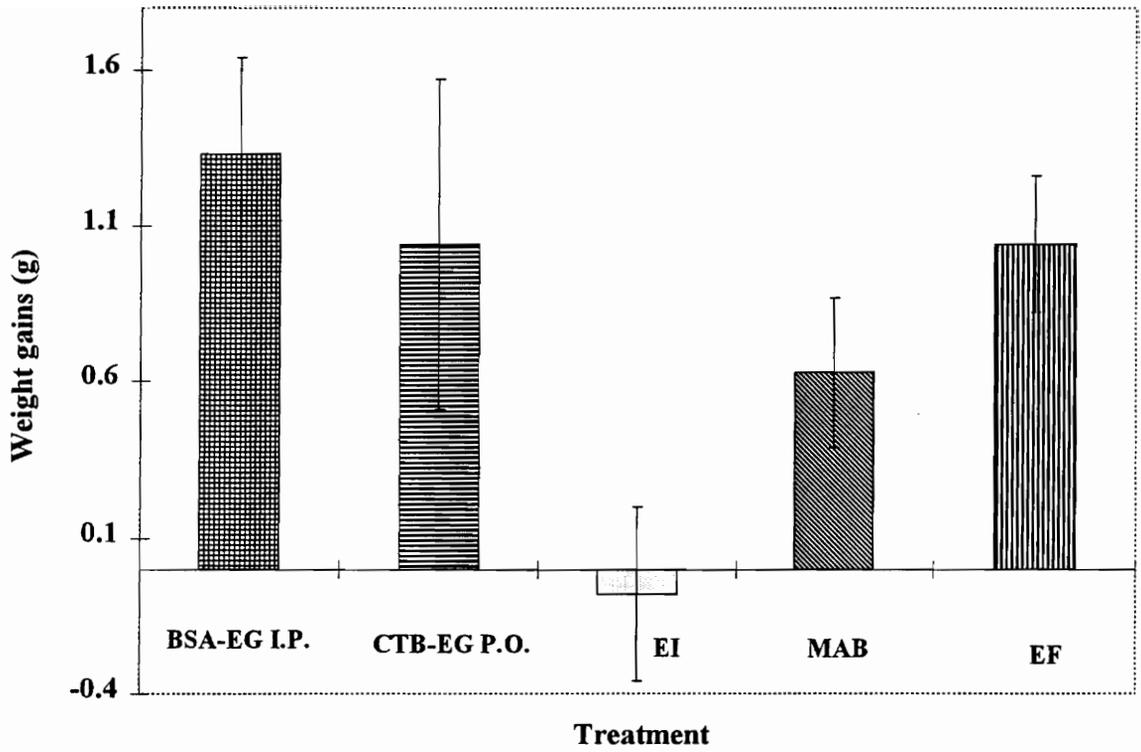


Figure 7:1. Average weight gains in mice during 13 day immunization study. n = 10 for BSA-EG IP, EI, and MAB groups; n = 7 for CTB-EG PO group; and n = 9 for EF group.

DISCUSSION

Immunized mice tended to have increased concentrations of prolactin, although prolactin concentrations were not different among groups. Hypoprolactemia in cattle grazing EI tall fescue is a fairly consistent finding. Mice of this age are resistant to bromocryptine-induced hypoprolactinemia (McMurray *et al.*, 1991). Perhaps, mice of this age were not the best model to demonstrate the effects of ergot alkaloids on prolactin concentrations in our study.

Active immunization increased systemic IgG antibodies against EG and increased levels of mucosal sIgA and IgG antibodies against EG. Mucosal sIgA antibodies against EG induced by IP administration of BSA-EG conjugate were increased during the course of the trial. Secretory IgA induction by IP immunization of BSA-EG was greater than that induced by oral administration of CTB-EG conjugate. Parenteral immunization usually does not stimulate sIgA immune response at mucosal surfaces because of the compartmentalization of systemic and secretory immune systems. Induction of sIgA by IP immunization possibly is a result of systemic absorption of the conjugate followed by biliary excretion into the intestines where a local mucosal immune response was elicited. Additionally, direct stimulation of mesenteric associated lymph nodes by IP administration of the conjugate may have induced specific committed B-cells against EG. Migration of sensitized B-cells to Peyer's patches may have resulted in sIgA production. The contribution of sIgA to the protection observed in weight gains in the parenterally

immunized group or the exacerbation of clinical indices cannot be determined from this study. Weight gain was correlated with titers. Perhaps protection in weight gain was because of the large molecular size of IgG-alkaloid immune complexes, which prevented EV from reaching receptors located in the central nervous system.

Passive immunization with specific anti-EV IgG antibodies tended to protect weight gains. Perhaps, systemic IgG anti-EV titers were low and may not have been sufficient to neutralize all the dietary EV absorbed.

The sIgA levels induced by oral immunization also tended to protect weight gains. Vaccination a third time with an oral dose of CTB-EG conjugate prior to the start of the feeding trial may have been beneficial, especially since mucosal anti-EG antibodies had already peaked. A higher oral dose of the CTB-EG conjugate may have resulted in higher specific sIgA production and protection. Vaccination of mucosal surfaces (oral or intranasal) is infrequently used in prevention of toxicosis and in the case of fescue toxicosis, further research is needed.

The ALP concentrations decreased in actively immunized and passively immunized mice. The ALP concentrations were negatively correlated with serum IgG antibodies against EG. Cholesterol concentrations tended to be lower in the EI, oral, parenteral, and passively immunized groups. Although lower serum ALP and cholesterol values may not have overt clinical significance, lower ALP and cholesterol values in immunized mice fed EI fescue diets demonstrated exacerbation of indices induced by fescue toxicosis. The decreases did not appear to be the result of an antigen depot effect

caused by the BSA-EG conjugate vaccine, because the effect of EV monoclonal antibodies was similar. Decreases in ALP and cholesterol concentrations were likely an effect of the presence of specific IgG antibodies binding antigen. Although the passively immunized group had a relatively low specific serum IgG titer, even a low serum IgG titer seemed to suppress ALP and cholesterol concentrations. The suppression of ALP and cholesterol appears to require systemic antibodies, since the effect was not observed in the orally immunized group.

As suggested previously in the case of sporidesmin, binding of anti-EG or anti-EV antibodies to EV may increase retention of EV in the body by altering elimination kinetics. Increasing elimination kinetics may allow higher concentrations of the alkaloid to accumulate in target organs. Ergotamine and systemic EG-immune complexes may inhibit ALP production by the liver. Serum ALP is usually of hepatic origin. Ergotamine has a high affinity for the liver after intravenous administration (Berde and Schild, 1978b). Production of specific antibodies to form alkaloid-antibody complexes may further increase alkaloid concentrations in the liver, because IgG and IgA immune complexes are cleared from circulation mainly by the liver and spleen. Ergotamine may decrease hepatic ALP levels by inhibition of cAMP.

In conclusion, EG conjugated to a protein carrier induced specific antibodies that protected weight gains in the murine model of fescue toxicosis. The tendency for decreased serum ALP and cholesterol concentrations, and decreased weight gains in the EI group versus the EF treatment group further validated the murine model in fescue

toxicosis. Titers required to provide protection in weight gains negatively influenced serum ALP concentrations, the implications of which are unknown. Although prolactin concentrations were not different between vaccinated and unvaccinated treatment groups, prolactin concentrations were positively correlated with titers. Passive immunization of mice with anti-EV antibodies did not increase prolactin concentrations as previously demonstrated in cattle intravenously infused with murine antibodies against lysergol. Further research in cattle is indicated to determine if cattle can mount a high enough titer to be protective against fescue toxicosis and whether the positive effects of vaccination on weight gains will translate into economic savings for producers. The possibility that vaccination, although beneficial for weight gains, may negatively impact effects of fescue toxicosis should be considered in future studies.

Chapter 8: General Discussion and Conclusions

In exploring the feasibility of a vaccine directed against fescue toxicosis, the first step was to discern whether cattle could mount humoral immune responses when grazing EI tall fescue. This research demonstrated that cattle with fescue toxicosis could mount humoral immune responses, despite hypoprolactemia. The findings are in agreement with previous research in cattle, but not with fescue research in rodents. The difference between species may be a result of dietary deficiencies present in 50 % fescue seed diets in rodent studies. Rodents have decreased antibody production if nutritionally stressed and a diet of 50 % fescue seed and 50 % rodent chow may not meet long term nutritional requirements.

Fescue toxicosis affects the immune-neuroendocrine axis. This is evident since fescue toxicosis decreases prolactin concentrations, affects immune responses, and is an alpha 2 adrenergic and dopaminergic agonist. One central theory to describe the action of fescue toxicosis on these interrelationships probably will not suffice, because these interrelations are very complex.

Humoral responses may be increased either by ergot alkaloids or by the endophytic fungus. Ergot alkaloids may act through alpha 2 adrenergic receptors, G proteins, cAMP and ALP. Prostaglandins did not appear to influence lymphocyte

blastogenesis *in vitro*, although further research is needed in this area, because of the small number of observations in the study.

Carbohydrates within the endophytic fungus may stimulate cytokine production (IL-1 or INF- γ) to enhance immune response. Growth hormone concentrations are not suppressed as is prolactin in cattle with fescue toxicosis. Growth hormone is similar in structure to prolactin; however, it remains to be shown if GH can bind prolactin receptors on bovine lymphocytes to exert prolactin-like actions. The cytokine profile produced by lymphocytes stimulated *in vitro* from mice fed EI fescue seed diets resembled those secreted by both T_{H1} and T_{H2} cells. Fescue toxicosis may favor the differentiation of T_{H0} cells into T_{H2} cells, which would augment humoral immune response. Further research is needed in cattle, because LTA and cytokine results in mice were not correlated with serum prolactin. It is well documented that hypoprolactemia decreases selected immune functions. The elucidation of how fescue augments humoral immune responses may have important ramifications in the field of immunostimulants. Factors in EI fescue may act similar to levamisole by increasing INF- γ production when T-cells are impaired, as they would be in hypoprolactemia.

In development of a vaccine against fescue toxicosis, conjugation of EG to protein carriers was necessary to induce specific antibodies. Several protein carriers were evaluated in this research as potential carriers. The Mannich reaction was used to conjugate EG to Con A, CT, and CTB protein carriers. Both CTB and Con A-EG conjugates induced systemic IgG anti-EG antibodies that had similar kinetics in mice. The CTB-EG conjugate did not prolong antibody titers as compared to other carrier-EG

conjugates, although smaller doses were needed to elicit titers. However, repeated parenteral vaccination with CTB-EG conjugate may have induced tolerance to EG. The use of Con A conjugated to EG will not provide any advantages as a mitogenic stimulant of T-cells, because activity of Con A is decreased by the Mannich reaction.

Revaccination of mice with CT-EG conjugate without adjuvants induced higher titers against EG than either CTB-EG or Con-EG conjugates in adjuvants. Encapsulation of BSA-EG conjugate into poly (DL-PLG) microspheres with a 50:50 ratio of lactide to glycolide did not prolong systemic IgG titers in mice.

Oral vaccination with the CTB-EG conjugate induced both mucosal IgA and IgG isotypes, in addition to a systemic IgG response. Parenteral revaccination of the CTB-EG conjugate in the orally vaccinated mice elicited an anamnestic mucosal immune response. The effect of repeated oral vaccinations of CTB-EG conjugate on immune responses was not investigated in this research.

A major disadvantage of CT or CTB as carrier proteins for use in vaccines is cost. Extraction of CTB from the holotoxin is a time consuming procedure with low yields, which limits availability and increases cost of vaccine production. Recently, plasmids that encode production of a homogenous recombinant CTB have been developed. The characterization of the recombinant CTB shows that it is: 1) essentially identical to the native toxin; 2) immunologically indistinguishable from vaccine preparations of native or recombinant CTB; 3) produced at significantly higher yields (1 gram per liter); and 4) readily purified from the growth medium (Lebens *et al.*, 1993). This improved ability to

produce CTB without purification from the holotoxin and the increase in amounts produced promote vaccine production and cost effectiveness.

Parenteral and oral immunization with protein carrier-EG conjugates against fescue toxicosis were evaluated in mice. Parenteral immunization with BSA-EG conjugate induced anti-EG antibodies that protected against adverse effects on weight gain in the murine model of fescue toxicosis. This data supports the use of EG as a hapten in the development of a vaccine and validates the murine model in fescue toxicosis. Although vaccination protected against adverse effects on weight gains in mice, it exacerbated selected indices associated with fescue toxicosis. Titers required to protect against adverse effects on weight gains in mice negatively influenced serum ALP concentrations, the implications of which are unknown. Neither anti-EG or anti-EV antibodies increased serum prolactin concentrations in mice fed EI fescue diets as previously demonstrated in cattle. However, serum prolactin concentrations were positively correlated with titers.

Parenteral vaccination with CTB-EG conjugate in FIA induced IgG titers against EG in cattle, but magnitude and duration of humoral responses were not determined. However, previous research indicates that inducing titers with prolonged duration may be difficult. The present research in mice indicates that to achieve an anti-EG antibody response of adequate magnitude and duration will require either frequent vaccine administration or an enhanced vaccine delivery system, such as microspheres.

In the development of a vaccine for fescue toxicosis, the route of vaccine administration may be important. Parenteral vaccination in the protection of cattle

against fescue toxicosis may not be the route of choice. Therefore, novel vaccination protocols against fescue toxicosis may be developed based on the induction of mucosal immune responses. This research confirmed the ability to induce anamnestic mucosal immune responses in mice orally sensitized against EG with parenteral vaccination.

Novel immunization protocols developed against fescue toxicosis could involve primary mucosal (oral or intranasal) immunization to induce anti-EG IgA effector and memory cell populations followed by subcutaneous immunization. Subcutaneous administration of microsphere-encapsulated vaccine may prolong mucosal and systemic titers for months to decrease the frequency of vaccination. Additionally, systemic anti-EG IgG antibodies induced by parenteral administration could neutralize any EV systemically absorbed.

Unfortunately, oral vaccination to induce mucosal immune responses is more complicated in cattle because of the complex nature of their gastrointestinal tract. An antigen administered orally in cattle must be resistant to severe changes in pH, resistant to degradation by the microflora of the rumenoreticulum, and resistant to proteolytic enzymes present in the abomasum. The limitations of inducing a mucosal immune response by oral vaccination in cattle may be overcome by the use of microspheres or aided by intranasal vaccination. Microspheres as the vaccine vehicle for an oral vaccine against respiratory infection caused by *Pasteurella haemolytica* decreased morbidity and mortality in calves (Bowersock *et al.*, 1994). Although not investigated in this research, surface coating microspheres with a mucoadhesive agent for intranasal and oral administration would enhance microsphere delivery to mucosal surfaces by extending

mucosal contact time and by increasing microsphere concentrations at mucosal surfaces. Additionally, surface-coated microspheres of the appropriate size given orally would adhere to the intestinal mucosal to allow the M cells of GALT more time to uptake the microspheres (Lehr *et al.*, 1992).

An oral or intranasal vaccine against fescue toxicosis would be advantageous to cattle producers. Oral or intranasal vaccination would eliminate the possibility of vaccine reactions in meat and could decrease time, labor and stress on animals. An oral vaccine could be added to feed at necessary intervals to induce sIgA responses in mucosal surfaces of the gastrointestinal tract to neutralize EV prior to systemic absorption and afford protection. Repeated oral vaccination would also prolong antibody production that will be necessary in cattle exposed to EI fescue forage throughout the grazing season. Cattle could be strategically vaccinated during the summer months when rate of gains in growing animals are most often affected.

In any oral vaccination protocol, the induction of IgE antibodies to normal dietary antigens present in the intestinal lumen may result in hypersensitivity to food antigens. However, CTB as a carrier for EG may not induce isotype switching to IgE (Whitmore *et al.*, 1991). Therefore, the incidence of food hypersensitivities may not be augmented with use of CTB as a carrier in an oral vaccine.

In conclusion, further research in cattle is indicated to determine if cattle can mount sufficient titers against EG to be protective against fescue toxicosis and whether the protective effects of vaccination on weight gains will translate into economic savings for producers.

Immunization against fescue toxicosis needs to be evaluated for its ability to reverse the adverse effects on CMI responses, so cattle losses incurred because of health problems can be minimized. Also, effects of anti-EG antibody production in cattle on CMI, expression of MHC II antigens, and free radical production need to be investigated to ensure these indices are not further compromised, as were ALP and cholesterol in immunized mice.

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Appendices

Appendix I

Preparation of Con A-EG Conjugates

Materials

- 20 mg Concanavalin A (Con A) type IV (Sigma)
- 2 mg ergotamine tartrate (Sigma)
- distilled water
- 3 M sodium acetate
- 37% formaldehyde
- methanol

Method

1. Dissolve Con A in 300 μL of distilled water and 600 μL of 3 M sodium acetate.
2. Add 1200 μL of 37% formaldehyde dropwise and stir for 5 minutes.
3. Dissolve ergotamine tartrate into 300 μL of methanol and 100 μL of water and add to protein solution. Stir mixture of Con A and ergotamine for 5.5 hours at room temperature in the dark.
4. Dilute reaction mixture to a final volume of 6 mL with distilled water and dialyze against distilled water for 4 days with frequent water changes.

Appendix II

Extraction of Fescue Plant Material and HPLC Analysis for Ergovaline Concentrations

Reference Hill, N. S., Rottinghaus, G. E., Agee, C. S., and Schultz, L. M., Simplified sample preparation for HPLC analysis of ergovaline in tall fescue, *Crop Sci.*, 33, 331, 1993.

Materials

- 1 g freeze dried fescue seed or grass
- 40 mL chloroform/ 0.01 M NaOH (9:1 v/v)
- ergotamine (4 µg/mL) (Sigma)
- methanol
- silica gel HL TLC plates (Analtech)
- Whatman 1PS filter paper
- 13 mm fritted disk
- 1 g of sodium sulfate
- 5 mL of acetone/chloroform (75:25 v/v).
- 2 mL chloroform
- 1 mL of ethyl ether
- 0.25 micron filter
- fluorescence spectrophotometer (excitation wavelength: 250 nm; emission wavelength: 420 nm)
- 3-µm, 7.5 cm x 4.2 mm C18 column

- 34% acetonitrile in a 200 mg/L aqueous solution of ammonium carbonate (degassed and filtered)

Method

1. Extract 1 g of ground fescue seed or freeze dried grass with 40 mL of chloroform/0.01 M NaOH (9:1 v/v) for 12 hours at 4 C. Also add 200 μ L of ergotamine (4 μ g/mL) in methanol as an internal standard.
2. Filter supernatant through a Whatman 1PS filter paper to remove residual water.
3. Construct a solid-phase clean-up column with TLC plates of silica gel HL (Analtech) containing an organic binder. Scrape silica gel from TLC plates and grind with a mortar and pestle. Prepare silica gel HL solid-phase chromatography columns by placing a 13 mm fritted disk in the bottom of a 6 mL disposable syringe barrel followed by 0.5 g of silica gel HL. After the silica gel layer, place a 13 mm disc followed by 1 g of sodium sulfate and another 13 mm disc to complete the column.
4. Prewash the solid-phase column with 2 mL of chloroform and add 20 mL of fescue filtrate to the column under vacuum.
5. Elute pigments from the column with 5 mL of acetone/chloroform (75:25 v/v). Remove residual acetone/chloroform with 1 mL of ethyl ether. Dry the column under vacuum.
6. Attach a 0.25 micron filter to the syringe bottom of the solid-phase column, then add 4 mL of methanol. Elute the methanol by slowly advancing the plunger manufactured for the syringe. If analyzing fescue seed, inject 20 μ L of filtered methanol extract into HPLC for analysis.
7. If analyzing fescue grass, evaporate the eluted methanol to dryness at 45C with nitrogen gas and reconstitute with 1 mL of methanol for HPLC analysis.
8. Prepare ergovaline and ergotamine working standard solutions ranging from 50 to 1000 ng/mL in methanol and store at -20 C.

9. Inject 20 μL of sample or standard into injection port of a liquid chromatograph equipped with a fluorescence spectrophotometer (excitation wavelength: 250 nm ; emission wavelength: 420 nm) and a 7.5 cm x 4.2 mm C18 3- μm column.
10. Use a mobile phase of 34% acetonitrile in a 200 mg/L aqueous solution of ammonium carbonate at a flow rate of 1.5 mL/min.
11. Record fluorescence responses to ergovaline and ergotamine as peak height with a reporting integrator.

Appendix III

Preparation of BSA-EG and Ovalbumin-EG Conjugates

Reference Kelley, V. C. and Shelby, R. A., Production and characterization of monoclonal antibody to ergovaline, in Proc. Int. Symp. on *Acremonium*/Grass Interactions Quisenberry, S. S. and Joost, R. E., Eds.. Louisiana Agricultural Experiment Station, Baton Rouge, LA., 1990, 83.

Materials

- 20 mg bovine serum albumin (BSA) fraction V (Sigma)
- 20 mg ovalbumin (Sigma)
- 2 mg ergotamine tartrate (Sigma)
- distilled water
- 3 M sodium acetate
- 37% formaldehyde
- methanol

Method

1. Dissolve either BSA or ovalbumin in 100 μL of distilled water and 200 μL of 3 M sodium acetate.
2. Add 400 μL of 37% formaldehyde dropwise and stir for 5 minutes.

3. Dissolve ergotamine tartrate into 300 μL of methanol and 100 μL of distilled water and add to the protein solution. Stir mixture of protein and ergotamine for 5.5 hours at room temperature in the dark.
4. Dilute reaction mixture to a final volume of 6 mL with distilled water and dialyze against distilled water for 4 days with frequent water changes.

Appendix IV

Preparation of CT-EG and CTB-EG Conjugates

Materials

- 2 mg cholera toxin subunit B (CTB) (Sigma)
- 2 mg cholera toxin (CT) (Sigma)
- 1 mg ergotamine tartrate (Sigma)
- distilled water
- 3 M sodium acetate
- 37% formaldehyde
- methanol

Method

- 1) Dissolve protein in 100 μ L of distilled water and 200 μ L of 3 M sodium acetate.
- 2) Add 400 μ L of 37% formaldehyde dropwise and stir for 5 minutes.
- 3) Dissolve 1 mg of ergotamine tartrate into 250 μ L of methanol:water (1:1, v/v).
- 4) Add ergotamine solution to the protein solution and stir for 5.5 h at room temperature in the dark.
- 5) Dilute reaction mixture to a final volume of 2 mL with distilled water and dialyze against distilled water for 4 days with frequent water changes.

Appendix V

Thin Layer Chromatography of Protein-EG Conjugates

Materials

- 250 μm , 5 X 20 cm thin-layer chromatographic plate of silica gel (EM Science)
- chloroform/methanol (9:1 v/v)
- ergotamine tartrate standard (1 $\mu\text{g}/1 \mu\text{L}$)

Method

1. Spot 5 μL of protein-EG conjugate and ergotamine tartrate standard (1 $\mu\text{g}/1 \mu\text{L}$) on a thin-layer chromatographic plate.
2. After spot drying, develop the plate in chloroform/methanol (9:1 v/v).
3. Allow the plate to dry and observe under a long wave lamp.
4. The protein-EG conjugates appear as a fluorescent spot at the origin, whereas free ergotamine migrates. Unconjugated protein remains at the origin and does not fluoresce when observed under a long wave lamp.

Appendix VI

BCA™ Protein Assay

Materials

- BCA™ Protein Kit (Pierce)
- 2 mg/mL BSA protein standard (Pierce)
- distilled water
- working reagent (Pierce)

Method

- 1) Prepare a set of protein standards with BSA (2 mg/mL) ranging from 7.8 µg to 2000 µg per mL.
- 2) Add 100 µL of protein-EG conjugate, protein standards, and water reference to 2 mL working reagent.
- 3) Incubate tubes at 37 C for 30 min, then cool to room temperature.
- 4) Measure absorbance of standards, protein-EG conjugate, and water reference at 562 nm with a double beam spectrophotometer (Gilford).
- 5) Construct a standard curve by plotting absorbance versus protein concentration.
- 6) Determine the equation of the line by linear regression. Determine protein content from the derived equation of the standard curve.

Appendix VII

Determination of Anti-Ergotamine IgG Antibodies in Mouse Serum by Indirect ELISA

Materials

- 96 well high binding immunoplates (Nunc Maxisorp®)
- ovalbumin-EG conjugate (Appendix III)
- 0.1 M sodium carbonate-bicarbonate buffer (pH 9.6).
- phosphate buffered saline with 0.02% Tween-80
- 0.3% (w/v) gelatin in PBST
- goat anti-mouse IgG horse radish peroxidase conjugate
- o-phenylenediamine (Sigma) and hydrogen peroxide substrate
- 8 N H₂SO₄.

Method

1. Coat wells of high binding immunoplates with 50 µL of ovalbumin-EG conjugate at 5 µg per well diluted in 0.1 M sodium carbonate-bicarbonate buffer (pH 9.6) overnight at 4 C.
2. Wash plates five times with PBST (PBS + 0.02% Tween-80). Block wells for 60 min at 37 C with 100 µL of 0.3% (w/v) gelatin in PBST. Wash plates five times with PBST.
3. Add to each well serially diluted serum (50 µL) and incubate for 30 min at 37 C. Wash plate five times with PBST.

4. Add to each well 50 μL of goat anti-mouse IgG horse radish peroxidase conjugate (diluted 1/600 in 0.3% gelatin-PBST). Incubate plates for 30 min at 37 C and wash five times with PBST.
5. Add 100 μL o-phenylenediamine and H_2O_2 substrate (10 mg o-phenylenediamine, 100 μL H_2O_2 , and 100 mL distilled water) to each well. Incubate plate for 30 min at room temperature in the dark.
6. Stop reaction with 25 μL per well of 8 N H_2SO_4 . Measure absorbance at 490 nm with a multiscan reader (Titertek[®], Molecular Devices Corp.).
7. Determine the titer of each serum as the maximum dilution where the absorbance is greater than the mean plus 3-fold the standard deviation of triplicates of the same dilution of pooled prevaccination sera.

Appendix VIII

Determination of Anti-Ergotamine Coproantibodies by Indirect ELISA

Reference DeVos, Theo and Dick, T. A., A rapid method to determine the isotype and specificity of coproantibodies in mice infected with *Trichinella* or fed cholera toxin, *J. Immunol. Methods*, 141, 285, 1991.

Materials

- 96 well high binding immunoplates (Nunc Maxisorp®)
- BSA-EG conjugate (Appendix III)
- cholera toxin subunit B (Sigma)
- 0.1 M sodium carbonate-bicarbonate buffer (pH 9.6).
- PBS + 0.02% Tween-80
- 0.5 % (w/v) gelatin in PBST
- goat anti-mouse IgG horse radish (HRPO) peroxidase conjugate
- goat anti-mouse IgA HRPO conjugate
- o-phenylenediamine and H₂O₂ substrate (Sigma)
- 8 N H₂SO₄
- fecal supernatant

Method

1. Coat wells of high binding immunoplates with 50 μL of BSA-ergotamine conjugate (5 μg per well) or cholera toxin subunit B (0.5 μg per well) in 0.1 M sodium carbonate-bicarbonate buffer (pH 9.6) overnight at 4 C.
2. Wash plates five times with PBST (PBS + 0.02% Tween-80). Block wells for 3 h at 37 C with 200 μL of 0.5 % (w/v) gelatin in PBST. Wash plates five times with PBST.
3. Pipet 100 μL of fecal supernatant to each well and incubate for 2 h at 37 C. Wash plate five times with PBST.
4. Add to each well 100 μL of goat anti-mouse IgG or IgA HRPO conjugate (diluted 1/600 in 0.5 % gelatin-PBST). Incubate plates for 2 h at 37 C and wash five times with PBST.
5. Add 200 μL of o-phenylenediamine and H_2O_2 substrate (10 mg o-phenylenediamine, 100 μL H_2O_2 , and 100 mL distilled water) to each well. Incubate plate for 30 min at room temperature in the dark.
6. Stop reaction with 25 μL per well of 8 N H_2SO_4 . Measure absorbance at 490 nm. with a multiscan reader (Titertek[®], Molecular Devices Corp.). Determine mucosal sIgA and IgG by measuring the optical density (OD) at wavelength of 490 nm.

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EDUCATION

Doctoral Candidate in Veterinary Medical Sciences (1991 to 1995), Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, 24061. Completion date: October, 1995. GPA: 3.8/4.0.

Dissertation Thesis: Induction of Anti-Ergotamine Antibodies in Mice and steers and Assessment of Protection Against Fescue Toxicosis in Mice.

- Awards: Vaughn Scholarship, University Instructional Fee Scholarship, Pauline Willson-Gunn Scholarship (1994 and 1995). Received second-place award for presentation of abstract at the Virginia-Maryland Regional College of Veterinary Medicine 7th Annual Research Symposium.
- Professional Membership: American Veterinary Medical Association.
- U.S.D.A. Accreditation

Doctor of Veterinary Medicine, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061 (1987 to 1991).

Date degree received: May 1991. GPA: 3.2/4.0.

- Awards: Old Dominion Kennel Club Scholarship.
- Activities: Co-founder and Coordinator/Treasurer of the VMRCVM Hill's College Feeding Program, member of the Student Chapter of American Veterinary Medical Association.

Bachelor of Science in Biology, Christopher Newport College, Newport News, Virginia 23666. Date degree received: May 1984. GPA: 3.6/4.0 (Magna Cum Laude).

- Minor Concentration: Chemistry.
- Activities: Alpha Chi National Honor Society.

EDUCATION (cont.)

High School, Holmes High School, Covington, Kentucky 41015.

Date of diploma: June, 1980.

- Awards: Bausch and Lomb Honorary Science Award, Most Outstanding Science Student Award, Covington Education Association Scholarship, City Council P.T.A. Ralph Eckler Scholarship.
- Activities: Vice President of the Latin Club, Student Council Representative, National Honor Society.

WORK EXPERIENCE

Research Assistant/Doctoral Candidate, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Duckpond Dr. Blacksburg, Virginia 24061. Dates worked: August 1991 to present.

- Tests and procedures performed include ELISA's in various configurations, hemagglutination assays, determination of an environmental toxin's effect on lymphocyte blastogenesis and interleukin production, protein-hapten conjugations to prepare immunogens against an environmental toxin, alkaloid extraction of fescue grass and seed with HPLC analysis for ergovaline, thin-layer chromatography, hybridoma cell culture, production and affinity column purification of monoclonal antibodies, radioimmunoassay determination of prolactin, protein determinations, production of microsphere-encapsulated vaccine, and oral and parenteral immunization of mice and cattle, acute LD50 oral dosing study in mice.
- Toxicology Board Eligible upon completion of Ph.D.
- Teaching Responsibility: Taught Veterinary Toxicology to veterinary students (1992 to 1995).
- Major Professors

Dr. Dennis Blodgett, D.V.M., Ph.D. A.B.V.T., Associate Professor of Veterinary

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- Manuscripts Submitted or in Preparation

Rice, R., Schurig, G., Swecker, W., Eversole, D., Thatcher, C., Blodgett, D., Use of Endophyte-Infected Fescue Pasture and Avoidance of Herbicide Pollution; in *Proceedings of the International Symposium on Nutrient Management of Food Animals to Enhance the Environment*, Blacksburg, Virginia, CRC Press, Lewis Publishing, 1995 (in press).

Rice, R., Schurig, G., Blodgett, D., Swecker, W., Thatcher, C., Eversole, D.,
Immunization of Mice against Fescue Toxicosis.

Rice, R., Schurig, G., Blodgett, D., Swecker, W., Fontenot, J. P., Allen, V.
G., Akers, R. M., Humoral Immune Responses of Cattle Maintained on
Fescue Pastures.

- Abstracts Presented

Rice, R., Schurig, G., Swecker, W., Eversole, D., Thatcher, C., Blodgett, D.,
Humoral Immune Responses of Cattle Maintained on Fescue Pastures.
Virginia-Maryland Regional College of Veterinary Medicine 7th Annual
Research Symposium, Blacksburg, Virginia, May 15 and 16, 1995.

Rice, R., Schurig, G., Blodgett, D., Swecker, W., Fontenot, J. P., Allen, V.
G., Akers, R. M., Humoral Immune Responses of Cattle Maintained on
Fescue Pastures. In Proc. of Southern Extension and Research Activity
Information and Exchange Group 8, Nashville, TN, Nov. 14-15, 1995.

Rice, R., Schurig, G., Blodgett, D., Swecker, W., Thatcher, C., Eversole, D.,
Immunization of Mice against Fescue Toxicosis, In Proc. of Southern
Extension and Research Activity Information and Exchange Group 8,
Nashville, TN, Nov. 14-15, 1995.

- Paper Presented

Rice, R., Schurig, G., Swecker, W., Eversole, D., Thatcher, C., Blodgett, D.,
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Relief Veterinarian, North Main Small Animal Clinic, 1407 North Main St.,
Blacksburg, Virginia 24060. Dates worked: July, 1991 to January, 1995.

- Owner: Dr. Mark Dallman D.V.M., Ph.D. (703) 951-1002
- Performed all professional services including surgery in a small animal clinic on a relief basis.
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Cardiovascular Technologist, Norfolk General Hospital, 600 Gresham Dr.,
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Duties: Operated the Intra-aortic Balloon Pump, monitored ECG and systemic pressures of patients undergoing heart bypass surgery. Corrected acid/base chemistry and heparinized the heart-lung pump. Measured arterial blood gases, activated clotting time, and determined sodium, potassium, glucose, and hematocrit readings.

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