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I am submitting herewith a dissertation written by Terry Lynn Bowersock entitled "Induction of Pulmonary Immunity to Pasteurella haemolytica in Cattle by Stimulation of the Gut-Associated Lymphatic Tissue." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

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INDUCTION OF PULMONARY IMMUNITY TO *PASTEURELLA HAEMOLYTICA* IN CATTLE BY STIMULATION OF THE GUT-ASSOCIATED LYMPHATIC TISSUE

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Terry Lynn Bowersock

June 1988

ACKNOWLEDGEMENTS

I would like to cite the excellent encouragement and down to earth advice of my advisor Dr. Robert N. Moore. Many thanks to my Committee members; Dr. M.A. Breider, Dr. F.M. Hopkins, Dr. A.T. Ichiki, and Dr. C.J. Wust for their support. I would like to thank Dr. M.D. McCracken for his assistance in the pathological studies; Ms. Dottie Fenner, for her assistance in the coagulation studies; Ms. Mary Jean Bryant and the Clinical Microbiology staff for their generous assistance and enthusiasm; J.A. Schneider and Dr. C.R. Reinemeyer for their assistance in the preparation of the statistics; and Kreis Weigel and Brian Rice for their assistance in the preparation of the figures. I would like to acknowledge Dr. R.D. Walker for his role in the early phases of this study. I would also like to thank Ms. Jan Grady for her patience and influence in the typing and preparation of this dissertation as well as for her editorial skills.

ABSTRACT

Pasteurella haemolytica is a leading cause of pneumonia in cattle. At present there is no suitable vaccine for the prevention of this economically important disease. The purpose of this investigation was to determine if pulmonary immunity to P haemolytica can be induced in cattle by stimulation of the gut-associated lymphatic tissue (GALT) by live bacteria or leukotoxin. In addition, the role of endotoxin and leukotoxin of P haemolytica in the pathogenesis of P haemolytica was investigated.

Immunogens were delivered to GALT through catheters surgically implanted in the proximal duodenum of calves. Immune responses of calves were subsequently boostered by a subcutaneous (SC) inoculation of killed P haemolytica or leukotoxin. Leukotoxin neutralizing antibodies (LNA) in serum and pulmonary lavage fluids were detected by a vital stain uptake assay using BL-3 cells. Serum IgG and IgG and IgA in pulmonary lavage fluids were detected by ELISA.

Live P haemolytica administered intraduodenally (ID) to calves resulted in increases in serum LNA, pulmonary LNA, IgG and IgA following a SC booster inoculation of killed bacterin. The ID administration of leukotoxin resulted in increases in pulmonary LNA and IgA. Subsequent SC inoculations of leukotoxin resulted in further increases in antibody titers but also resulted in an endotoxic shock reaction in calves.

The role of ID priming, endotoxin, and leukotoxin in the pathophysiology of pasteurellosis was determined by inoculating calves

ID with leukotoxin and endotoxin (LE) or RPMI-1640 and boostering them SC with LE or heat treated LE. Results indicated that calves inoculated SC with heat treated LE had less pathophysiological changes than calves inoculated SC with LE regardless of previous ID exposure.

The results of these experiments suggest that endotoxin and leukotoxin of P haemolytica act in a separate but additive manner in inducing the pathophysiology of pasteurellosis. The production of oral vaccines to stimulate GALT appears to be an effective way to induce LNA and reduce the lesions of pasteurellosis in calves.

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LIST OF ABBREVIATIONS

BALT--bronchus-associated lymphatic tissue

BL-3--bovine lymphocyte-3 cell line

BRDC--bovine respiratory disease complex

CCL--concentrated crude leukotoxin

CFU--colony forming units

CMI--cell mediated immunity

EDTA--ethylenediaminetetraacetic acid

ELISA--enzyme linked immunosorbent assay

FAE--follicle-associated epithelium

FCA--Freund's complete adjuvant

FKB--formalin killed bacteria

GALT--gut-associated lymphatic tissue

ID--intraduodenal

IFA--incomplete Freund's adjuvant

IM--intramuscular

KSCN--potassium thiocyanate

LE--leukotoxin and endotoxin

ln--natural logarithm

LNA--leukotoxin neutralizing antibodies

LPS--lipopolysaccharide

MALT--(common) mucosal associated lymphatic tissue

MNL--mononuclear leukocytes

NBTR--nitroblue tetrazolium reduction

PAM--pulmonary alveolar macrophages

PBL--peripheral blood leukocytes

PBSS--phosphate buffered saline solution

PMN--polymorphonuclear cells

SC--subcutaneous

sIgA--secretory immunoglobulin A

Tcs--T contra-suppressor lymphocyte

Th--T helper lymphocytes

Ts--T suppressor lymphocyte

WBC--white blood cells

INTRODUCTION

Pasteurella haemolytica is the most important bacterial isolate associated with the bovine shipping fever complex. It produces substantial monetary losses in the cattle industry due to deaths, the cost of medical treatment, and chronic poor health in animals. Vaccines to control this disease have depended primarily on killed bacterins. Bacterins, however, can actually increase pathology associated with the disease. More recent vaccines using live organisms have had varied success but require properly timed injections to be effective. Present management practices discourage immunization protocols using these products. Experimental procedures using aerosolized live organisms have produced consistent immunity but are commercially impractical and unacceptable to producers. Therefore, at present there is no commercially acceptable, effective vaccine to control Pasteurella pneumonia.

Aerosol immunization induces a pulmonary mucosal immune response capable of neutralizing the ruminant-specific leukotoxin of P haemolytica. Generation of such neutralizing antibodies is felt to be the advantage of this route of vaccination. An alternative method of stimulating mucosal immunity could utilize the interaction of the common mucosal associated lymphatic tissue. The gut-associated lymphatic tissue (GALT) is the primary target of this method of immunization. Antigens in the intestines enter the Peyer's patches where they stimulate the lymphatic tissue. Plasma cells enter the general circulation through the lymph and migrate preferentially to

other mucosal sites in the body including the bronchus-associated lymphatic tissue (BALT) of the lung. Investigations concerning GALT in sheep have used simple antigens to demonstrate GALT interaction with BALT and mammary tissues. The GALT-BALT interaction in bovine species has not been investigated. Virtually nothing is known about the interaction of microbial antigens and GALT in ruminants.

The overall intent of this research was to investigate the role of leukotoxin and endotoxin as virulence factors of P haemolytica, and to evaluate a novel way of inducing mucosal immunity in ruminants by stimulating GALT. The specific purposes of this series of experiments were: (1) to determine if a pulmonary antibody response could be induced following an intraduodenal stimulation by live P haemolytica or its leukotoxin and endotoxin; (2) to determine what effect a subsequent parenteral inoculation by a killed bacterin of P haemolytica would have following ID inoculation; (3) to evaluate the immunogenicity of chemically and heat treated leukotoxin preparations injected into rabbits; (4) to determine what adverse effects the endotoxin and leukotoxin exert on the coagulation factors and peripheral leukocytes of calves, and (5) to determine if stimulation of GALT by live bacteria could result in reduced pathology in calves challenged with live P haemolytica.

CHAPTER 1

LITERATURE REVIEW

Bovine respiratory disease complex (BRDC) (i.e. shipping fever) is an important cause of death in feedlot animals causing tremendous economic loss to producers. The most common bacterial isolate associated with the disease is Pasteurella haemolytica A1. Efforts to control this complex disease by vaccination have not been successful. Killed bacterins were used for many years until research showed they could actually increase pneumonic pathology.[1] Newer modified live vaccines require handling of animals prior to shipment at times not amenable to current management practices.

Host defense against certain pathogens depends as much upon mucosal as humoral immunity.[2] This applies to respiratory infections acquired by inhalation of pathogens such as P haemolytica. Mucosal immunity consists of cellular mediated defenses eg., alveolar macrophages, as well as the presence of antibodies such as secretory immunoglobulin A (sIgA). Mucosal antibody levels depend primarily on local production in most species as well as upon addition of a secretory piece to dimeric molecules (IgA) which can be derived principally from intestinal lymphoid tissue in certain species eg., sheep.[3]

Immunity at the mucosal level can be very important in preventing colonization and invasion by pathogenic organisms. Local immunity depends on nonspecific factors (such as interferons, mucous, etc.), cell mediated factors (natural killer cells, alveolar macrophages,

etc.), and specific antibodies. The type of antibody present in the respiratory tract of calves varies depending on the level of airway examined. In general, the upper airways (nares) contain almost exclusively IgA while the proportion of IgG to IgA increases proportionately for the lower airways.[4] The relative amount of IgA to IgG is still controversial with various researchers reporting different ratios. Allan et al.[5] reported IgA producing cells to predominate in the subepithelial tissue of bronchoalveolar tissue in healthy calves while IgG_1 predominates in pneumonic calves. Walker et al.[6] reported IgA to predominate in bronchoalveolar washings in contrast to Butler[7] who reported that IgG predominates. Controversy also pertains to other mucosal sites.[8] Chang et al. found IgG_1 levels to be greater than IgA in mammary secretions and attributed this to local production of IgG_1. Both IgG and IgA are important antibodies in mucosal secretions of ruminants. The contribution of GALT stimulated antibodies to other mucosal sites in ruminants consists of IgA and possibly IgG.

Part of the controversy concerning mucosal antibody levels is related to disagreement about what proportion of antibodies found in local secretions is produced locally and what amount is transported from the blood. This problem is illustrated by the great variability of amounts of immunoglobulin producing cells adjacent to lavage areas. It therefore seems likely that levels of antibodies secreted at the mucosal level depend on local production, the availability of classes of immunoglobulins in adjacent tissue and blood, and the ability of (respiratory) epithelium to transport these antibodies.[4]

Other factors such as age and species differences can also affect the level of antibodies present on mucosal surfaces. Colostrum-deprived neonates have no antibodies on their nasal mucosa. In colostrum competent pigs IgA is the primary antibody secreted;[9] in calves IgG_1 predominates.[10] Route of administration of inoculum can also affect the class of antibody produced. Repeated parenteral inoculations[11] and the use of adjuvants[12] can result in the elevation of IgG in the lungs of mice[13] and calves.[12] Direct stimulation of the respiratory tract can result in elevated IgG or IgA depending on the level of the site stimulated.

Local stimulation of the lung can result in a more widespread immune response.[14] Particulate and soluble antigens can be readily taken up by the lung resulting not only in a local immune resopnse, but a systemic response also. Direct stimulation results in IgG, IgA and cell mediated immune (CMI) responses. The CMI response is restricted locally with no effect on systemic CMI. By the same token systemic stimulation usually results in a systemic CMI response with no mucosal CMI.[15]

The efficacy of the mucosal cell mediated response depends on how well the system is stimulated and modulated. Research has shown that viable microbial organisms are a better stimulant of mucosal immunity than killed organisms.[14] Immunological memory of the CMI can be long lived. Direct mucosal introduction of an antigen at a much later date leads to rapid reexpression of the CMI no matter how separated in time the two exposures may have been.[15]

Macrophages play an important modulating effect on lymphocytes in CMI. This is a species specific reponse with pulmonary macrophages in the dog, rat, rabbit and calf having an inhibitory effect while having a stimulatory effect in the mouse and guinea pig. The inhibitory effect of macrophages can be overcome if the lungs are persistently stimulated. This can then lead to a population of macrophages that enhance the CMI response.[16] Appropriate stimulation and effective modulation are very important to the effector function of the immune system. At least with some pathogens evidence suggests that CMI and immunoglobulins found in respiratory secretions are more effective in affording protection than immune factors found in serum.[14]

It is not always practical to directly stimulate mucosal sites in order to achieve local antibody production. However, these antibodies have advantages to the host. For example, sIgA against common intestinal and respiratory pathogens is transferred to the mammary gland where it is absorbed by the nursing neonate who is then protected.[17] This phenomenon exemplifies the transfer of mucosal antibodies by a common mucosal associated lymphatic tissue (MALT) system. Central to MALT is the gut-associated lymphatic tissue (GALT) composed of Peyer's patches and less obvious areas of antigen sampling referred to as follicular-associated epithelium (FAE) tissue.[18] These areas are characterized by their lack of goblet cells, lack of villi, and the presence of M cells or macrophages which take up antigen and present it to underlying follicular areas of B lymphocytes which are separated by areas of T cells.

Any antigen taken up by GALT is processed by dendritic cells and T lymphocytes. Stimulated B cells are taken up by the lymphatics, transported via the thoracic duct to the general circulation, seed the central lymphatic tissue (including spleen) and then home randomly to all mucosal sites in the body.[19] This is true for lymphocytes stimulated at any mucosal site. However, there is some preference of these cells to home to their original site of origin and to inhabit the appropriate lamina propria.[20] In most species investigated there appears to be close ties between GALT and bronchus-associated lymphatic tissue (BALT) whereby these two sites tend to take up most antigens a host is exposed to. Their lymphocytes also have a greater affinity to migrate to either site.[21] In the ruminant this takes on a different significance. Evidence suggests that BALT is poorly developed in sheep.[22] Yet, IgA cells are in numerous supply in lung tissue and sIgA is a primary antibody of upper airways (above alveoli-lower bronchioles). It is felt that a significant amount of IgA in sheep lung is derived from GALT to BALT migration perhaps due to this inherent tendency.

GALT stimulation to enhance this trafficking of primarily IgA labelled lymphocytes has many advantages. Antigen preparations do not have to be well purified to be given orally compared to parenteral vaccines. Patient compliance would be better with less side effects or pain accompanying administration. Mucosal immunity appears to remain active well into old age and does not lose its potency.[23] For these reasons many oral vaccine preparations are being studied for a wide range of antigens.

Early evidence of homing of MALT derived lymphocytes to mucosal sites resulted from injecting MALT lymphocytes into irradiated hosts.[20] Results confirmed that peripheral node lymphocytes home to peripheral node sites whereas mucosal originated lymphocytes tended to home primarily to their tissue of origin, as well as other mucosal sites. It was later deduced that most of these lymphocytes produce sIgA,[21] a fact enhanced by evidence of a great number of T helper cells in GALT sites. It is presumed one role of these T helper cells is to stimulate the isotype switch of lymphocytes to IgA production.

There are now numerous examples of how GALT may be manipulated to induce mucosal immunity to pathogens at distant sites. Experiments exposing rats or humans to Streptococcus mutans by gastric lavage led to salivary IgA antibodies specific for S mutans and evidence of a decrease in dental caries in challenged animals.[25] Intraperitoneal injections of soluble antigen in Freund's complete adjuvant (FCA) is an effective means of stimulating GALT tissue (by serosal absorption of antigen following FCA induced inflammation).[26] In sheep this has been an effective method of inducing sIgA in mammary glands at certain times of mammary gland development. It has also been a useful way to increase sIgA cells in the respiratory tract of animals subsquently stimulated by homologous or heterologous antigens intrabronchially.[27] These examples demonstrate that GALT-BALT interaction exists in ruminants (sheep).

An interesting finding in sheep is that mammary mucosal IgG_1 is increased in these animals at the same time as IgA levels increase in

GALT. Whether this is due to stimulation of the central immune system by the antigen or due to plasma cells of mucosal origin is unsure. Brandtzaeg[28] has suggested that GALT stimulation results in production of a primordial isotype lymphocyte. This type of lymphocyte does not differentiate further until stimulation of memory cells by the stimulating antigen at a mucosal site at a later time. Most cells convert to IgA production but some IgG and IgM producing cells remain. IgG titers in sheep at mucosal sites tend to be approximately 1/6 of those found in serum samples.

Evidence of GALT-BALT interaction in sheep is encouraging especially due to BALT being a poor source of IgA in this species. Research suggests that approximately 19% of IgA in sheep respiratory secretions comes from GALT even though 81% of IgA of lung is produced locally.[24] However, due to a loss of intestinal surface antigen markers, GALT-derived IgA may actually be much greater. It appears that in the sheep, the gut does make a significant contribution to ruminant respiratory mucosal antibody levels.

P haemolytica is an organism that could be controlled by stimulation of mucosal immunity. This organism possesses several virulence factors that could be targeted by mucosal immunity. P haemolytica possesses a thick capsule which is most prevalent in the log phase of growth.[29] With prolonged growth the capsule decreases in prevalence. This is one explanation for the apparent improved immunogenicity of log phase organisms. The capsule is also useful in serotyping different P haemolytica organisms.[30,31] The capsule of P

haemolytica is believed to be a potent virulence factor allowing the bacterium to avoid phagocytosis in the nasal mucosa where it is considered to be part of the normal flora. The capsule may also allow the bacterium to avoid antibacterial host defenses in the pulmonary alveoli including complement-antibody interaction.

At least 6 different antigens have been identified from the capsule of P haemolytica.[30,32] These vary in protein and polysaccharide content and in their ability to induce antibody response in cattle. Characterization of the polysaccharide antigen in ovine strains has revealed a teichoic acid structure which is highly unusual for a gram negative organism. Ovine strains vary widely in their immunogenicity. The purified polymer is immunogenic for sheep but not for rabbits. Strains of crude capsular extracts vary in protein content and some are poor immunogens for sheep (eg, strain A2).[33] Similar results have been demonstrated in laboratory animals for this and other strains.[34]

Various vaccine preparations with crude capsular extracts indicate that the potassium thiocyanate (KSCN) method of extraction is slightly superior to salycilate extraction and even more so than saline extraction techniques.[35] Extracts adjuvanted with incomplete Freund's adjuvant (IFA) and aluminum hydroxide (Al_2O_3) together appear to induce an optimal immune response in laboratory animals and sheep. These extracts have been administered subcutaneously (SC), intramuscularly (IM) or by aerosol to mice, hamsters, sheep and calves. Protection has been shown for all routes of administration with SC or IM superior to

aerosol exposure.[36] Attempts to stimulate mucosal surfaces directly have been the least successful route of administration, but are encouraging. Protection has been determined by decreases in mortality, clinical signs of illness, and the occurrence of fibrinous pneumonia in calves. There was also decreased bacterial isolation from the lungs in protected calves and decreased mortality in laboratory animals. Protected animals have had variable antibody responses to capsular antigens in these experiments.[37,38,39] These seem to indicate a possible correlation between decreased incidences of pneumonia and antibodies against capsule. More recent experiments provide a stronger suggestion that antibodies to capsular antigens may be required for protection.[40] Antibodies to other virulence factors may be equally or more necessary to reduce disease.

The laboratory animal and ovine studies have clearly indicated that protection can only be induced to the homologous strain of capsular extract. No cross protection is conveyed to other strains.[33,34]

Viable log phase P haemolytica A1 produce an extracellular glycoprotein toxin which is specific in activity for ruminant leukocytes. Heat killed or formalin treated P haemolytica do not possess this toxin.[41] Stationary growth phase bacteria produce significantly less toxin.[42] This toxin is O_2 and N_2 stable, heat labile, water soluble, pH stable, non-hemolytic, and is reported to have a molecular weight of 57,000 to 300,000.[43,44,45] The wide range reported in molecular weight may be due to aggregation with other

serum proteins or due to polymer formation. Antibodies made to crude supernatant toxin cross reacts with all 12 serotypes of P haemolytica[46] but not to other (eg., Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa) species of gram negative bacteria.[47]

Benson et al.[48] first suspected P haemolytica produced an exotoxin when they exposed pulmonary alveolar macrophages (PAM) to live and heat-killed P haemolytica. Heat killed P haemolytica were readily phagocytosed by PAM, but live bacteria were not. The live bacteria caused rounding up of PAM, loss of pseudopodia and death of the leukocytes. Berggren et al.[42] investigated the effects of crude supernatants on PAM and demonstrated transmission electron microscopic (TEM) evidence of cell toxicity. Polymorphoneucleated cells (PMN) did not phagocytose live log phase bacteria and showed changes in cytoplasmic organelles and membranes attributed to toxicity. Heat treated P haemolytica were readily phagocytosed and degraded by PMNs.

The leukotoxin is highly immunogenic and is able to induce an antibody response in calves, rabbits and other laboratory animals.[46,49] Sera from adult cattle and calves hyperimmunized with live bacteria are able to neutralize the effects of toxin on leukocytes in vitro. Shewen et al.[49] showed that cattle with naturally occurring serum leukotoxin neutralizing antibodies (LNA) have less deaths due to fibrinous pneumonia than cattle who have no LNA.

Gentry et al.[50] showed that cattle immunized with formalin killed P haemolytica had somatic antibody titers similar to live P haemolytica vaccinated animals. However, only calves that received live organisms

possessed serum LNA. Furthermore, calves with LNA had a much better correlation with decreased lung lesions when challenged than calves vaccinated with killed bacterins. Cho et al.[51] confirmed these results and determined that protected animals also had detectable LNA in nasal washings and pulmonary lavage washings. Moore et al.[47] showed that calves exposed to aerosolized P haemolytica develop LNA in pulmonary washings. Immunoprecipitation and separation of isotypes from pulmonary lavage fluids showed that IgA, IgG_1, and IgG_2 possess LNA capabilities, especially IgG_2 which is the primary antibody found in lavage fluids.

Previous investigations by Friend et al.[1] and Markham et al.[52] had suggested that opsonizing antibodies actually increased pulmonary lesions perhaps by increasing macrophage activity in the presence of leukotoxin. Lysis of PAM and PMN then release enzymes that cause pulmonary damage. However, experiments with LNA suggested that the class of antibody produced is not as important as the specificity of the antibody induced. In the experiments of Friend et al.[1] and Markham et al.[52] animals were inoculated with formalin killed bacteria which do not induce LNA production. Apparently IgG antibodies specific for somatic antigens do not prevent pneumonia.

Most research on leukotoxin is done with crude culture supernatant extracts. Characterization of physical properties have been performed on partially purified preparations that are unstable and impure. Recombinant DNA technology has led to genetic cloning techniques to identify and purify specific protein groups. Lo et al.[53] have

identified several genetic clones of P haemolytica that react to antibodies made against crude supernatant leukotoxin preparations. Gel electrophoresis has further confirmed that the proteins generated by some of these clones are identical to proteins present in crude supernatants at a molecular weight consistent with the suspected size of P haemolytica leukotoxin. Cloned leukotoxin can now be much better characterized and its role as a virulence factor confirmed.

P haemolytica produces significant amounts of endotoxin or lipopolysaccharide (LPS).[54] This endotoxin causes widespread serious effects on the vascular, pulmonary and coagulation systems of calves including leukopenia and (decreased) leukocyte migration to the lungs; serious changes in pulmonary arterial pressure, decreased cardiac output, and (late) systemic hypotension in sheep;[55] vascular thrombosis, platelet aggregation and degranulation, and dermonecrocity for rabbits.[54,56] These effects also appear following the rapid growth and lysis of P haemolytica in the lungs of affected animals.[56]

Lipopolysaccharides are generally not very immunogenic. The toxic properties of P haemolytica endotoxin are not necessarily neutralized by homologous antibodies. Confer et al.[57] found no correlation of antibodies directed against P haemolytica LPS with lung lesion scores in calves inoculated with phosphate buffered saline solution (PBSS), formalin killed P haemolytica or live bacteria. However, active systemic vaccination with P aeruginosa LPS has conferred protection on guinea pig lungs from Pseudomonas pneumonia.[58] This suggests that endotoxin can be immunogenic. Further investigation of the immunogenicity of P haemolytica's endotoxin is required.

The mitogenic/adjuvant effects of LPS have been well documented.[59] Oral stimulation with LPS can suppress or prime the mucosal immune system of mice depending on previous exposure and the genetic make-up of the animal. It is suspected that the immune response in these mice depends on the balance of stimulation of helper T lymphocytes (Th), contrasuppressor T lymphocytes (Tcs) and suppressor T lymphocytes (Ts) in the GALT. Orally administered thymic dependent antigens can induce Th pathways and prime the immune system for an anamnestic response. This can result in higher IgA levels depending on the level of endogenous or environmental LPS. The mitogenic effect of LPS can drive B lymphocyte antibody production and also induce an antibody isotype switch of B cell clones towards IgA production.[59,60]

Little work has been done with subunit vaccines of P haemolytica derived strictly of somatic antigens. Tadayon et al.[35] found that mice and hamsters injected with cellular debris recovered from the remains of cells used to extract LPS and capsular antigens had increased survival when challenged by P haemolytica. This contrasts to studies in cattle which evaluated the immune response to somatic antigens of formalinized bacteria. Confer et al.[38] and Cho et al.[51] found no correlation of antibody titers to somatic antigens of P haemolytica with resistance to pneumonia in calves vaccinated with these preparations. Friend et al.[1] and Markham et al.[52] found calves with antibodies to somatic antigens (vaccinated with formalinized bacteria) actually had worse pulmonary lesions. The effect of subunit somatic antigens from cells devoid of capsular and LPS antigens in cattle remains unknown.

Control of P haemolytica pneumonia depends on the improved management of cow-calf herds as well as feedlot operations. Research to develop practical vaccination protocols is essential. Understanding more fully the pathogenesis of bovine respiratory diseae complex and learning how to modulate the host defense mechanisms to eliminate the pathogens without further damaging the host are important considerations in studying this disease.

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CHAPTER 2

STIMULATION OF THE GUT-ASSOCIATED LYMPHATIC TISSUE OF CALVES BY LIVE *PASTEURELLA HAEMOLYTICA*

Pasteurella haemolytica is the most common bacterial isolate associated with bovine shipping fever complex. This disease complex causes substantial monetary loss to the cattle industry each year and is a primary concern of cattle producers. Past attempts to prevent and control *P haemolytica* with killed bacterins have been unsatisfactory.[1,2] Recognition of the importance of mucosal immunity in the prevention of bacterial infection has led to the development of experimental aerosol vaccination protocols.[a,3] Calves exposed to aerosolized live *Pasteurella multocida* have reduced pulmonic lesions following challenge exposure. Calves exposed to aerosols of *P haemolytica* have increased levels of pulmonary serum leukotoxin neutralizing antibodies.[3] Apparent resistance to *P haemolytica* has been associated with the presence of antibodies capable of neutralizing the leukocyte specific exotoxin, an important virulence component of *P haemolytica* in serum.[4,5]

Although effective, aerosol inoculation to induce mucosal immunity to *P haemolytica* is unsatisfactory at the commercial level. The procedure is both cumbersome and time consuming. An alternative method for inducing pulmonary mucosal immunity could utilize the common

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[a]Panciera RJ, Corstvet RE, Walker RD, et al. Induction of pulmonary lesions in cattle with *Pasteurella multocida* (abst.) 57th Annual Meeting Research Workers Animal Disease, No. 12, 1976.

mucosal associated lymphatic tissue (MALT) in an oral vaccine. The ability of precursor (primed) lymphocytes from Peyer's patches to migrate to other mucosal surfaces where they elaborate antibodies in response to a "second antigen" stimulation has been well described.[6-9]

The purpose of this portion of the study was to determine if intraduodenal exposure of calves to live P haemolytica could prime the animals for a secondary pulmonary antibody response to P haemolytica somatic antigens or its leukotoxin following a subcutaneous challenge with killed bacteria.

Materials and Methods

Calves--Hereford or Hereford-Angus crossbred calves (150 to 250 kg) were purchased from local farms. Calves had negative cultures for P haemolytica on nasal and tracheal swabs and had serum anti-P haemolytica titers equal to or less than 1:16 for ELISA.

Bacterium--P haemolytica (isolate 12216, biotype A, serotype 1) was originally isolated from a pneumonic lung of a calf. This isolate was passed three times in calves by intrabronchial inoculation and the reisolated organism suspended in skim milk and stored at -70 C.

Pulmonary lavage--The presence of antibodies in pulmonary lavage fluids was determined on samples collected on a weekly basis. Lavages were performed once a week beginning 3 days prior to intraduodenal inoculation and continuing throughout the experiment. Blood was collected from the jugular vein on the same dates as pulmonary

lavages. Blood was allowed to clot, centrifuged and serum was separated and frozen in aliquots at -20 C. Pulmonary lavage of calves was performed as described by Walker et al.[10] with modifications listed below. Briefly, each calf was restrained in a head chute with the head elevated and a wooden speculum in its mouth. A dependent lobe was catheterized with a Foley-type catheter[b] inserted through a endotracheal tube and the airway to that lung occluded. The occluded lung lobe was lavaged by infusing 50 ml aliquots of sterile buffered glucose-supplemented saline (pH 7.2) followed immediately by aspirating the solution. The infusion-aspiration was continued until 500 ml of lavage fluid was collected. This required approximately 800 ml of saline. The lavage fluid was clarified by centrifugation at 500 x g for 10 minutes. Clarified lavage fluids (500 ml/calf) were concentrated 100-fold by ultrafiltration using 100,000 MW exclusion membranes (Minitan cassette concentrator)[c] followed by further concentration on a stirred cell concentrator[d] using a 30,000 MW exclusion membrane. Resultant concentrated lavage fluids were frozen in several aliquots at -20 C until assayed.

Intraduodenal inoculation--Calves were inoculated with live bacteria in the log phase of growth suspended in phosphate buffered saline solution (PBSS) by injection into the proximal duodenum through

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[b] Bivona, Inc., Gary, IN.

[c] Minitan, Millipore Corp., Bedford, MA.

[d] Amicon Corp., Lexington, MA.

a catheter. Intraduodenal catheterization of calves with a plastic[e] tubing (inside diameter 1.5 mm by 3.0 mm outside diameter) was achieved by surgical intervention. Calves were maintained in lateral recumbancy under halothane[f] general anesthesia. Aseptic technique was strictly adhered to and approach to the proximal duodenum was obtained by a right flank incision. The catheter was inserted 3 to 5 cm distal to the pylorus with 12.5 cm of catheter positioned so the tip was directed distally in the duodenum. A 1.5 cm diameter button of silastic adhesive[e] was affixed to the catheter and sutured in the intestine to prevent slippage. Catheters were tunneled subcutaneously from the duodenum to exit in the right paralumbar fossa of the calf. A three-way stopcock on a blunted 14 ga. needle was affixed to the catheter and used to flush the catheter, and to administer the P haemolytica inoculum. Calves were allowed to recover for 10 days post-surgery before exposure to the inoculum.

Following complete recovery from surgery calves received an intraduodenal inoculation of either live P haemolytica or PBSS. Logarithmic growth phase P haemolytica grown as described below in brain-heart infusion (BHI) broth[g] (with 1% fetal calf serum[h] added) were suspended in 0.01 M PBSS (pH 7.2). The inoculum was suspended to

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[e] Silastic, Dow Corning Corp., Medical Division, Midland, MI.

[f] Fluothane, Fort Dodge Laboratories, Inc., Fort Dodge, IA.

[g] Gibco Laboratories, Madison, WI.

[h] Hyclone Laboratories, Inc., Logan, UT.

approximately 1.0 x 10^{10} colony forming units (CFU)/ml, and 100 ml of this inoculum was injected via the catheter to each experimental calf (group 1). Controls received an equivalent volume of PBSS (group 2). The inoculum was flushed immediately post-injection with 50 ml of PBSS to clear the catheter. Two weeks following intraduodenal inoculation of P haemolytica each Group 2 calf received 100 ml of 10^{10} CFU/ml of log phase growth P haemolytica killed with 0.45% buffered neutral formalin. The solution of killed bacteria was injected subcutaneously in the neck in 4 sites, 2 on each side. Calves in group 1 received the killed bacterin 5 weeks later when serum titers were determined to have returned to a baseline level.

Leukotoxin production and assay--P haemolytica leukotoxin supernatant fluids were prepared with slight modications of the procedure described by by Shewen et al.[12] Briefly, 4 to 5 colonies of P haemolytica from an overnight culture on blood agar (enriched with yeast extract[g] and horse serum[h]) were inoculated into brain heart infusion broth and incubated for 4 1/2 hours in a shaker water bath[i] at 37 C. This culture was centrifuged at 4500 x g for 20 minutes. The pellet was resuspended in a volume of RPMI-1640 equal to the original volume with 7% horse serum added and incubated for 1 hour in a shaker water bath[i] at 37 C. This culture was centrifuged at 10,000 x g for 20 minutes and the supernatant filtered through 0.2 um filters[j] to remove

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[i]Orbit Environ-Shaker, Lab-Line Instruments, Inc., Melrose Park, IL.

[j]Nalge Company, Division of Sybron Corp., Rochester, NY.

all bacteria. The resultant leukotoxin was stored at -70 C until used. Bacteria pelleted in this centrifugation were suspended in PBSS and used as inoculum for intraduodenal stimulation.

Leukotoxin activity of the supernatant was determined by a modification of the techniques described by Moore et al.[3], using the vital stain uptake assay. Briefly, 2 x 10^5 BL-3 cells (a bovine lymphoid cell of B cell origin initially provided by Dr. G. Thielan, University of California, Davis) grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 0.03% L-glutamine, 10 mg/ml gentamicin[k] and 5 ug/ml Amphotericin B[l] were suspended in 100 ul of medium in each well of 96 well round bottom microplates.[m] The plates were centrifuged at 500 x g for 10 minutes and 65 ul of medium was removed from each cell. The leukotoxin supernatant was diluted in RPMI-1640 in half log dilutions. One hundred ul of each dilution was added in triplicate to wells of the microtiter plate. The plate was incubated at 37 C in 5% CO_2 for 2 hours. One hundred ul of 0.036% neutral red[n] in normal saline was added to each well. The plate was then incubated at 37 C for 20 minutes. Following this incubation the plate was centrifuged at 500 x g for 10 minutes. The stain plus medium was removed from each well with care taken not to disturb the pellet of cells. The plate was

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[k]Gentocin, Schering Corp., Kenilworth, NJ.

[l]Fungizone, E.R. Squibb and Sons, Inc., Princeton, NJ.

[m]Corning Glass Works, Corning, NY.

[n]Sigma Chemical Co., St. Louis, MO.

washed with 200 ul of normal saline per well; the washes were pipetted rapidly to mix thoroughly each pellet of cells and the plate was centrifuged at 500 x g for 10 minutes. This was done two times, and after the second wash, 100 ul of a lysis buffer composed of 50% ethanol in 0.02 M PBSS was added to each well. The plate was exposed to -70 C for a rapid freeze thaw to complete stain release and each well was thoroughly mixed by pipette to complete cell lysis. The plate was then centrifuged at 500 x g for 10 minutes and 65 ul of medium from each well transferred to a 96 well flat bottom plate. The absorbance of each well of this microtiter plate was then read at 590 nm using a spectrophotometer.[o] The minimal dilution of leukotoxin which killed 100% of cells present was then used for determination of cytotoxin neutralizing antibodies in serum and pulmonary lavage samples.

To determine leukotoxin neutralizing antibody (LNA) activity, serial 2-fold dilutions of concentrated lavage fluids and of serum samples were prepared in RPMI-1640 medium. Each serial dilution was then mixed with an equal volume of P haemolytica leukotoxin. This produced a minimal dilution of leukotoxin capable of killing 100% of BL-3 cells. Serial dilutions plus leukotoxin were incubated at 4 C for 2 hours and then added to BL-3 cells on microtiter plates. A leukotoxin neutralization assay was then performed as described above. All assays were performed in triplicate. The neutralizing titer was reported as the reciprocal dilution of serum or lavage fluids that

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[o]EIA Autoreader, Model EL-310, BioTek, Industries, Winooski, VT.

protected 50% of the cells as determined from the linear portion of the dose response curve.

Immunoglobulin assays--Anti-P haemolytica IgG titers for serum and IgG and IgA titers for concentrated lavage samples were determined using an enzyme linked immunosorbent assay (ELISA). The technique was slightly modified as described by Voller et al.[11] Briefly, plates were prepared using microtiter platesP with 50 ul per well of whole cell P haemolytica suspended at a concentration of 2 x 10^9 CFU/ml in carbonate-bicarbonate buffer at pH 9.6. The plates were dried overnight at 37 C and stored at -20 C until used.

Serial 2-fold dilutions of serum or concentrated lavage fluids were incubated for 3 hours at 22 to 25 C on a shaker platform. Each sample was run in triplicate. After washing with PBSS-tween, plates used for IgA assays were incubated with rabbit anti-bovine IgA serum for 3 hours and then washed with PBS-tween. Horseradish peroxidase-conjugated anti-bovine IgG or anti-rabbit IgAq was added to each well and incubated for another 2 hours. After washing, the substrate, O-phenylenediamine plus H_2O_2, was added to each well and incubated for 30 minutes. The reaction was stopped by addition of 2.5 M H_2SO_4. Plates were read at 490 nm using a spectrophotometer.n The antibody titer was defined as the last dilution that gave a strong color reaction and had an absorbance value 2 times or greater than the next

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PImmulon 2, Dynatech Laboratories, Alexandria, VA.

qBethyl Laboratories, Montgomery, TX.

dilution. The absorbance cutoff was compared to a positive control to minimize variability in dilution technique and differences between plates. The absorbance cutoff was adjusted if necessary to meet both criteria.

Statistical analysis--Mean antibody titers pre-exposure, post-ID, and post-SC within each group and between groups were compared by the Waller-Duncan's analysis of variances test. Significance levels were reported at $p<0.16$ even though this is not usually recognized as an acceptable level. This was done to indicate cases where titers were increased 4-fold within a group and are thereby considered immunologically significant. It was also meant to indicate trends in cases where the small number of subjects per group and the wide variation normally seen in cattle[12] make statistical interpretation difficult.

Procedure

A summary of the protocols used in this experiment is shown in Table 2.1. Following initial screening to determine the level of serum antibodies to P haemolytica, and confirmation of negative tracheal and nasal cultures for the presence of P haemolytica, calves had intraduodenal catheters placed under general anesthesia.

The calves were allowed to recover for 10 days at which time each calf underwent the first pulmonary lavage and collection of 10 cc of blood. This procedure was repeated at weekly intervals until the end of the experiment. Each pulmonary lavage sample was concentrated,

Table 2.1. Experimental procedures performed.

Procedure	Pulmonary Lavage		Blood Sample		Inoculation	
Calf Group	1	2	1	2	1	2
Time in Weeks						
0	PL	PL	BS	BS		
0.5					ID Live Bacteria	ID PBSS
1	PL	PL	BS	BS		
2	PL	PL	BS	BS		
2.5						SC Killed Bacterin
3	PL	PL	BS	BS		
4	PL	PL	BS	BS		
5	PL	PL	BS	BS		
5.5					SC Killed Bacterin	
6	PL	ND	BS	ND		
7	PL	ND	BS	ND		
8	PL	ND	BS	ND		

PL = Pulmonary lavage BS = Blood sample ID = Intraduodenally
SC = Subcutaneously ND = Not done

aliquoted and frozen at -20 C until assayed. Serum was harvested from each blood sample, aliquoted, and frozen until assayed. Calves were inoculated intraduodenally 3 days after the first lavage with either PBSS or live bacteria. Calves that received live bacteria were injected with a subcutaneous booster dose of killed bacterin five weeks later. When primary antibody response levels had reached their peak and were declining, calves that received PBSS initially were injected subcutaneously two weeks later with a killed bacterin. The presence of IgG in serum and IgA and IgG in lavage fluids was determined by ELISA. Leukotoxin neutralizing antibodies in serum and lavage fluids were measured by a vital stain uptake assay.

Results

Serum antibody responses--Serum IgG titers specific for somatic *P haemolytica* antigens in group 2 calves increased by 4-fold following intraduodenal PBSS (Table 2.2). These same calves had a further 4 to 8-fold rise in titer following a booster injection of killed bacterin. This increase in titer was rather moderate never exceeding 128. These increases were significant ($p<0.05$).

Serum IgG titers specific for *P haemolytica* somatic antigens in group 1 calves increased 4 to 8-fold following intraduodenal (ID) priming (Table 2.2). Group 1 calves' titers increased 16 to 32-fold with titers peaking at 512 to 2024 following subcutaneous boostering with a killed bacterin. This is 4 to 8-fold higher than seen in the sham primed group 2 calves that had received only the killed bacterin.

Table 2.2. Serum IgG titers of calves stimulated by an intraduodenal dose of live P haemolytica or PBSS and boostered by a subcutaneous injection of a killed bacterin.

	Calf No.	Pre-exposure*	Post-ID	Post-SC
Live bacteria/	54	16	64	512
FKB**	56	4	256	2024
(group 1)	57	8	64	512
	Mean†	9.3§	128.0§§	1016§§§,#
	std. dev.	6.1	110.8	873
PBSS/FKB**	348	8	32	128
(group 2)	349	16	64	128
	350	16	64	128
	Mean	13.3¶	53.3¶¶	128¶¶¶
	std. dev.	4.6	18.5	0

* ELISA titer pre-exposure, following intraduodenal stimulation and following subcutaneous killed bacterin.

† Mean and standard deviation of each group.

§ Groups with different number of ¶ are significantly different ($p<0.05$).

¶ Groups with different number of # are significantly different ($p<0.10$).

Mean is significantly different from the post-SC mean in group 2 ($p<0.17$).

** Group 1 calves received 10^{12} CFU log phase P haemolytica intraduodenally followed in 2 weeks by 10^{12} CFU of formalin (0.45%) killed bacterin. Group 2 calves received phosphate buffered saline solution intraduodenally followed 2 weeks later by 10^{12} CFU of formalin (0.45%) killed bacterin.

ID = Intraduodenally SC = Subcutaneously

Increases following the first inoculation and following the booster, represent a significant difference ($p<0.10$).

The mean peak post-SC serum IgG titer in group 1 calves was 8-fold greater than the mean peak post-SC titer in group 2 calves. This difference was significant only at $p<0.16$ due to the wide variation in titers of these calves. Wide variation in experimental values is typical of many cattle experiments.[12]

Serum leukotoxin neutralizing antibodies (LNA) did not increase demonstrably in calves primed intraduodenally with PBSS or live bacteria (Table 2.3). Group 2 calves had less than a 4-fold increase in titer following subcutaneous boostering with the killed bacterin. One calf in this group (#350) showed a greater increase in titer following the killed bacterin booster. Although the titer did increase to a moderate level, it was lower than the other calves in this group and is 10-fold less than the titer exhibited by group 1 calves (Table 2.3). The post-SC increase in titer for group 2 was not significant.

Group 1 calves showed an 8 to 22-fold increase in LNA titer following the killed bacterin booster (Table 2.3). These calves had peak titers that ranged from 700 to 1024 following the booster injection. The post-SC injection titers were significantly different from the previous titers in this group ($p<0.05$). The mean peak post-SC serum LNA titer in group 1 was significantly greater than the mean peak post-SC serum LNA titer of group 2 ($p<0.05$). The group 2 titer was greater than 8-fold higher than the group 1 titer.

Table 2.3. Serum leukotoxin neutralizing antibody titers of calves following intraduodenal inoculation with live *P haemolytica* or PBSS.

	Calf No.	Pre-exposure*	Post-ID	Post-SC
Live bacteria/	54	128	128	1024
FKB**	56	16	32	700
(group 1)	57	128	128	1024
	Mean†	90.7§	96.0§	916§§,#
	std. dev.	64.5	55.4	187
PBSS/FKB**	348	70	75	90
(group 2)	349	40	75	130
	350	0	0	75
	Mean	36.7	50.0	98.0
	std. dev.	35.0	43.0	28.0

* Leukotoxin neutralizing antibody titer as determined by vital stain uptake assay.

† Mean and standard deviation of each group.

§ Groups with a different number of § are significantly different ($p<0.05$).

\# Mean is significantly different from post-SC mean of group 2 ($p<0.05$).

** Group 1 calves received 10^{12} CFU log phase *P haemolytica* intraduodenally followed by 10^{12} CFU of formalin (0.45%) killed bacteria subcutaneously. Group 2 calves received phosphate buffered saline solution intraduodenally followed 2 weeks later by 10^{12} CFU of formalin (0.45%) killed bacteria subcutaneously.

ID = Intraduodenally SC = Subcutaneously

Pulmonary antibody responses--Pulmonary IgA titers to P haemolytica did not increase following the initial inoculation in either group 1 or group 2 calves (Table 2.4). No calf in group 2 responded to a subcutaneous booster of killed bacterin. The calves in group 1 showed a 2 to 8-fold increase in IgA titers following the killed bacterin booster. These titers were very low but typical of titers previously reported.[3] The post-SC titer in group 1 represents a significant increase ($p<0.05$) compared to pre-exposure titers. The mean pulmonary IgA titer of group 1 was significantly greater than the mean peak titer of group 2 ($p<0.10$).

Pulmonary IgG antibodies to P haemolytica somatic antigens were not detectable in group 2 calves (Table 2.5) following intraduodenal inoculation. There was no significant change for group 2 following ID exposure. Group 2 calves did not have any detectable pulmonary IgG titer to P haemolytica following the subcutaneous booster with a killed bacterin. Group 1 calves showed an 8 to 32-fold increase in titer following the killed bacterin booster. This increase was significant compared to pre- and post-ID titers ($p<0.10$). The mean peak post-SC pulmonary IgG titer of group 1 was 16-fold greater than the mean peak titer in group 2. Due to the large variance in the means of titers, this difference was significant only at ($p<0.16$).

Pulmonary LNA increased by no more than 2-fold in calves in group 1 or 2 following ID priming with either PBSS or live bacteria (Table 2.6). Group 2 calves showed no further increase in pulmonary neutralizing antibodies following exposure to the killed bacterin

Table 2.4. Pulmonary IgA titers of calves following intraduodenal inoculation with live P haemolytica or PBSS.

	Calf No.	Pre-exposure*	Post-ID	Post-SC
Live bacteria/ FKB** (group 1)	54	4	4	8
	56	0	0	8
	57	0	0	4
	Mean†	1.3§	1.3§	6.7§§,#
	std. dev.	2.3	2.3	2.3
PBSS/FKB** (group 2)	348	0	0	0
	349	0	0	0
	350	0	0	0
	Mean	0	0	0
	std. dev.	0	0	0

* ELISA titer pre-exposure, following intraduodenal stimulation and following subcutaneous killed bacterin.

† Mean and standard deviation of each group.

§ Means with a different number of § are significantly different ($p<0.05$).

\# Mean is significantly different from post-SC mean of group 2 ($p<0.10$).

** Group 1 calves received 10^{12} CFU log phase P haemolytica intraduodenally followed by 10^{12} CFU of formalin (0.45%) killed bacteria subcutaneously. Group 2 calves received phosphate buffered saline solution intraduodenally followed 2 weeks later by 10^{12} CFU of formalin (0.45%) killed bacteria subcutaneously.

ID = Intraduodenally SC = Subcutaneously

Table 2.5. Pulmonary IgG titers of calves following intraduodenal inoculation with live P haemolytica or PBSS.

	Calf No.	Pre-exposure*	Post-ID	Post-SC
Live bacteria/ FKB** (group 1)	54	0	0	8
	56	0	4	32
	57	0	0	8
	Mean†	0§	1.3§	16§§,#
	std. dev.	0	2.3	13.9
PBSS/FKB** (group 2)	348	0	0	0
	349	0	0	0
	350	0	0	0
	Mean	0	0	0
	std. dev.	0	0	0

* ELISA titer pre-exposure, following intraduodenal stimulation and following subcutaneous killed bacterin.

† Mean and standard deviation of each group.

§ Means with a different number of § are significantly different ($p<0.10$).

\# Mean is significantly different from post-SC mean of group 2 ($p<0.16$).

** Group 1 calves received 10^{12} CFU log phase P haemolytica intraduodenally followed by 10^{12} CFU of formalin (0.45%) killed bacteria subcutaneously. Group 2 calves received phosphate buffered saline solution intraduodenally followed 2 weeks later by 10^{12} CFU of formalin (0.45%) killed bacteria subcutaneously.

ID = Intraduodenally SC = Subcutaneously

Table 2.6. Pulmonary LNA titers of calves following intraduodenal inoculation with live P haemolytica or PBSS.

	Calf No.	Pre-exposure*	Post-ID	Post-SC
Live bacteria/ FKB** (group 1)	54	9	8	50
	56	2	4	50
	57	8	26	50
	Mean†	6.3§	9.3§	50§§,#
	std. dev.	3.8	6.1	0
PBSS/FKB** (group 2)	348	2	4	4
	349	2	0	0
	350	2	5	3
	Mean	2	3.0	2.3
	std. dev.	0	2.7	2.1

* Leukotoxin neutralizing antibody titer as determined by vital stain uptake assay.

† Mean and standard deviation of each group.

§ Means with a different number of § are significantly different ($p<0.05$).

\# Mean is significantly different from the post-SC mean of group 2 ($p<0.05$).

** Group 1 calves received 10^{12} CFU log phase P haemolytica intraduodenally followed by 10^{12} CFU of formalin (0.45%) killed bacteria subcutaneously. Group 2 calves received phosphate buffered saline solution intraduodenally followed 2 weeks later by 10^{12} CFU of formalin (0.45%) killed bacteria subcutaneously.

ID = Intraduodenally SC = Subcutaneously

booster. However, group 1 calves showed a dramatic 25-fold increase in titer following exposure to the killed bacterin booster. This increase was significantly different ($p<0.05$). The mean peak post-SC pulmonary LNA titer for group 1 was significantly greater than the mean peak post-SC titer of group 2 ($p<0.05$).

Discussion

P haemolytica leukotoxin is considered a primary virulence factor for this organism in bovine lungs. The toxin is an immunogenic factor that induces neutralizing antibodies in rabbits.[13] In addition, antibodies in the nasal and lung washings of calves also possess the ability to neutralize the toxin.[3,4] Prevention of fibrinous pneumonia caused by P haemolytica appears to depend on the presence of leukotoxin and neutralizing antibodies.[4] Parenteral killed bacterins used to date have not been effective in inducing LNA. Evidence suggests that serum antibodies induced by killed bacterins against somatic antigens can actually increase pulmonary lesions.[1,2] Aerosolization of live bacteria is an effective means of inducing leukotoxin neutralizing antibodies in serum and pulmonary lavage fluids of calves,[3] but is impractical as a commercial method of immunization. If mucosal antibody production could be primed with the common mucosal immune system, subsequent booster injections might lead to protective mucosal antibody production.

Viable log phase P haemolytica appear to be more capable of inducing neutralizing antibodies than killed bacterin.[14] Calves in

this study given a dose of 10^{12} CFU of live P haemolytica intraduodenally did not show a resultant increase in LNA in the serum or lavage concentrates. However, a subcutaneous booster injection of a killed bacterin resulted in an increase in serum IgG and LNA as well as pulmonary IgG, IgA and LNA.

These results suggest that the live bacteria primed gut-associated lymphoblasts that subsequently circulated to other mucosal tissues. No antibodies were produced in pulmonary mucosal sites until the migrated lymphoblasts were stimulated by the killed bacterin. The fact that IgG and IgA were detected in pulmonary lavage fluids supports the suggestion by Brandtzaeg[8] that migrated lymphoblasts are polyclonal. Classical MALT immunology states that only IgA is produced by the MALT system.[15,16,17] Brandtzaeg[8] however, has suggested that lymphoblasts become activated and are coded for J chain production at the Peyer's patches, but the immunoglobulin isotype is not determined until memory lymphoblasts are stimulated a second time at seeded mucosal sites. This allows mucosal antibodies to be produced in a manner consistent with direct stimulation of the same mucosa.

The increase in serum IgG following gut priming with live P haemolytica without any increase in leukotoxin neutralizing antibodies is another interesting finding. This could be explained by the production of antibodies by Peyer's patches. It is known that Peyer's patches contain a small number of cells capable of antibody production although its primary function is antigen presentation and lymphocyte priming.[8] These antibodies could also be due to the digestion and

absorption of different antigenic components into the vascular system. The leukotoxin in the lumen may be destroyed or broken down so that it is not recognized in the portion that is absorbed and induces a central reticuloendothelial IgG response to somatic antigens alone. The leukotoxin of P haemolytica is a protein that is rather unstable and quite labile.[18,19] Digestion of the toxin in this manner would therefore be possible. The leukotoxin may also damage the intestinal mucosa allowing access of bacteria to the lamina propria where lymphocytes could be directly stimulated to produce IgG. Antibodies would then enter the lymph and enter the general circulation.

A more likely explanation for the immune response seen in this experiment is the mixture of antigens. Previous studies have indicated that soluble and particulate antigens are handled quite differently by GALT. Soluble antigens may be absorbed directly into the blood stream via GALT or via other sites in the lamina propria to stimulate a systemic immune response.[20] Particulate antigens are more likely to be processed by GALT to stimulate a mucosal immune response. Depending on previous parenteral exposure to soluble antigens, subsequent gut stimulation can induce tolerance or amplify an existing level of immunity. The presence of preexisting parenteral antibodies usually results in an amplified systemic immune response following gut stimulation with soluble antigens. Lack of any previous exposure to an antigen may result in systemic tolerance to that antigen following gut stimulation. In this experiment the mixture of bacterial antigens known to consist of many soluble antigens eg., capsular, leukotoxin,

cell wall proteins, apparently resulted in an increased systemic response following ID stimulation. The particulate antigens appeared to prime the mucosal immune system for a later antibody response following the subcutaneous booster inoculation.

The increase in serum IgG in group 2 calves may have been due to natural infection to P haemolytica concomitant to the start of this experiment or due to laboratory error in reading ELISA titers of preexposure samples. Calves inoculated with PBSS reacted with similar increase in titers as calves inoculated with live bacteria. This subclinical infection or laboratory error may have been responsible for the increase in IgG titer to somatic antigens without a concurrent increase in leukotoxin neutralizing antibodies.

It seems that priming of the antigen presenting cells of the Peyer's patches leads to the pulmonary antibody response since control calves receiving the killed bacterin alone did not show a pulmonary antibody response. The presence of leukotoxin neutralizing antibodies indicates this method of stimulation can induce the type of antibody associated with decreased pneumonic lesions. Parenteral vaccination with killed bacterins cannot induce these antibodies.[5,14]

Calves that received a priming dose of PBSS responded initially with no increase in antibody titers in serum or pulmonary lavage concentrates. A booster injection of a killed bacterin did not stimulate measurable pulmonary antibody production and only a very weak serum response compared to calves primed with live bacteria.

The antibody responses in this study are consistent with the

pattern of antibody responses seen by aerosolization of live P haemolytica to calf lungs by Moore et al.[3] The response time is also similar to Moore's previous study with peak antibody responses seen 10 to 14 days after injection of the booster dose of bacterin. In other words, stimulation of mucosal surfaces with live bacteria, whether in the gut or in the lung, can result in similar antibody responses provided that gut stimulation is boostered with a parenteral injection of a killed bacterin.

The most notable finding in this experiment is the secondary immune response of serum and pulmonary leukotoxin and neutralizing antibodies following gut priming by live P haemolytica. The induction of pulmonary leukotoxin antibodies is associated with concurrent increases in pulmonary IgG and IgA. The very high serum LNA and IgG could be crossing into the alveoli and represent a major source of pulmonary antibodies. Previous studies have shown that IgG_1, IgG_2 and IgA each possess the ability to neutralize the leukotoxin of P haemolytica.[3] The IgA and IgG titers in this experiment appear to be locally (mucosally) produced, at least in part, due to their appearance only in calves gut-primed with live bacteria. These results support the findings of Chang et al.[21] who found both IgA and IgG_1 in bovine mammary gland following intestinal stimulation by an antigen. Further studies are needed to better identify the relative importance of each antibody isotype induced by this method of inoculation.

This experiment suggests that even though killed bacterins are not efficient in inducing leukotoxin neutralizing antibodies associated

with decreased P haemolytica pneumonic lesions, they are acceptable as a "second signal" for mucosal lymphoblasts primed by Peyer's patches' processing of similar antigens. The increase in serum LNA and IgG following inoculation of killed bacterin in group 2 calves may have been partially due to a natural infection early in the experiment leading to increased serum IgG antibodies. A more likely explanation is the presence of significant leukotoxin in the bacterin preparation which induced both LNA and an IgG response in these calves. While intraduodenal priming, as used in this study is clearly useful only as an experimental vaccination approach, the results suggest inclusion of antigens in vehicles such as by-pass proteins may have potential for oral immunization of cattle.

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CHAPTER 3

ANTIGENICITY OF *PASTEURELLA HAEMOLYTICA* LEUKOTOXIN IN RABBITS

Pasteurella haemolytica is the most common bacterium isolated from feedlot cattle affected with the bovine respiratory disease complex, a a major cause of economic loss to both dairy and beef cattle producers. *P haemolytica* A-1 is the most common biotype isolated from pneumonic pasteurellosis. This organism produces a heat labile, ruminant specific leukotoxin that may be a virulence factor in the production of pneumonic lesions in calves.

The leukotoxin produced by *P haemolytica* is very labile being sensitive to heat, changes in pH, and protein splitting enzymes.[1-3] Efforts to purify the toxin have been unsuccessful. However, serum antibodies capable of neutralizing crude supernatant preparations of the toxin have been detected in cattle vaccinated with live bacteria and in cattle naturally exposed to *P haemolytica*.[4,5] The presence of serum leukotoxin neutralizing antibodies seems to be associated with decreased pulmonary lesions and mortality.

There is much interest in investigating the immunogenicity of the leukotoxin to produce a possible subunit vaccine for this organism. Investigations in laboratory animals have indicated that rabbits are capable of producing leukotoxin neutralizing antibodies to all pathogenic serotypes of *P haemolytica*.[6] These antibodies appear to be heterologous in their neutralizing activity although less so than to the homologous strain. Other investigations have examined most of the other important antigens of *P haemolytica* in mice and guinea pigs.[7]

These studies concluded that capsular antigens made the best immunogen. However, leukotoxin was not examined.

The purpose of this experiment was: (1) to determine the immunogenicity of P haemolytica's leukotoxin after treatment with heat or formalin; (2) to determine if heat or formalin treatment of the leukotoxin of P haemolytica affects its toxicity for bovine leukocytes in vitro, and (3) to compare the immunogenicity of viable log phase P haemolytica, killed P haemolytica, leukotoxin and LPS when injected into rabbits.

Materials and Methods

Animals

Experiment 1--Sixteen 10 to 14-week old female New Zealand white rabbits[a] were used. These were specific pathogen-free (including Pasteurella multocida) animals.

Experiment 2--Eight 10 to 14-week old conventionally-reared rabbits[b] were used. Both groups of rabbits were negative for the presence of P multocida prior to experimentation.

Bacterium and antigens--P haemolytica A-1 isolate 12216 originally cultured from a clinical case of pneumonia in a calf was used as a source for all antigens.

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[a] Hazelton Laboratories, Denver, PA.

[b] Myrtle's Rabbitry, Nashville, TN

Preparation of live log phase and formalin killed bacteria was as previously described (Chapter 2).

Leukotoxin for Experiment 1 was produced as previously described (Chapter 2) with the following modification. Culture medium used to grow the bacteria was supplemented with 1% rabbit serum[c] instead of calf serum. Once the bacteria-free supernatants were obtained, they were dialyzed against 3 changes of an 8-fold volume of ultrapure H_2O for 24 hours. The dialyzed supernatants were then lyophilized and stored at 4 C until used. Total protein content of the crude supernatant was determined to be 3.0 mg/ml. Total toxicity was determined to be 30 toxic units by use of the vital stain uptake assay as previously described (Chapter 2).

For injection purposes lyophilized toxin was reconstituted in RPMI-1640 to the desired total protein levels. The 1% total protein toxin preparation contained 100 toxic units as determined by the vital stain uptake assay previously described. The 2% solution contained 200 toxic units of activity.

Formalin treated toxin was reconstituted as described above with 1% buffered neutral formalin (BNF) added, held at 4 C for 24 hours and then dialyzed prior to usage against 100X RPMI-1640 for 24 hours with a total of 3 changes in medium.

Heat treated leukotoxin was reconstituted as described above and placed in a 56 C water bath for 30 minutes.

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[c] Gibco Laboratories, Madison, WI.

P haemolytica endotoxin was prepared as described by McIntyre et al.[8] It was suspended in phosphate buffered saline solution (PBSS) for injection to a concentration of 200 ug/cc or 350 ug/cc.

Control animals received phosphate buffered saline solution (PBSS) pH 7.4 in an equal volume.

Leukotoxin for Experiment 2 was prepared in a manner slightly different from that previously described in order to achieve 100-fold concentration. Toxin supernatants were prepared from cultures as described above. The crude supernatants were then concentrated using a ultrafiltration unit[d] with a 30,000 MW exclusion membrane with further concentration on a ultrafiltration stirred cell[e] with 10,000 MW exclusion membrane. Both units were sterilized prior to use with 1% NaOH. This 100-fold volume was then used as the stock solution to make all dilutions for each group of rabbits. The concentrated leukotoxin was stored at -70 C until used.

The first three injections for all rabbits were from the same preparation of toxin. The last two injections for all rabbits was derived from two other batches of toxin prepared in an identical manner and aliquoted so that each rabbit was given an exact amount of the same toxin preparation.

Biologic activity of treated leukotoxin--The biologic activity was evaluated for reconstituted lyophilized leukotoxin which was treated by

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[d] Minitan, Millipore Corp., Bedford, MA.

[e] Amicon Corp., Lexington, MA.

1% BNF, placed in 56 C water bath for 30 minutes, or dialyzed. The leukotoxin was reconstituted to its original protein content (3 mg/ml) in RPMI-1640 culture medium. One aliquot was reconstituted with 1% BNF added, mixed well, allowed to sit at 4 C for 24 hours at which time it was dialyzed against a 100-fold volume RPMI-1640 for 24 hours with 3 changes of medium. Another aliquot was placed in a 56 C water bath for 30 minutes, placed in 4 C for 24 hours, and then dialyzed as described above. Another aliquot was placed in 4 C for 24 hours and then dialyzed for 24 hours. A fourth aliquot was reconstituted and held at 4 C for 48 hours.

Biologic activity was determined for crude leukotoxin supernatants that had been stored at -70 C and treated by 1% BNF, placed in 56 C water bath for 30 minutes, or dialyzed. The leukotoxin was brought to 4 C and treated in the same manner as the reconstituted lyopholized leukotoxin.

Biologic activity of the leukotoxin was measured by the ability of the treated leukotoxin to kill BL-3 target cells. The BL-3 cells are a bovine lymphocyte cell line provided by Dr. G. Thielan, University of California. Cytotoxicity for BL-3 cells was measured by the vital stain (neutral red) uptake assay as previously described (Chapter 2).

Antibody assays--Rabbits were bled from the marginal ear vein prior to the first injection and at the time of each inoculation beginning with the third inoculation. Seven days after the last inoculation all rabbits were deeply tranquilized with Ketamine[f] and

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[f] Ketaset , Bristol Laboratories, Syracuse, NY

xylazine[g] and sacrificed by exsanguination. Assay of serum IgG antibody titers against a P haemolytica whole cell preparation was performed by ELISA as previously described with goat anti-rabbit antibody conjugated with horseradish peroxidase[h] used as the conjugate. The presence of leukotoxin neutralizing antibodies was determined by the neutral red uptake assay as previously described.

Procedure

Rabbits were divided into groups containing 2 to 3 rabbits each. Groups were inoculated as designated in Table 3.1. In Experiment 1 each rabbit received 3 doses of antigen at 4 day intervals. Each dosage was inoculated half intradermally and half subcutaneously. To stimulate higher titers antigen preparation dosages were doubled 19 days later as indicated in Table 3.1. Each rabbit was inoculated with 1% leukotoxin at day 56 as a final booster. This inoculation was also given to non-responders to determine if they would respond anamnestically to this antigen.

In Experiment 2 each group of rabbits received a different dose of leukotoxin as shown in Table 3.2. Each dosage level of toxin was administered half subcutaneously and half intradermally in three doses 4 days apart. A fourth injection was given seven days after the third injection and six days later a fifth dose was given. The 100X toxin

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[g] Rompum , Haver-Lockhart, The Baynet Division of Miles Laboratories, Shawnee, KS.

[h] Miles Laboratories, Inc., Elkhart, IN.

Table 3.1 Immunization schedule and dosages of immunogens for rabbits in Experiment 1.

	Day 4	Day 8	Day 12	Day 31	Day 56
PBSS*	1cc	1cc	1cc	1cc	Leukotoxin† 1% TP
LPS§	200ug/cc	200ug/cc	200ug/cc	350ug/cc	Leukotoxin 1% TP
Killed¶ Bacteria	$1.2x10^6$ CFU/cc	$2x10^6$ CFU/cc	$1x10^8$ CFU/cc	$1x10^8$ CFU/cc	Leukotoxin 1% TP
Live Bacteria	$1.2x10^6$ CFU/cc	$2x10^6$ CFU/cc	$1x10^8$ CFU/cc	$1x10^8$ CFU/cc	Leukotoxin 1% TP
Heat treated Leukotoxin	1% TP	1% TP	1% TP	2% TP#	1% TP
BNF treated Leukotoxin	1% TP	1% TP	1% TP	2% TP	1% TP
Leukotoxin	1% TP	1% TP	1% TP	2% TP	1% TP

* Phosphate buffered saline pH 7.4

† Lyophilized leukotoxin 10 mg/cc total protein reconstituted in RPMI-1640

§ Lyophilized lipopolysaccharide reconstituted in PBSS

¶ Inactivated by 0.45% buffered neutral formalin

TP = Total protein; CFU = Colony forming units; BNF = Buffered neutral formalin

All injections were 1 cc in volume given half intradermally and half subcutaneously.

Table 3.2. Immunization schedule for rabbits in Experiment 2.

	Day 0	Day 4	Day 8	Day 15	Day 21
Leukotoxin 100X*	1 cc†	1 cc	1 cc	1 cc	1 cc
Leukotoxin 33X	1 cc	1 cc	1 cc	1 cc	1 cc
Leukotoxin 10X	1 cc	1 cc	1 cc	1 cc	1 cc

* Leukotoxin prepared as described in Materials and Methods and concentrated by ultrafiltration. Toxin was concentrated 100-fold and diluted in RPMI-1640 to 33X or 10X.

† Each animal received a 1 cc dosage of leukotoxin injected half intradermally and half subcutaneously.

contained 44 mg/ml total protein and 5000 toxic units/ul. The concentrated toxin was diluted in RPMI-1640 to 33X and 10X concentrations so that each rabbit received an equal volume of inoculum.

Results

Results of IgG antibody responses for Experiment 1 are shown in Figures 3.1 and 3.2. In Experiment 1 each rabbit tested had no preexposure IgG specific for P haemolytica. Control rabbits exposed to PBSS had no increase in IgG titers for the entire experiment. One rabbit had an 8-fold increase in titer following 3 injections of LPS. Each rabbit treated with the killed bacterin had a 32-fold increase in titer with a peak of 128 or 256. The rabbits exposed to live bacteria had a 16 or 64-fold increase in IgG with peak titers of 128 or 256. Rabbits inoculated with heat treated leukotoxin had a 32 or 128-fold increase in IgG with peak titers of 128 or 256. Each rabbit inoculated with BNF treated leukotoxin had a 64-fold increase of IgG with a peak titer of 128, 256 or 512. Rabbits inoculated with unadulterated leukotoxin had an increase of 32, 64 or 128-fold with peak titers of 128, 256 and 512.

Results of LNA titers for Experiment 1 are shown in Figure 3.3. Groups inoculated with PBSS, LPS and killed bacterin had no detectable levels of LNA at any time. The group inoculated with live bacteria had a very mild but detectable increase in LNA following the third injection of bacteria. Animals injected with toxin treated with BNF,

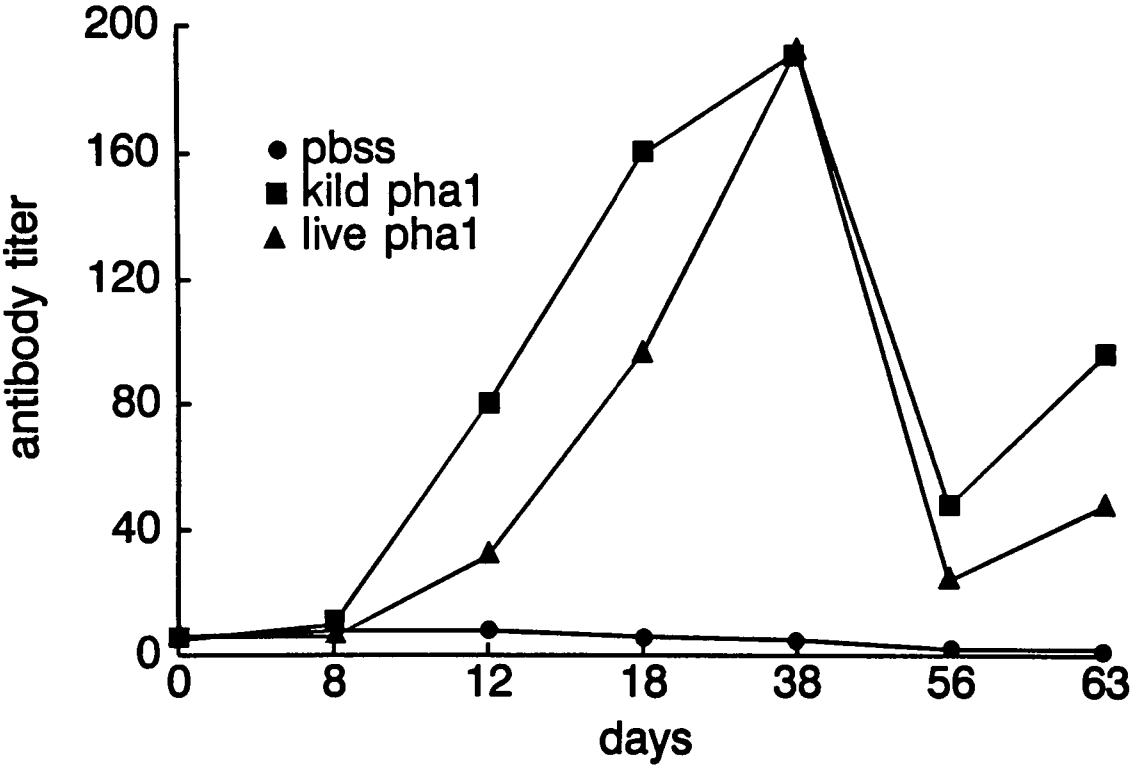

Figure 3.1. Serum IgG titers of rabbits inoculated with P haemolytica. Each group of rabbits was inoculated at day 4, 8 and 12 with an appropriate antigen, day 31 with a 2-fold strength preparation of the appropriate antigen, and at day 56 each rabbit in every group was inoculated with a 1% TP solution of leukotoxin. pbss = phosphate buffered saline solution; kild pha1 = formalin killed P haemolytica; live pha1 = log phase viable P haemolytica.

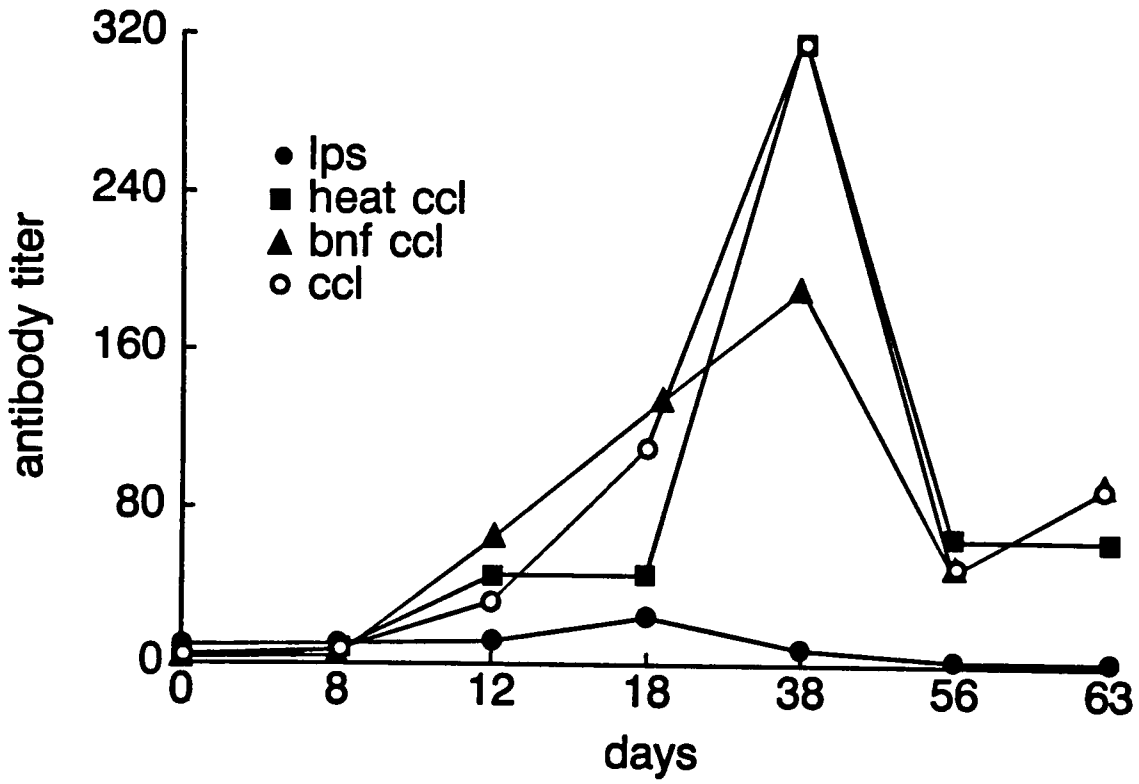

Figure 3.2. Serum IgG titers of rabbits inoculated with toxin preparations of P haemolytica. Each group of rabbits was inoculated at day 4, 8 and 12 with an appropriate antigen, day 31 with a 2-fold strength preparation of the appropriate antigen, and at day 56 each rabbit in every group was inoculated with a 1% TP solution of leukotoxin. lps = lipopolysaccharide; heat ccl = crude concentrated leukotoxin warmed to 56 C for 30 minutes; bnf ccl = crude concentrated leukotoxin rehydrated in 1% buffered neutral formalin; ccl = crude concentrated leukotoxin.

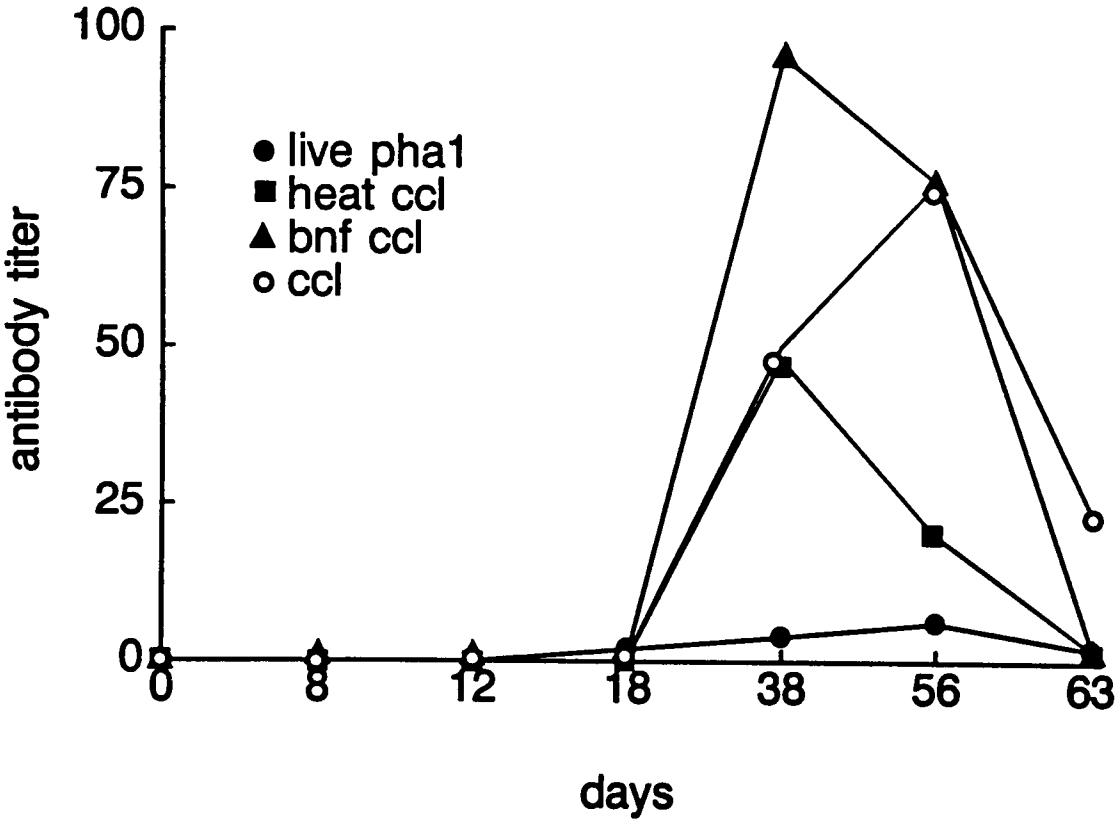

Figure 3.3. Serum leukotoxin neutralizing antibody titers of rabbits inoculated with P haemolytica or its leukotoxin. Each group of rabbits was inoculated at day 4, 8 and 12 with an appropriate antigen, day 31 with a 2-fold strength preparation of the appropriate antigen, and at day 56 each rabbit in every group was inoculated with a 1% TP solution of leukotoxin. live phal = log phase viable P haemolytica; heat ccl = crude concentrated leukotoxin warmed to 56 C for 30 minutes; bnf ccl = crude concentrated leukotoxin treated with 1% buffered neutral formalin; ccl = crude concentrated leukotoxin.

heat or nothing responded with sharp increases in titer only after the inoculation dosage was doubled. All titers peaked seven days after receiving the double dosage of toxin and remained elevated above pre-immunization levels for 18 days post injection. Inoculation with another dosage of 1X toxin did not result in any further increase in LNA levels.

Figure 3.4 compares the geometric mean of peak serum IgG responses to peak LNA. Very low to nondetectable levels of LNA were found in rabbits that had very large increases in serum IgG (killed and live bacteria). The largest LNA titer was in rabbits inoculated with BNF treated leukotoxin. Rabbits injected with untreated or heat treated leukotoxin had very good LNA responses.

In Experiment 2 serum IgG peaked at a higher level in the 100X group versus 33X or 10X (the lowest) (Fig. 3.5). All had very large increases in titer (10 to 50-fold). LNA titers peaked in a similar manner (Fig. 3.6). The 100X group had the highest titer followed by the 33X and 10X groups of rabbits with detectable titers 4 days following the first injection for the 100X and 33X groups. Animals in the 10X group had very little response until day 15 and had a noticeably lower peak response.

The results of the in vitro biologic activity of lyophilized toxin is shown in Figure 3.7. Treatment of reconstituted lyophilized leukotoxin by heat or dialysis resulted in loss of toxic activity. The toxin reconstituted to its original protein content contained 28 toxic units/ul. A toxic unit is defined as the inverse dilution of toxin

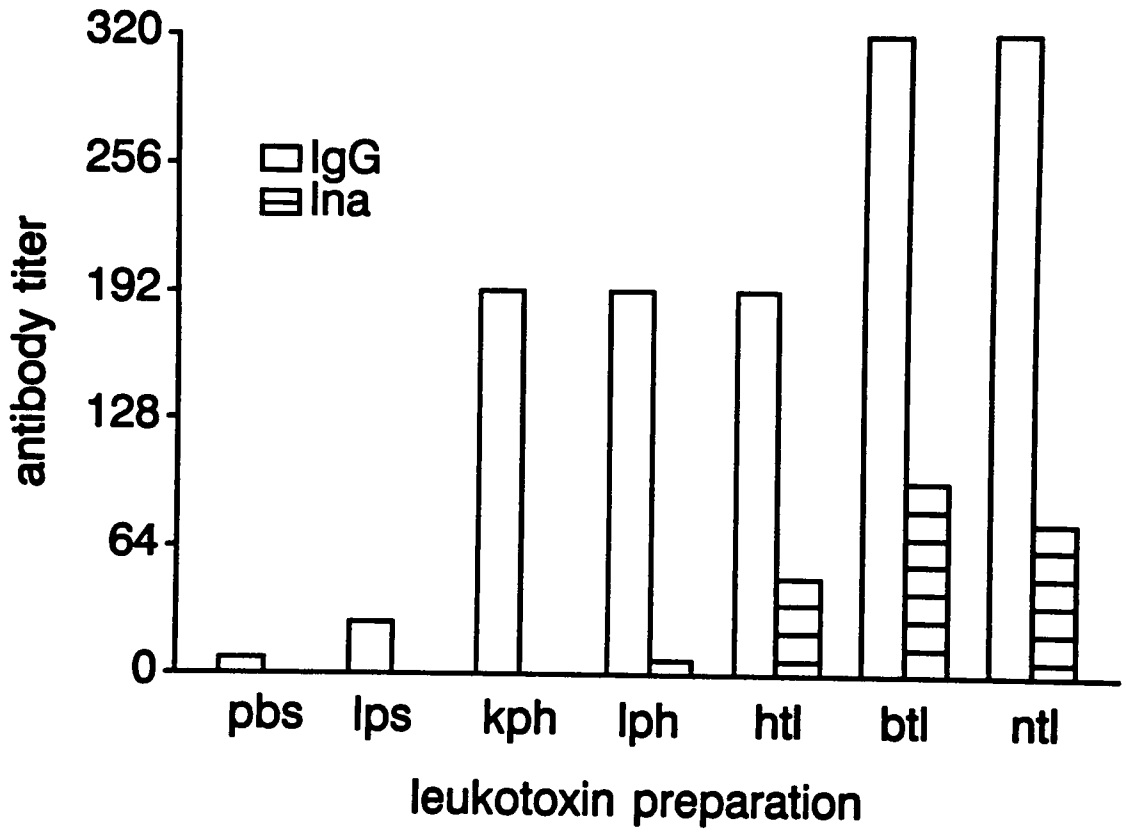

Figure 3.4. Peak mean serum IgG and leukotoxin neutralizing antibody titers of rabbits inoculated with P haemolytica or its leukotoxin. IgG = serum IgG; lna = leukotoxin neutralizing antibody; pbs = phosphate buffered saline; lps = lipopolysaccharide; kph = formalin killed P haemolytica; lph = log phase viable P haemolytica; htl = crude concentrated leukotoxin (heat treated); btl = crude concentrated leukotoxin (buffered neutral formalin treated); ntl = crude concentrated leukotoxin (untreated).

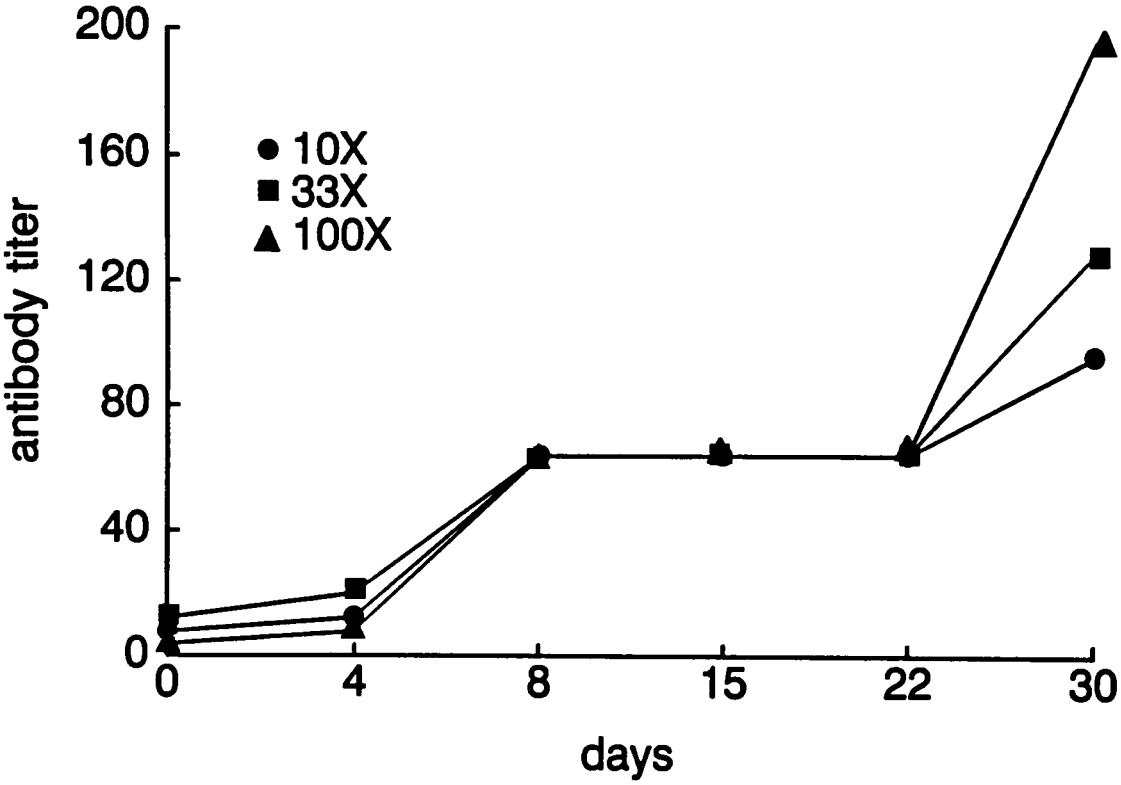

Figure 3.5. Serum IgG titers of rabbits inoculated with various dilutions of crude concentrated leukotoxin of P haemolytica. Rabbits in each group were inoculated at day 0, 4, 8, 15, and 21. 10X = 10-fold; 33X = 33-fold; 100X = 100-fold concentration by volume of crude leukotoxin supernatants.

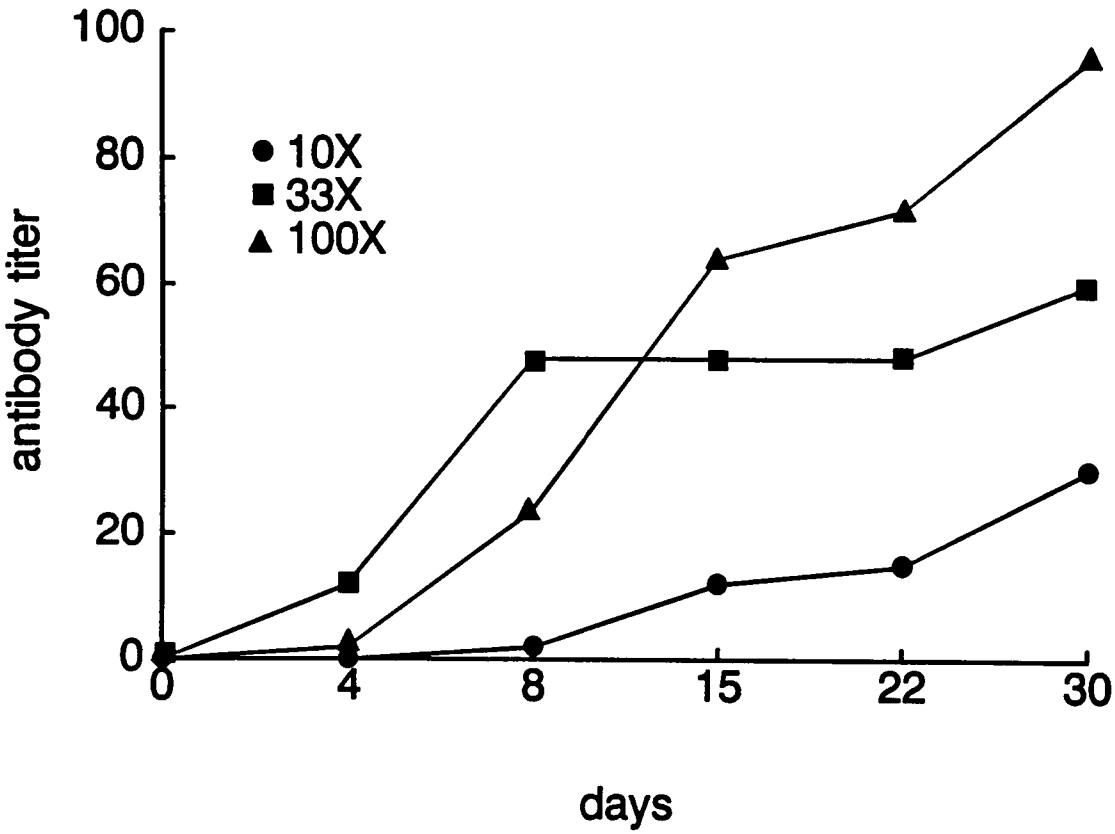

Figure 3.6. Serum leukotoxin neutralizing antibody titers of rabbits inoculated with various dilutions of crude concentrated leukotoxin of P haemolytica. Rabbits in each group were inoculated on day 0, 4, 8, 15 and 21. 10X = 10-fold; 33X = 33-fold; 100X = 100-fold concentration by volume of crude leukotoxin supernatants.

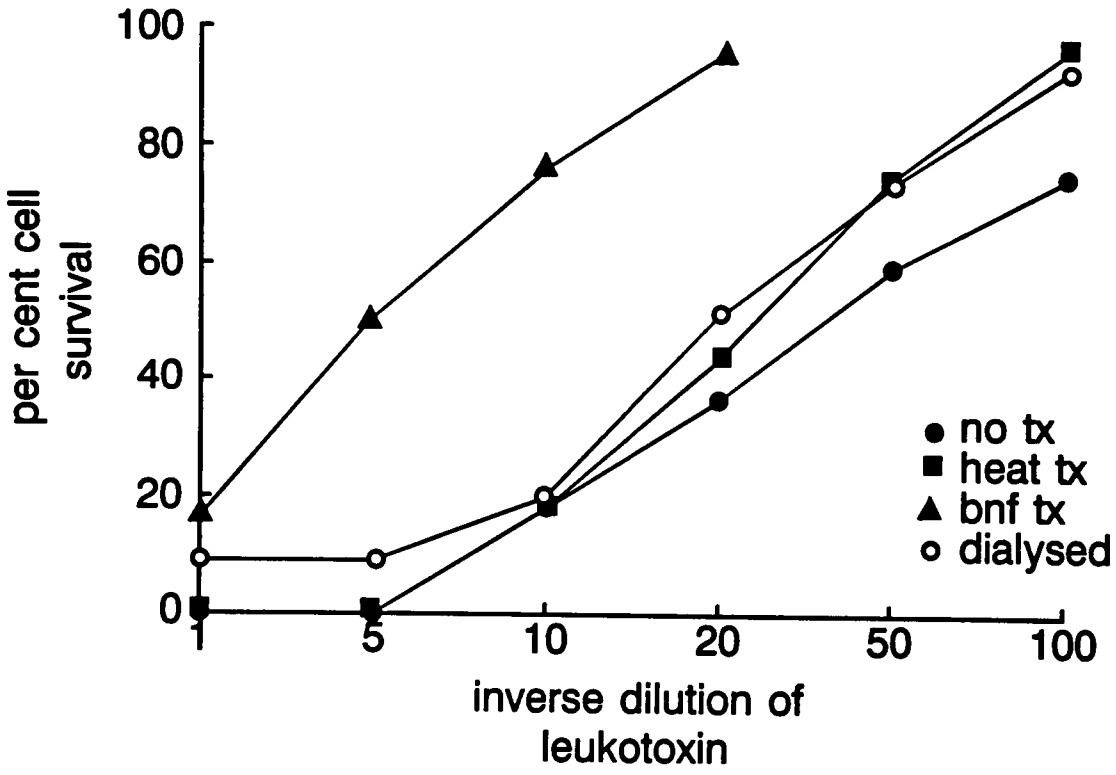

Figure 3.7. Leukotoxic activity of reconstituted lyophilized leukotoxin of P haemolytica. Percent BL-3 lymphocytes that survived exposure to serial dilutions of lyophilized leukotoxin reconstituted to the level of protein present in the crude supernatant state (3 mg/ml). no tx = reconstituted leukotoxin with no treatment; heat tx = reconstituted leukotoxin placed in 56 C water bath for 30 minutes; bnf tx = leukotoxin reconstituted in culture medium containing 1% buffered neutral formalin; dialysed = reconstituted leukotoxin dialysed against RPMI-1640.

capable of killing 50% of target cells. Treatment of reconstituted leukotoxin by dialysis against RPMI-1640 resulted in a reduction of its activity to 18 toxic units of activity. Exposure of the lyophilized toxin to 56 C water bath for 30 minutes resulted in a reduction of activity to 21 toxic units. The greatest reduction in activity was induced by 1% BNF which had 3.1 toxic units.

Results of treatment of the crude leukotoxin supernatants are shown in Figure 3.8. Untreated leukotoxin had 15 toxic units activity. Dialysis at 4 C reduced the activity to 4 toxic units, a 73% loss of activity. Incubation of leukotoxin at 56 C for 30 minutes reduced the toxic activity to 3 units which is an 80% loss of activity. Treatment by 1% BNF reduced the toxic activity to 0 with no less than 66% viability of target cells for the undiluted toxin.

Discussion

The leukotoxin of P haemolytica is postulated to be a primary virulence factor of the organism. Antibodies capable of neutralizing this leukotoxin have been associated with decreased pulmonary lesions and decreased morbidity in feedlot situations as well as challenge experiments.[4,5] In cattle the presence of humoral IgG against somatic antigens of P haemolytica does not appear to correlate well with the presence of neutralizing antibodies.[5] IgG directed against somatic antigens has been shown to actually increase pulmonary lesions.[9,10]

Supernatants of P haemolytica culture contain considerable amounts

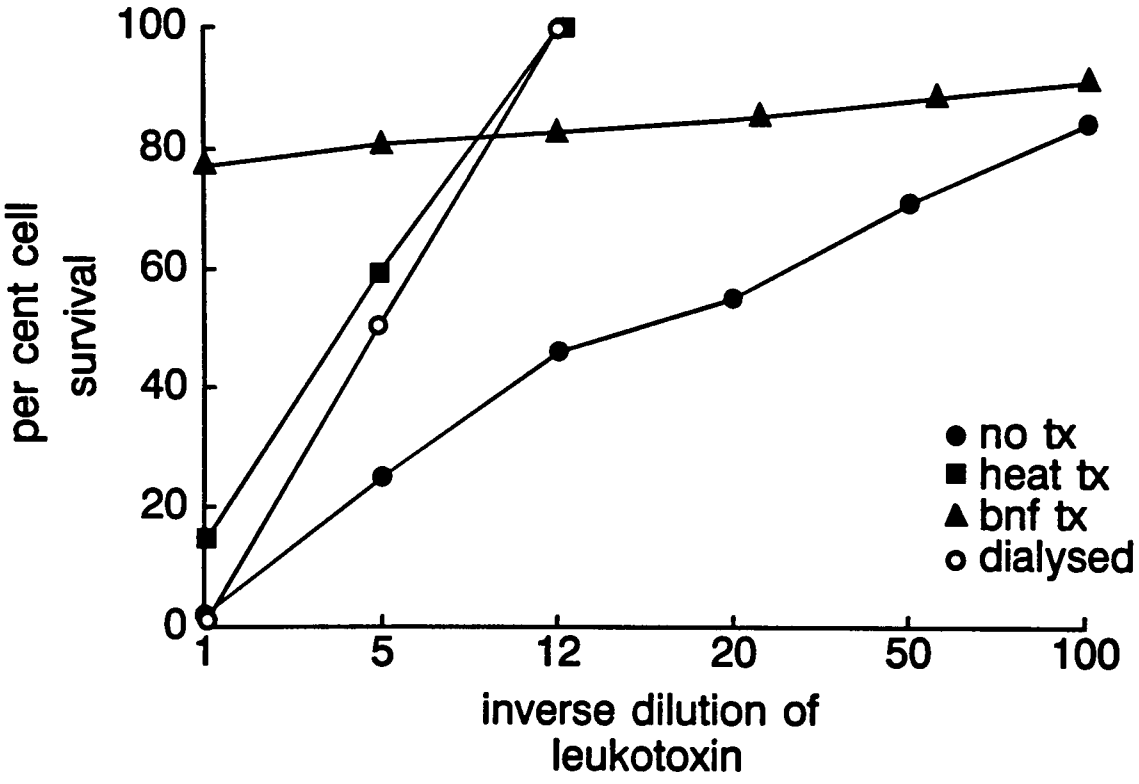

Figure 3.8. Leukotoxic activity of crude supernatant leukotoxin of P haemolytica. Percent BL-3 lymphocytes that survived exposure to serial dilutions of crude supernatant leukotoxin. no tx = untreated leukotoxin supernatants; heat tx = supernatant leukotoxin placed in 56 C waterbath for 30 minutes; bnf tx = supernatant leukotoxin with 1% buffered neutral formalin added; dialysed = supernatant leukotoxin dialysed against RPMI-1640.

of leukotoxin activity. Shewen et al.[4] have shown that the leukotoxin of various serotypes of P haemolytica can induce neutralizing antibody production in rabbits. However, no investigation of toxoid preparations has ever been undertaken in laboratory animals. Lipopolysaccharide constitutes a large proportion of the P haemolytica cell.[11] Little information is available concerning the immunogenicity of this component of P haemolytica although one study has indicated that anti-LPS antibodies have no correlation with lung lesions.[12]

This study confirmed that LPS is a poor inducer of specific antibodies when administered in doses that are normally quite toxic to rabbits. No rabbit responded adversely to any injection, including LPS, when given intradermally. This may indicate that rabbits were not sensitive to the LPS of P haemolytica. The antibodies induced in the rabbits injected with LPS had no capacity to neutralize leukotoxin. It is possible that these dosages were too great and depressed the immune system instead of having a mitogenic effect. The immunomodulatory effects of endotoxin are dose dependent.[13]

The presence of a good humoral IgG response to killed bacteria and live bacteria is not suprising as these have been well documented in cattle.[5,10] Killed bacteria induced no LNA which is consistent with reports in cattle, but the poor response to live bacteria contradicts results of cattle experiments. Perhaps this is due to an inability of the organism to proliferate as well in rabbits and therefore less leukotoxin was elaborated in vivo resulting in a poorer response. P haemolytica is not considered pathogenic for rabbits suggesting an inherent host resistance to this organism.

Equally good IgG and LNA antibody responses were found in rabbits inoculated with BNF treated or untreated leukotoxin. The serum IgG titer was similar between these antigens with BNF treated leukotoxin actually inducing greater LNA titers. This may be due to a change induced by BNF in the structure of leukotoxin exposing more immunogenic epitopes or due to a change in the leukotoxin configuration that rendered it more immunogenic. Both these preparations were better than heat treated leukotoxin in the induction of IgG and LNA. It is somewhat surprising that heat treated leukotoxin had any immunogenic effect since its biologic activity has been shown to be drastically reduced following heat treatment as used in this experiment.[14] The loss of the biologic activity suggests that the epitopes recognized immunogenically are somewhat different (or more protected) than the structured components that induce toxicity in receptor cells. The heat treatment of lyopholized leukotoxin and primary leukotoxin confirmed a decrease in biologic activity (Fig. 3.7 & 3.8). However, lyophilized leukotoxin had a much smaller decrease in activity following heat treatment than the primary leukotoxin. This suggests that the rigorous dialysis and/or freeze drying process resulted in protection of leukotoxicity in some manner perhaps by increasing binding of other serum proteins present in the media to the leukotoxin.

The level of toxin used in Experiment I was approximately 3 times greater than the primary isolated toxin. Injection of rabbits with 3-fold concentrated leukotoxin induced higher levels of LNA than any of the more concentrated toxin preparations used in Experiment 2. All of

these preparations were capable of inducing the production of LNA and IgG in rabbits. The production of similar levels of antibody caused by widely variable amounts of leukotoxin could be due to several factors. There is some difference in leukotoxin activity between batches of toxin produced. These experiments used different batches of toxin which could explain some difference in antigenicity. However, assays to determine toxic units present in each batch showed batches to be consistent in activity (data not shown). The animals used in Experiment 2 may have had preexisting cross reacting antibodies to P multocida or other gram negative bacteria. Although P multocida was not isolated from these rabbits, they could have been exposed previously. These antibodies may have partially blocked recognition of P haemolytica antigens. The method of concentration of toxin was also different. In order to attain a 100X concentration, toxin was concentrated at 4 C by ultrafiltration since lyophilized toxin could not be reconstituted greater than 3X. These differences in toxin and blocking antibody may explain why Experiment 1 rabbits had much higher antibody titers than rabbits in Experiment 2.

The effects of Experiment 2 suggest that there was a dose dependent response of rabbits with this antigen preparation. Rabbits inoculated with 33X or 100X leukotoxin had an increasingly better leukotoxin and IgG response (Fig. 3.5 & 3.6). This could be due to several factors. It is known that this toxin adversely affects leukocytes only of ruminants.[15] The effects on rabbits and other laboratory animals is not known. Although cells of other species

(human, pig, horse)[15] appear to be resistant to this toxin, perhaps at higher dosages inhibition of lymphocytes occurs. In this experiment there appears to be a somewhat selective inhibition with a greater effect on lymphocytes producing antibodies specific for somatic antigens as opposed to the leukotoxin itself. This could be explained by the dose dependent effect of LPS on lymphocytes where low doses stimulate antibody production and higher doses inhibit antibody production.[13] It would be very interesting to note if the same phenomenon occurs in ruminants exposed to a large dosage of leukotoxin.

In summary, leukotoxin antigen preparations appear to be more capable than LPS or bacteria of inducing a LNA response in rabbits with BNF and untreated toxin preparations superior to a heat treated toxin. Concentrated toxin preparations of 33X or 100X are comparable to lesser concentrations in their ability to induce LNA production. Although killed or live bacteria and LPS can stimulate an IgG response in rabbits, there does not appear to be any correlation with serum IgG and the presence of LNA. Any subunit cattle vaccine must take into consideration the ability of the preparation to induce antibodies capable of neutralizing specific virulence factors and not just a response to somatic antigens of P haemolytica. Handling of this toxin is critical as heat or the method of concentration can adversely affect immunogenicity. Certain treatments of the leukotoxin such as BNF can possibly increase immunogenicity of this leukotoxin and should be taken into consideration in any toxoid preparation for testing in cattle.

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CHAPTER 4

USE OF CONCENTRATED CRUDE LEUKOTOXIN OF PASTEURELLA HAEMOLYTICA AS AN INTRADUODENAL AND SUBCUTANEOUS INOCULANT IN CALVES

Previous experiments demonstrated the efficacy of priming the gut-associated lymphatic tissue (GALT) with viable Pasteurella haemolytica and boostering subcutaneously with formalin killed bacteria (Chapter 2). A more commercially appealing method to immunize animals by GALT priming would require using non-viable organisms or perhaps a subunit virulence factor. The importance of the leukotoxin as a virulence factor of P haemolytica in killing ruminant macrophages, lymphocytes and neutrophils has been established.[1,2] The importance of antibodies capable of neutralizing this toxin in reducing pulmonary pathology has been suggested by Gentry et al.[3] and Cho.[4]

Investigations to determine the pathogenicity of the leukotoxin in cattle have determined that direct administration of this toxin to the lungs can induce pulmonary edema.[a] The administration of killed P haemolytica transtracheally to the lung has also been associated with induction of pneumonic lesions in goats.[5] Parenterally administered dermonecrotic toxin of another organism (Pasteurella multocida) to swine has induced severe physiological changes including turbinate

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[a]Slocombe RF, Derksen FJ, Robinson NE. Pathogenic effects of intratracheally administered Pasteurella haemolytica cytotoxin on neutropenic and normal dairy calves. (abst.) Proceedings 14th World Congress on Diseases of Cattle, pp. 567-572, 1986.

atrophy associated with atrophic rhinitis.[b] These studies suggest that the leukotoxin of P haemolytica may be an important virulence factor in the pathogenesis of pneumonic pasteurellosis.

Administration of leukotoxin intraduodenally could be a way to avoid the adverse systemic or pulmonary effects of this toxin. Antigens delivered in this manner may become more immunogenic even though, and perhaps because, gastric enzymes may alter the antigens.[6] Injection of rabbits with preparations of P haemolytica leukotoxin has shown it to be dermonecrotic.[7] Investigations by this laboratory resulted in minimal dermonecrocity in rabbits while inducing leukotoxin neutralizing antibodies. Based on these data, the purpose of this experiment was to prime calves with concentrated crude leukotoxin (CCL) intraduodenally and then booster them subcutaneously two weeks later with CCL to determine the efficacy of a leukotoxin preparation to induce pulmonary leukotoxin neutralizing antibodies in calves.

Materials and Methods

Calves--Calves were purchased locally as previously described. Intraduodenal catheters were surgically implanted as described earlier Chapter 2).

Bacterial immunogen--Supernatants of P haemolytica were prepared as previously described (Chapter 2) and concentrated 100-fold using a

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[b]Williams PP, Hall MR, Rimler RB. Host response to Pasteurella multocida toxin in pseudorabies-infected swine (abst.) 67th Annual Meeting Research Workers in Animal Disease, No. 70, 1986.

Minitan ultrafiltration[c] system with 30,000 MW exclusion membranes. Several batches of leukotoxin were prepared with each containing approximately 50 toxic units of leukotoxin activity per ml prior to concentration. These batches of concentrated toxin were pooled prior to administration to calves so that each animal received an identical dose of leukotoxin both intraduodenally and subcutaneously. Each dosage contained 1.5 ug lipopolysaccharide (LPS)/kg body weight of calf as deterimined by limulus lysate assay and 3000 toxic units leukotoxin/kg body weight of calf as determined by neutral red uptake assay. Calves received a total volume of 25 cc of CCL intraduodenally on day 4. Intraduodenal inoculation was followed by 50 cc flush of phosphate buffered saline. On day 18 of the experiment each calf received 25 cc of CCL subcutaneously in the right cervical region.

Pulmonary lavage--Pulmonary lavage and blood samples were acquired on a weekly basis beginning 4 days prior to intraduodenal exposure to the CCL. Lavage samples and blood were processed as previously described (Chapter 2).

Antibody assays--Serum was assayed for the presence of IgG specific for P haemolytica somatic antigens as well as for the presence of leukotoxin neutralizing antibodies (LNA). Lavage samples were assayed for the presence of IgA, IgG and LNA. All assays used were the same as described in Chapter 2.

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[c]Minitan, Millipore Corp., Bedford, MA.

Pulmonary lavage concentrates were assayed for the presence of IgG and IgA specific in activity to P haemolytica and for leukotoxin neutralizing antibodies.

Immunization schedule--Leukotoxin inoculants were prepared as previously described (Chapter 3, Experiment 2). A schedule of immunizations and procedures performed on calves is shown in Table 4.1.

Procedure

Calves were purchased and acclimated for 10 days prior to surgery. Calves were divided into 2 groups with 3 calves per group. Intraduodenal (ID) catheters were placed under general anesthesia; animals were allowed to recover 10 days post surgery. On day 0 of the experiment preexposure pulmonary lavages and blood samples were drawn. Four days later each calf received an ID inoculation of either 25 cc of CCL or phosphate buffered saline solution (PBSS). Two weeks later the calves that received an ID dose of CCL were inoculated subcutaneously (SC) with 25 cc of CCL. Calves that received an ID injection of PBSS were inoculated subcutaneously with 10^{12} CFU of formalin killed bacterin. Pulmonary lavages were performed and blood samples were taken weekly throughout the experiment.

Following the SC inoculation of CCL, two of three calves died. A complete necropsy was performed on these calves at this time. The surviving calf was euthanized at the end of the experiment and a complete necropsy was performed.

Antibody titers were determined by ELISA (IgG, IgA) and vital stain uptake assays (leukotoxin neutralizing antibodies = LNA).

Table 4.1. Schedule of procedures and immunizations of calves.

Procedure	Pulmonary Lavage*	25cc CCL or PBSS ID†	Pulmonary Lavage	25cc CCL or FKB SC§	Pulmonary Lavage	PM Exam
Day	0	4	7,14	18	21,28,35,42	43

* Blood serum was collected each day a pulmonary lavage was performed.

† One hundred-fold concentrated crude leukotoxin or phosphate buffered saline solution administered intraduodenally.

§ One hundred-fold concentrated crude leukotoxin or formalin killed bacterin administered subcutaneously.

CCL = Concentrated crude leukotoxin PBSS = Phosphate buffered saline solution ID = Intraduodenally FKB = Formalin killed bacterin SC = Subcutaneously PM = Post mortem

Results

Pathology--All three experimental calves demonstrated clinical signs of endotoxemia following the subcutaneous inoculation of concentrated leukotoxin. Within 45 minutes all three calves exhibited clinical signs of tachypnea, sweating, elevated temperatures of 103 to 105 F, salivation, and diarrhea. One calf died 6 to 10 hours following inoculation and another died 18 hours post-inoculation.

Gross lesions of calf #326 indicated the presence of mild pleural petechiation, ecchymoses of the thoracic aorta adventitia, widespread pulmonary edema with intralobular septal edema, and the presence of moderate amounts of serosanguineous fluid in bronchi. Histopathological lesions were mostly confined to the lung. Interlobular septa and lymphatics were distended due to edema; intraalveolar edema with fibrin was also present in some areas. In areas of extensive edema numerous microthrombi were noted within alveolar capillaries. Extensive necrosis in the kidneys of the thick loops of Henle was detected.

In calf #327 gross lesions indicated the presence of disseminated petechia and ecchymoses. The cause of death in this animal could be attributed to a diffuse subdural cerebellar hemorrhage. Gross lesions observed include desseminated petechial and ecchymotic hemorrhages including adventitia of the esophagus, pleura of the pericardial sac, epicardium, testes, serosal surface of rumen and reticulum, and masseter muscles; diffuse subdural hematoma into the cerebellum; and marked congestion of the lung. Histological lesions noted include

microthrombi and congestion of interalveolar septa; alveoli distended with fibrin; mild intraalveolar infiltrates of neutrophils, macrophages and erythrocytes; edema of interlobular septa and lymphatics. Renal glomeruli contained microthrombi with minimal necrosis of the thick segments of the loops of Henle. The epicardium and myocardium contained extensive hemorrhage. Lesions were consistent with early fibrinopurulent pneumonia and disseminated intravascular coagulopathy (DIC). No microbiological cultures were performed due to the absence of gross lesions suggestive of pneumonia.

Calf #328 survived the subcutaneous injection of leukotoxin and was euthanized and necropsied at the end of the experiment four weeks later. The only gross lesion evident was an area of pneumonia in the right caudal lung lobe, 3cm x 3cm in size. No bacteria or mycoplasma were isolated from the lesion. Histologically the affected part of the lung suggested an acute inflammatory process was present. This was characterized by filling of the alveolar spaces with fibrin, edema fluid, macrophages, eosinophils and neutrophils. Areas adjacent to this lesion and other sections of lung demonstrated mild to moderate atelectasis and moderate parabronchial hyperplasia.

Immune responses--Immune responses of experimental calves are shown in Tables 4.2 through 4.6. Two of three calves inoculated ID with CCL followed by a subcutaneous booster of CCL (CCL/CCL) demonstrated a 4-fold increase in serum IgG, lavage IgG, and lavage IgA following intraduodenal stimulation with the leukotoxin. Serum LNA titers increased from 4 to 32-fold in each calf. Lavage LNA titers increased 3 to 8-fold in two of three calves.

Table 4.2. Serum IgG titers of calves inoculated intraduodenally with concentrated crude leukotoxin or PBSS.

	Calf No.	Pre-exposure*	Post-ID	Post-SC
CCL/CCL† (group 1)	326	16	64	ND
	327	32	128	ND
	328	64	64	1024
	Mean§	37.3	85.3	1024
	std. dev.	24.4	37.0	ND
PBSS/FKB** (group 2)	348	8	32	128
	349	16	64	128
	350	16	64	128
	Mean	13.3	53.3	128
	std. dev.	4.6	18.5	0

* ELISA titers preexposure, after intraduodenal inoculation and after the subcutaneous booster inoculations.

† Concentrated crude leukotoxin intraduodenally/concentrated crude leukotoxin subcutaneously.

§ Mean and standard deviation.

** Phosphate buffered saline intraduodenally/formalin killed bacterin subcutaneously.

ND = Not done

Table 4.3. Pulmonary IgG titers of calves inoculated intraduodenally with concentrated crude leukotoxin or PBSS.

	Calf No.	Pre-exposure*	Post-ID	Post-SC
CCL/CCL† (group 1)	326	0	8	ND
	327	0	8	ND
	328	0	0	16
	Mean§	0	5.3	16
	std. dev.	0	4.6	ND
PBSS/FKB** (group 2)	348	0	0	0
	349	0	0	0
	350	0	0	0
	Mean	0	0	0
	std. dev.	0	0	0

* ELISA titers preexposure, after intraduodenal inoculation and after the subcutaneous booster inoculations.

† Concentrated crude leukotoxin intraduodenally/concentrated crude leukotoxin subcutaneously.

§ Mean and standard deviation.

** Phosphate buffered saline intraduodenally/formalin killed bacterin subcutaneously.

ND = Not done

Table 4.4. Serum LNA titers of calves inoculated intraduodenally with concentrated crude leukotoxin or PBSS.

	Calf No.	Pre-exposure*	Post-ID	Post-SC
CCL/CCL† (group 1)	326	50	180	ND
	327	16	380	ND
	328	64	256	700
	Mean§	43.3	272.0	700
	std. dev.	24.3	100.0	ND
PBSS/FKB** (group 2)	348	70	75	90
	349	40	75	130
	350	0	0	75
	Mean	36.7	50.0	98.0
	std. dev.	35.0	43.0	28.0

* Leukotoxin neutralizing titer determined by vital stain uptake assay.

† Concentrated crude leukotoxin intraduodenally/concentrated crude leukotoxin subcutaneously.

§ Mean and standard deviation.

** Phosphate buffered saline intraduodenally/formalin killed bacterin subcutaneously.

ND = Not done

Table 4.5. Pulmonary LNA titers of calves inoculated intraduodenally with concentrated crude leukotoxin or PBSS.

	Calf No.	Pre-exposure*	Post-ID	Post-SC
CCL/CCL† (group 1)	326	4	12	ND
	327	3	24	ND
	328	6	8	16
	Mean§	4.3	14.7	16
	std. dev.	1.7	8.3	ND
PBSS/FKB** (group 2)	348	2	4	4
	349	2	0	0
	350	2	5	3
	Mean	2	3	2.3
	std. dev.	0	2.7	2.1

* Leukotoxin neutralizing titer determined by vital stain uptake assay.

† Concentrated crude leukotoxin intraduodenally/concentrated crude leukotoxin subcutaneously.

§ Mean and standard deviation.

** Phosphate buffered saline intraduodenally/formalin killed bacterin subcutaneously.

ND = Not done

Table 4.6. Pulmonary IgA titers of calves inoculated intraduodenally with concentrated crude leukotoxin or PBSS.

	Calf No.	Pre-exposure*	Post-ID	Post-SC
CCL/CCL† (group 1)	326	0	4	ND
	327	0	16	ND
	328	0	0	0
	Mean§	0	6.7	0
	std. dev.	0	8.3	ND
PBSS/FKB** (group 2)	348	0	0	0
	349	0	0	0
	350	0	0	0
	Mean	0	0	0
	std. dev.	0	0	0

* ELISA titers preexposure, after intraduodenal inoculation and after the subcutaneous booster inoculations.

† Concentrated crude leukotoxin intraduodenally/concentrated crude leukotoxin subcutaneously.

§ Mean and standard deviation.

** Phosphate buffered saline intraduodenally/formalin killed bacterin subcutaneously.

ND = Not done

For control calves inoculated ID with PBSS and followed by a subcutaneous booster of formalin killed bacterin (FKB) (PBSS/FKB) the only notable increase in titer following intraduodenal inoculation was in serum IgG.

Two of three experimental calves died following the subcutaneous booster dose of leukotoxin. The surviving calf (#328) demonstrated a 16-fold increase in serum IgG compared to no increase following ID stimulation. This calf also experienced a further 3-fold increase in LNA in addition to the 4-fold increase following ID stimulation. The lavage IgG titer in this calf increased 8-fold while the LNA increased another 2-fold. The post-SC increases in titer for serum IgG, pulmonary lavage IgG, and serum LNA were all increased compared to were preexposure or post ID titers. The pulmonary LNA titer was greater than the preexposure titer. There was no change in lavage IgA which remained at undetectable levels the duration of the experiment for this calf.

Post-SC serum and pulmonary IgG titers in calves inoculated with an ID dose of CCL were different from calves inoculated ID with PBSS. Post-ID and post-SC serum LNA titers were greater in CCL/CCL calves compared to PBSS/FKB calves. Post-ID and post-SC pulmonary LNA and pulmonary IgG titers were greater in CCL/CCL calves compared to PBSS/FKB calves.

The control calves (PBSS/FKB) had increased serum IgG following SC inoculation by the killed bacterin. The serum LNA titers also increased moderately. There were no detectable levels of pulmonary IgG

or IgA at any time during the experiment. Pulmonary LNA titers remained at barely detectable levels throughout the experiment.

Discussion

Although the presence of leukotoxin neutralizing antibodies has been suspected of playing an important role in preventing P haemolytica induced pneumonia, calves have not been immunized with concentrated dosages of this subunit component. Exposure of rabbits to the leukotoxin has indicated it is capable of inducing a neutralizing antibody response.[9] The present investigation sought to examine the induction of LNA humorally and at mucosal surfaces following stimulation of GALT by concentrated leukotoxin.

Stimulation of GALT resulted in a 4-fold increase in serum IgG titers in 2 of 3 calves within 4 days of receiving the leukotoxin. The titer obtained (64 to 256) was similar to levels seen when calves were inoculated intraduodenally with viable bacteria (Chapter 2). The secondary serum IgG response of the surviving calf also increased to a titer similar to that seen following stimulation by live bacteria (1024 vs 700).

Each of the animals inoculated intraduodenally with CCL in this study demonstrated a 4-fold or greater increase in serum LNA following the GALT stimulation. Calves that received live bacteria intraduodenally also demonstrated increases in serum LNA following the booster inoculation (Chapter 2). The titers increased quite dramatically to 700 to 1024. A similar titer (1024) was seen in the one surviving calf in this study following the booster injection.

Calves inoculated ID with CCL in this experiment had greater serum (IgG and LNA) and pulmonary (IgG, IgA, LNA) antibody titers than calves inoculated with PBSS. These results suggest that even though inoculation by the formalin killed bacterin did result in elevated leukotoxin neutralizing antibodies, the calves inoculated with CCL had much greater titers. Previous studies[3] have suggested that formalin killed bacterins do not induce serum LNA. This apparent contradiction is probably the result of the method of preparation of the bacterins. In this study bacteria with log phase of growth were used. These organisms were grown in broth culture, centrifuged and resuspended in PBSS without any steps taken to work the cells further. It is highly likely that some leukotoxin was still being elaborated by these organisms at the time of formalin treatment. Inoculation of rabbits with formalin treated crude leukotoxin did not adversely affect the immune response to leukotoxin (Chapter 3). These results indicate that use of leukotoxin as an ID inoculum can induce significant LNA antibody responses, both in serum and pulmonary lavage fluids. At this time the role of pulmonary lavage LNA in protection from disease is unknown. Further studies are required to determine what level of LNA titers are necessary for protection.

The 4-fold increase in serum IgG in calves inoculated ID with PBSS is an unexpected finding. If this were the result of a natural or accidental exposure to P haemolytica, increases in other antibody titers would have been expected over the same time period. There were no other increases in any titer including LNA.

The pattern of antibody response in this experiment suggests that these animals responded to an immunogen composed of particulate and soluble components. The classical GALT response to particulate antigens is usually a priming or direct secretion of IgA which was evident in all three calves. The elevated pulmonary IgA response in this experiment is consistent with observed GALT responses in other species as well as sheep.[10] Furthermore, these data are compatible with studies of Chang et al.[11] in cattle which noted an elevation in mammary and humoral IgG following GALT stimulation. This is often seen with soluble antigens that can be readily absorbed. These results could be due to the concentrated crude leukotoxin containing both particulate and soluble antigens. The antigens may be stable in the duodenum, at least in the time it takes to reach the follicle associated epithelium to be taken up, processed by macrophages to stimulate lymphocyte response, and then enter into the general circulation.

These data also support the observation that certain orally administered immunogens can booster a systemic immune response to the same antigen.[12] This appears to occur when mucosal surfaces are stimulated by pathogenic factors that can readily pass mucosal barriers and adversely affect the host. P haemolytica possesses capsular components that are easily dissolved as well as several proteins in its outer cell wall. The capsule, LPS, leukotoxin and other surface proteins may be equally capable of being readily absorbed across mucosal surfaces.

Postmortem lesions in calves that died in this experiment were compatible with those seen in experimental animals exposed to LPS. Analysis of concentrated leukotoxin preparations used in this experiment detected the presence of 1.5 ug/kg body weight of LPS. Although it has been accepted that LPS is a considerable component of the mass of P haemolytica,[7] detectable levels are inconsistently found in crude leukotoxin preparations. Ultra-concentration of leukotoxin apparently enhanced the levels of LPS in the immunogenic preparation to detectable levels. It appears that the LPS was a major contributory factor in the demise of these two animals.

Histologic examination of the third calf suggested the presence of eosinophils, macrophages and neutrophils in the lymph node and lung at necropsy. Assays of the pulmonary fluids and serum of all three calves by another laboratory[d] unaware of this diagnosis detected demonstrable increases of serum and pulmonary IgE in these calves prior to the second injection of CCL. This suggests that the death of these calves could be attributed in part to a hypersensitivity reaction. The presence of increased levels of serum and pulmonary IgG in the two calves that died may also indicate hypersensitivity as a result of elevated opsonizing antibodies that activated complement and may also have accelerated the usage of fibrinogen in the coagulation cascade.

Results of this experiment suggest that the crude leukotoxin supernatants retain their immunogenicity when introduced to the host

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[d]Dr. Laurel Gershwin, University of California, Department of Veterinary Microbiology and Immunology, Davis, CA.

via the intraduodenal route; at the same time severe physiological effects appear to be avoided. However, the role of systemic or mucosal priming of IgE by stimulation of GALT is unknown at this time. There were no clinical signs of any adverse reaction including no increase in body temperature in any calf following intraduodenal administration of the leukotoxin. This is in sharp contrast to the effects following subcutaneous injection.

Future experiments will evaluate the role of the route of administration of P haemolytica leukotoxin in causing hypersensitivity reactions, inducing pneumonic lesions in calves, and causing disseminated intravascular coagulopathy, and whether subcutaneous booster injections of formalin killed bacterin can stimulate anamnestic responses in calves primed with CCL.

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CHAPTER 5

IMMUNE RESPONSES OF CALVES STIMULATED INTRADOUDENALLY BY LEUKOTOXIN OF PASTEURELLA HAEMOLYTICA FOLLOWED BY A SUBCUTANEOUS INJECTION OF KILLED BACTERIN

Previous studies demonstrated that Pasteurella haemolytica administered introduodenally can stimulate the gut-associated lymphatic tissue resulting in increased serum and pulmonary antibodies capable of neutralizing the leukotoxin of P haemolytica. Intraduodenally administered viable bacteria appear to prime the bronchus associated lymphatic tissue for a secondary response to a subsequent parenterally administered killed bacterial stimulation of the immune system (Chapter 2). Concentrated leukotoxin administered intraduodenally increased serum and pulmonary neutralizing antibodies as well as pulmonary IgG and IgA. However, concentrated leukotoxin administered subcutaneously resulted in severe systemic reactions in calves obviating its usage as a parenteral toxoid until this effect can be altered (Chapter 4). The reaction shown by these calves appeared to be due to an endotoxic shock reaction with a possible hypersensitivity type I component also (Chapter 4).

The purpose of this experiment was to determine if these problems could be circumvented by using leukotoxin concentrated 100-fold as an intraduodenal inoculatant followed by a subsequent subcutaneous injection of a formalin killed bacterin of P haemolytica.

Materials and Methods

Calves--Calves were procured and intraduodenal catheters were surgically implanted as previously described (Chapter 2).

Bacterium and antigen preparation--*P haemolytica* preparations for viable log phase organisms, and preparations of killed bacterin were prepared as described (Chapter 2). A preparation of 100-fold concentrated leukotoxin was used as previously described (Chapter 3).

Pulmonary lavage--Pulmonary lavages were performed weekly as previously described.

Immune assays--Detection of serum IgG, leukotoxin neutralizing antibodies, pulmonary lavage IgG, IgA and leukotoxin neutralizing antibodies (LNA) was performed with assays previously described (Chapter 2).

Statistical analysis--Mean antibody titers were compared within each group and between groups by the Waller-Duncan analysis of variance test.

Procedure

Six calves were purchased, acclimated, and surgically implanted with intraduodenal catheters. Calves were divided into 2 groups of 3 calves each. Assays of pulmonary lavage fluids and serum were performed to determine baseline antibody levels. Four days later each experimental calf received 25cc of the 100-fold concentrated leukotoxin delivered directly to the duodenum through the catheter. Weekly pulmonary lavage and serum samples were collected. Five weeks after

receiving an intraduodenal (ID) dose of concentrated leukotoxin, each calf was inoculated subcutaneously (SC) with 100cc of 2 x 10^{10} CFU/ml of P haemolytica killed with 0.45% buffered neutral formalin. Pulmonary lavage fluid and serum were collected for 3 weeks following the booster inoculation. Antibody assays were performed on each sample of pulmonary lavage fluids and serum. A synopsis of procedures used is shown in Table 5.1.

Control calves were given phosphate buffered saline solution (PBSS) intraduodenally followed two weeks later by a SC dose of formalin killed P haemolytica. Collection of blood serum and pulmonary lavages were performed weekly beginning 4 days prior to ID exposure and continued once weekly throughout the experiment. A synopsis of procedures is shown in Table 5.2.

Results

The serum IgG antibody responses of experimental calves following intraduodenal inoculation with concentrated leukotoxin are shown in Table 5.3. Each calf had a 2-fold increase in serum IgG following ID inoculation. This level was not significantly different from preexposure levels but was maintained until the administration of a subcutaneous dosage of killed bacterin 5 weeks later. Following the booster injection of killed bacterin, the IgG titer increased 2-fold in two calves and 4-fold in the other. This titer remained elevated for approximately 3 weeks. This was a statistically significant increase compared to the preexposure titer or compared to the titer following

Table 5.1. Schedule of procedures performed on experimental calves.

Procedure	Pulmonary Lavage*	25cc CCL IDt	Pulmonary Lavage	100 cc FKB SC§	Pulmonary Lavage
Day	0	4	7,14,21,28,35	39	42,49,54

* Blood (serum) was collected each day pulmonary lavage was performed.

† One hundred-fold concentrated crude leukotoxin administered intraduodenally.

§ A total of 10^{12} CFU of buffered neutral formalin (0.45%) killed of P haemolytica.

Table 5.2. Schedule of procedures performed on control calves.

Procedure	Pulmonary Lavage*	25cc PBSS IDt	Pulmonary Lavage	100 cc FKB SC§	Pulmonary Lavage
Day	0	4	7,14	18	21,28,35

* Blood (serum) was collected each day pulmonary lavage was performed.

† Phosphate buffered saline solution administered intraduodenally.

§ A total of 10^{12} of buffered neutral formalin (0.45%) killed of *P haemolytica*.

Table 5.3. Serum IgG titers of calves following intraduodenal stimulation by leukotoxin or PBSS and subsequent booster by a killed bacterin of P haemolytica.

	Calf No.	Preexposure*	Post-ID	Post-SC
CCL/FKB†	801	32	64	256
	802	32	64	128
	803	32	64	128
	Mean§	32¶	64¶	170.7¶¶
	std. dev.	0	0	73.9
PBSS/FKB††	348	8	32	128
	349	16	64	128
	359	16	64	128
	Mean	13.3¶	53.3¶¶	128¶¶¶
	std. dev.	4.6	18.5	0

* ELISA titers preexposure, after the intraduodenal inoculation and after the subcutaneous booster inoculation.

† Experimental calves received concentrated crude leukotoxin intraduodenally followed 2 weeks later by 10^{12} CFU of formalin killed P haemolytica subcutaneously.

§ Mean and standard deviation.

¶ Means with different number ¶ are significantly different ($p<0.05$).

††Phosphate buffered saline solution intraduodenally followed 2 weeks later by 10^{12} CFU of formalin killed P haemolytica subcutaneously.

the first injection ($p<0.05$). This increase in titer would not be considered immunologically significant since it is less than a 4-fold change.

Serum IgG titers in control calves increased 4-fold in each calf following ID exposure to PBSS. Titers were increasing by four days post-inoculation of PBSS and were still increasing 10 days post exposure. Serum IgG titers increased 4-fold in each control calf following the SC injection of killed bacterin. The increase in titers following ID and again following SC injection was significantly different ($p<0.05$).

The serum leukotoxin neutralizing antibody (LNA) titer increased less than 2-fold in 2 of 3 experimental calves following intraduodenal stimulation with leukotoxin (Table 5.4). No calf demonstrated an increase in titer following the subcutaneous injection of killed bacterin. These increases were not significant compared to preexposure titers.

Control calves had no increase in serum LNA following ID injection of PBSS. Titers did increase 2 to 3-fold in 2 calves and 25-fold in a third calf following SC injection of the killed bacterin. The mean titer for this group did not represent a significant increase from preexposure levels.

The mean post-ID titer of experimental calves was significantly greater than the mean post-ID titer of control calves ($p<0.05$).

Pulmonary lavage IgG titers increased 4-fold in one experimental calf following the intraduodenal inoculation of concentrated

Table 5.4. Serum leukotoxin neutralizing antibody titers of experimental and control calves.

	Calf No.	Preexposure*	Post-ID	Post-SC
CCL/FKB†	801	16	16	16
	802	100	180	100
	803	130	180	130
	Mean §	82.0	125.0#	82.0
	std. dev.	59.0	95.0	59.0
PBSS/FKB††	348	70	75	90
	349	40	75	130
	359	0	0	75
	Mean	36.7	50.0	98.0
	std. dev.	35.0	43.0	28.0

* ELISA titers preexposure, after the intraduodenal inoculation and after the subcutaneous booster inoculation.

† Experimental calves received concentrated crude leukotoxin intraduodenally followed 2 weeks later by 10^{12} CFU of formalin killed P haemolytica subcutaneously.

§ Mean and standard deviation.

Mean titer is significantly different compared to post-ID titer of PBSS/FKB group ($p<0.05$).

††Phosphate buffered saline solution intraduodenally followed 2 weeks later by 10^{12} CFU of formalin killed P haemolytica subcutaneously.

Table 5.5. Pulmonary IgG titers of experimental and control calves.

	Calf No.	Preexposure*	Post-ID	Post-SC
CCL/FKB†	801	0	0	0
	802	0	4	0
	803	0	0	8
	Mean §	0	1.3	2.7
	std. dev.	0	2.3	4.6
PBSS/FKB††	348	0	0	0
	349	0	0	0
	359	0	0	0
	Mean	0	0	0
	std. dev.	0	0	0

* ELISA titers preexposure, after the intraduodenal inoculation and after the subcutaneous booster inoculation.

† Experimental calves received concentrated crude leukotoxin intraduodenally followed 2 weeks later by 10^{12} CFU of formalin killed P haemolytica subcutaneously.

§ Mean and standard deviation.

††Phosphate buffered saline solution intraduodenally followed 2 weeks later by 10^{12} CFU of formalin killed P haemolytica subcutaneously.

leukotoxin (Table 5.5). One of three calves demonstrated an 8-fold increase in titer compared to its preexposure titer following the subcutaneous dosage of killed bacterin. As a group these did not represent a significant increase in titer. Control calves had no detectable IgG in pulmonary lavage fluids at any time during the experiment.

Pulmonary lavage IgA titers increased 4-fold in 2 of 3 experimental calves following intraduodenal exposure to the leukotoxin (Table 5.6). One of three experimental calves demonstrated a 4-fold increase in titer following the subcutaneous dosage of killed bacterin. These did not represent significant changes compared to preexposure titers. Control calves had no detectable IgA in pulmonary lavage fluids at any time during the experiment. The mean post-SC titer in experimental calves was significantly greater than the mean post-SC titer in control calves ($p<0.10$).

Pulmonary lavage LNA titers increased in 2 of 3 experimental calves following intraduodenal inoculation with the leukotoxin (Table 5.7). This was a significant change compared to preexposure titers ($p<0.10$). No increase in toxin neutralizing antibody titer occurred following the subcutaneous injection of killed bacterin. There was no detectable increase in LNA in pulmonary lavage fluids of control calves during this experiment. The mean post-ID titer in experimental calves was significantly different than the mean post-ID titer in control calves ($p<0.10$).

Table 5.6. Pulmonary IgA titers of experimental and control calves.

	Calf No.	Preexposure*	Post-ID	Post-SC
CCL/FKB†	801	0	4	0
	802	0	8	8
	803	4	0	8
	Mean §	1.3	4.0	5.3#
	std. dev.	2.3	4.0	4.6
PBSS/FKB††	348	0	0	0
	349	0	0	0
	359	0	0	0
	Mean	0	0	0
	std. dev.	0	0	0

* ELISA titers preexposure, after the intraduodenal inoculation and after the subcutaneous booster inoculation.

† Experimental calves received concentrated crude leukotoxin intraduodenally followed 2 weeks later by 10^{12} CFU of formalin killed P haemolytica subcutaneously.

§ Mean and standard deviation.

Mean titer is significantly different from mean titer post-SC of PBSS/FKB group ($p<0.10$).

††Phosphate buffered saline solution intraduodenally followed 2 weeks later by 10^{12} CFU of formalin killed P haemolytica subcutaneously.

Table 5.7. Pulmonary leukotoxin neutralizing antibody titers of experimental and control calves.

	Calf No.	Preexposure*	Post-ID	Post-SC
CCL/FKB†	801	0	0	0
	802	0	24	0
	803	3	32	4
	Mean §	1.0¶	18.7¶¶,#	1.3¶
	std. dev.	1.7	16.7	2.3
PBSS/FKB††	348	2	4	4
	349	2	0	0
	359	2	5	3
	Mean	2	3	2.3
	std. dev.	0	2.7	2.1

* ELISA titers preexposure, after the intraduodenal inoculation and after the subcutaneous booster inoculation.

† Experimental calves received concentrated crude leukotoxin intraduodenally followed 2 weeks later by 10^{12} CFU of formalin killed *P haemolytica* subcutaneously.

§ Mean and standard deviation.

¶ Means with a different number of ¶ are significantly different ($p<0.10$).

\# Mean is significantly different compared to post-ID titer of PBSS/FKB group ($p<0.10$).

††Phosphate buffered saline solution intraduodenally followed 2 weeks later by 10^{12} CFU of formalin killed *P haemolytica* subcutaneously.

Discussion

This group of calves had very different immune reactions even though treated with a toxin preparation prepared in a manner similar to that used previously (Chapter 4). The experimental calves in this experiment did not have any detectable increase in serum IgG titers following intraduodenal inoculation with the concentrated toxin. This immune response contrasts with that in calves in Chapter 4 in which 2 of 3 calves had demonstrable increases in antibodies following intraduodenal exposure to concentrated leukotoxin.

A poor antibody response was also seen in this group of calves following a subcutaneous booster with killed bacterin. Calves primed by ID stimulation with live bacterin or PBSS responded to a SC injection of killed bacterin with a 4-fold or greater increase in serum IgG titers. These calves had much lower responses than any other group of calves.

The poor immune response of these calves was unexpected. There could be several explanations for this. Suppression of orally induced immune responses has been seen in laboratory animals previously vaccinated parenterally[1]. The vaccination history of these calves was unknown. It is possible that these animals had been vaccinated with P haemolytica just prior to purchase or that they underwent natural infection after purchase. Evidence to support this is suggested by the higher than usual preexposure IgG titers of 32. These same calves had serum IgG titers of 8 at the time of purchase which was three weeks prior to the time blood was drawn for the preexposure titer.

Experimental calves also had preexposure LNA titers of approximately 100 which is quite high. Natural exposure could have occurred although no bacteria were ever found in nasal or tracheal swabs, nor were any Pasteurella organisms ever cultured from the weekly pulmonary lavage fluids.

This does not explain the lack of immune response following the killed bacterin injection. Animals previously exposed to a gut associated lymphatic tissue (GALT) inoculation usually respond very well to this injection with increases in serum and pulmonary LNA as well as serum IgG and IgA (Chapter 2). This lack of response could be explained by immunosuppression of GALT by bovine viral diarrhea (BVD) virus or by other viruses. BVD has been shown to greatly diminish immune responses in cattle.[2] Calf number 801 had a demonstrable increase in titer to adenovirus 7; calves 802 and 803 both seroconverted with high titers to BVD. The poor response could also be due to alteration of the toxin in the duodenum prior to uptake by the mucosa. Since surgery and catheter placement were consistent with previous cases this is unlikely although these catheters were not examined at post mortem to confirm accurate catheter placement. Different batches of leukotoxin were used and inasmuch as there is likely some inherent variation in levels of toxicity of batches, assays confirmed the presence of approximately equal amounts of biologic units of toxin present in each batch. Several batches of toxin were pooled to eliminate any batch effects for this experiment.

A major factor in the different antibody response in this

experiment could be the difference between the priming and booster antigen preparations. Previous experiments used inoculation regimens that consisted of either an inoculation of live organisms followed by an injection of killed organisms, or an inoculation of leukotoxin followed by an injection of leukotoxin. In each of those studies the second antigen was identical to or closely identified to the primary antigen. The present study deviated from that model and used a priming antigen preparation that was more different from the booster antegen. Perhaps the reason there was poor priming of lymphoid tissue was that there was not enough leukotoxin in the killed bacterin to initiate an anamnestic immune response.

The titers that showed the greatest change for the experimental group of calves were post-ID and post-SC pulmonary IgA and post-ID pulmonary LNA. These responses appear to represent direct GALT stimulation with migration of IgA plasmacytes to other mucosal sites as expected from studies in other species.[3,4,5], The post-SC titer of pulmonary IgA was significantly different from the control group suggesting that considerable priming of memory IgA cells also occurred. Moore et al.[6] has shown that IgA has considerable leukotoxin neutralizing capabilities. The increased pulmonary LNA may be directly related to IgA levels in these calves. Pulmonary IgG titers were very low except in one calf (#803). Serum LNA and IgG represent relatively poor responses. Therefore, it seems unlikely that crossover of serum IgG to pulmonary lavage fluids contributed to the pulmonary neutralizing antibodies in these calves. These responses are even more promising considering the strong suggestion of immunosuppression due to

the possibility of active BVD virus infection. Mucosal immunity can be stimulated at the same time systemic tolerance is induced indicating separate control of these immune processes.[7] This study suggests that BVD may also affect the mucosal immune system differently than the systemic system. Concurrent infection by BVD virus at the time of inoculation could drastically reduce systemic immunity with a possible sparing of GALT and associated mucosal immunity.

In summary, stimulation of the GALT in calves by concentrated leukotoxin of P haemolytica resulted in low increases in serum and moderate increases in lavage LNA and IgA. Anamnestic responses following a subcutaneous booster with a killed bacterin resulted in no further immune responses in serum. However, there was a further moderate increase in pulmonary lavage fluid IgA titers. These poor responses could be explained by the presence of immunosuppressive viruses, existing antibodies induced by a previous parenteral inoculation that inhibited GALT stimulation, poor antigen relationship between the primary and secondary antigen preparations, or due to a combination of these factors.

Although conclusive, pulmonary IgA increases following intraduodenal stimulation by leukotoxin appear to be associated with the elevated pulmonary LNA titers. These results suggest that even in the face of systemic immunosuppression by BVD virus infection, the common lymphatic tissue response can still remain active. Inoculation of GALT by respiratory vaccines may therefore be a way to induce mucosal immunity while avoiding the systemic immune suppression of BVD.

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CHAPTER 6

A CHALLENGE STUDY OF CALVES INOCULATED INTRADUODENALLY WITH PASTEURELLA HAEMOLYTICA OR ITS LEUKOTOXIN

Calves exposed to Pasteurella haemolytica appear to have reduced pulmonary lesions if they possess humoral leukotoxin neutralizing antibodies (LNA).[1,2] Calves exposed to capsular antigen preparations of P haemolytica also have increased LNA titers and decreased pulmonary pathology.[3] Confer et al.[4], however, reported that calves with humoral LNA do not always have reduced pulmonary lesions. This could be due to several factors. Protection could be related to immunity to virulence factors such as capsular antigens rather than leukotoxin. Protection could also depend on immunity to both factors. Alternatively, other immune factors could be important. Moore et al.[5] have shown that calves exposed by aerosolization of live P haemolytica have elevated titers of LNA in serum and pulmonary lavage fluids. Mucosal immunity to leukotoxin may be more important than systemic immunity in preventing pasteurellosis.

The presence of serum LNA does not necessarily imply the presence of pulmonary LNA. Calves inoculated with a formalin killed bacterin may have increased serum LNA without increases in pulmonary LNA (Chapter 2). Pulmonary LNA can also be present in the absence of increased serum LNA. Calves exposed to an intraduodenal inoculation of concentrated crude leukotoxin had elevated pulmonary IgA and LNA without an increase in serum LNA (Chapter 4). To date no studies have evaluated the role of LNA in pulmonary lavage fluids in preventing pneumonic lesions of P haemolytica.

There are means of inducing pulmonary mucosal immunity other than by aerosolization. The stimulation of the gut associated lymphatic tissue (GALT) induces immunity at other mucosal sites by several mechanisms. First, there can be direct stimulation of IgA producing cells with immediate sIgA detected at other sites. Second, memory lymphocytes may be primed and migrate to other mucosal sites. These cells may later respond to antigenic signals resulting in the production of antibodies. Third, in the ruminant there is evidence that IgG_1 producing cells may be routed from GALT to other mucosal sites.[6]

The purpose of this experiment was to evaluate the efficacy of several regimens of inoculation of calves to prevent P haemolytica pneumonia and, to determine if there was any correlation of antibody titers to the amount of pulmonary pathology in challenged calves.

Materials and Methods

Calves--Ten calves aged 4 to 6 months were selected as previously described (Chapter 2). Three calves were assigned to each experimental group and one calf was treated as a control.

Bacterium--Actively growing P haemolytica was prepared as previously described for the GALT inoculation, challenge dosages and to make the bacterin (Chapter 2).

Pulmonary lavage--Calves were bled from the jugular vein and pulmonary lavage was performed weekly on each calf for the duration of the experiment. These procedures were performed by the same protocol previously described (Chapter 2). The last pulmonary lavage was always

performed 4 days or more prior to the challenge exposure to P haemolytica.

Immunoglobulin assays--Antibody assays for the presence of IgG in serum, and IgG and IgA in pulmonary lavage fluids were performed by ELISA as previously described (Chapter 2). Leukotoxin neutralizing antibody titers were determined by the vital stain (neutral red) uptake assay as previously described (Chapter 2).

Transtracheal challenge--Viable log phase P haemolytica were prepared as described for the inoculation protocol (Chapter 2). These bacteria were suspended in phosphate buffered saline solution (PBSS) to a concentration of 2×10^9 CFU/ml. Each calf was restrained in a head chute, intubated, and catheterized by a Foley tubing.[a] The right caudal lobe was then inoculated with 25 cc of the challenge inoculum, the catheter was flushed with 25 cc of PBSS, and the catheter held in place for 1 minute. The cuff of the catheter was deflated and the catheter was then withdrawn. Calves were returned to their pen for observation.

Pathological examination--Calves were euthanized 96 hours following challenge by intravenous injection of T-61[b] solution. A complete post mortem examination was performed. Gross lesions of lung and thoracic cavity were carefully evaluated. The primary lesion at the site of challenge inoculation in the right caudal lung lobe was measured three-dimensionally. The percentage of each lung lobe

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[a] Bivona, Inc., Gary, IN.

[b] Euthanasia solution, American Hoechst Corp., Somerville, NJ.

affected by pneumonia was recorded. The gross lesions were scored by parameters shown in Table 6.1. Lesions were scored as severe = 3, moderate = 2, mild = 1, and none = 0. Histopathological lesions were scored by parameters shown in Table 6.2. Severity of lesions was scored as for gross lesions with severe = 3, moderate = 2, mild = 1, none = 0.

Statistical analysis--Mean gross and histopathological lesion scores were compared between groups by the Waller-Duncan analysis of variance test. Comparison of lesion scores to antibody titers was performed by determination of correlation coefficients.

Procedure

The schedule of inoculations for each group of calves is indicated in Table 6.3. Calves were exposed to either GALT stimulation with live bacteria followed by subcutaneous (SC) exposure to a killed bacterin, GALT stimulation by live bacteria followed by a subcutaneous booster with PBSS, GALT stimulation by PBSS followed by a subcutaneous stimulation by formalin killed bacterin, or GALT stimulation with PBSS followed by a subcutaneous stimulation by PBSS. Serum and pulmonary lavage specimens for antibody titer were collected at weekly intervals beginning at day 0 and concluding 4 days prior to challenge. ELISA assays were performed on serum for the presence of IgG and pulmonary lavage specimens for IgG and IgA specific for somatic antigens of *P haemolytica*. The presence of leukotoxin neutralizing antibodies in serum and lavage concentrates was determined by the neutral red uptake

Table 6.1. Parameters evaluated to determine gross lesion scores for the lung.

Presence of Fibrin	Transudate/Exudate
Interlobular septa	Interlobular edema
Pleural surfaces	Lobular congestion/consolidation
Airways	Exudate in airways

Table 6.2. Parameters evaluated to determine histopathological lesion scores for the lung.

Infiltrates	Transudates	Inflammation
Interlobular septal	Alveolar congestion	Acute parenchymal
Intraalveolar	Alveolar hemorrhage	Chronic parenchymal
• exudate		
• fibrin		
• fibrin clots		

Table 6.3. Schedule of inoculations of calves prior to challenge.

Inoculant	Group A	Group B	Group C	Group D
Intraduodenal inoculation Day 0	Live log phase *P haemolytica*	Live log phase *P haemolytica*	Phosphate buffered saline solution	Phosphate buffered saline solution
Subcutaneous inoculation Day 14	Formalin killed bacterin of *P haemolytica*	Phosphate buffered saline solution	Formalin killed bacterin of *P haemolytica*	Phosphate buffered saline solution

assay. Eleven days following the last inoculation each calf was challenged by a transtracheal dosage of live P haemolytica. Each calf in a group received a challenge inoculation of bacteria at the same time although groups were challenged at different times. Each bacterial inoculum varied slightly with respect to total CFU/ml (Table 6.4). Following challenge, calves were evaluated daily for clinical signs of disease by the parameters described by Potgieter et al.[6] Animals that demonstrated suffering were euthanized. All surviving calves were euthanized 96 hours following challenge. Complete gross and histopathological post mortem examinations were performed on each calf. Lesions were scored and compared to antibody titers.

Results

Gross Pathologic Lesions of the Lungs

Group A--The pneumonic lesion was restricted to the right caudal lung lobe in the area that received direct exposure to the challenge dose of bacteria. The size of the primary lesion is indicated in Table 6.5, and comprised 25 to 30% of this lobe. The pneumonic area was identified by its increased firmness and darker red color. No fibrin or edema was present in the interlobular septa of any calf. One calf exhibited mild amounts of granular fibrinous exudate on the surface of the right caudal lobe with moderate amounts of exudate present in airways when this area was incised. The increased firmness of the challenge area was severe in one calf, moderate in one and mild in the third calf. A comparison of gross pathological lesion scores for each calf is shown in Table 6.6.

Table 6.4. Pulmonary lesions of challenged calves.

	Calf Group			
Tissue Cultured	Group A	Group B	Group C	Group D*
LUNG				
Right anterior cranial	-	+++	-	+
Right post cranial	-	+++	-	+
Right medial	-	+++	-	+
Right intermediate	-	+++	-	+
Right caudal	+	+++	+++	+
Left anterior cranial	-	+++	-	+
Left post cranial	-	+++	-	+
Left caudal	-	+++	-	+
Heart blood	-	+++	-	+
Liver	-	+++	-	+
Tracheal bronchial lymph node	-	+++	-	+
Mesenteric lymph node	-	+++	-	+
Challenge dose P haemolytica used CFU/ml	$2x10^9$	$2x10^{10}$	$2x10^9$	$4x10^9$

* Group A = Live log phase P haemolytica intraduodenally and formalin killed P haemolytica subcutaneously 14 days later; Group B = Live log phase P haemolytica intraduodenally and phosphate buffered saline solution subcutaneously 14 days later; Group C = Phosphate buffered saline solution intraduodenally and formalin killed P haemolytica subcutaneously 14 days later; Group D = Phosphate buffered saline solution intraduodenally and phosphate buffered saline solution subcutaneously 14 days later. (-) = No P haemolytica isolated; (+) = 0 to 10 P haemolytica CFU/plate; (+++) = Greater than 100 P haemolytica CFU/plate.

CFU = Colony forming units

Table 6.5 Gross and histopathological lesion scores of the right caudal lung lobe of calves.

% Lung Lobe Affected*	Calf Group			
	Group A	Group B	Group C	Group D†
Right Anterior Cranial	0§	10.0§ ±10.0	0§	60§§
Right Posterior Cranial	0§	15.0§ ±18.0	0§	50§§
Right Medial	0§	23.3§ ±32.2	0§	60§
Right Intermediate	0§	8.3§ ± 7.6	0§	40§§
Left Anterior Cranial	0	20.6 ± 5.7	0	30
Left Posterior Cranial	0§	28.0§§ ±20.3	0§	0§
Left Caudal	0	3.3 ± 5.8	0	0
Lesion size Right caudal (cm^3)	472§,§§ ±279	253§§ ±114	1233§ ±624	304§§

* Mean and standard deviation of each group.

† Group A = Live log phase P haemolytica intraduodenally and formalin killed P haemolytica subcutaneously 14 days later; Group B = Live log phase P haemolytica intraduodenally and phosphate buffered saline solution subcutaneously 14 days later; Group C = Phosphate buffered saline solution intraduodenally and formalin killed P haemolytica subcutaneously 14 days later; Group D = Phosphate buffered saline solution intraduodenally and phosphate buffered saline solution subcutaneously 14 days later.

§ Means in the same row with a different number of § are significantly different ($p<0.09$).

cm^3 = Cubic centimeters

Table 6.6. Antibody responses of calves exposed to different methods of inoculation.

	Group A	Group B	Group C	Group D*
Gross lesion score (Mean ± standard deviation)	3.67±3.0†	9.3±1.2††	7.33±1.7†,††	18.0†††
Histopathological lesion score (Mean ± standard deviation)	12.67±0.9§	17.7±1.2§§	15.67±1.9§,§§	15.0§,§§

* Group A = Live log phase P haemolytica intraduodenally and formalin killed P haemolytica subcutaneously 14 days later; Group B = Live log phase P haemolytica intraduodenally and phosphate buffered saline solution subcutaneously 14 days later; Group C = Phosphate buffered saline solution intraduodenally and formalin killed P haemolytica subcutaneously 14 days later; Group D = Phosphate buffered saline solution intraduodenally and phosphate buffered saline solution subcutaneously 14 days later.

† Means with a different number of † are significantly different ($p<0.01$).

§ Means with a different number of § are significantly different ($p<0.06$).

Group B--The pneumonic area was restricted to the right caudal lobe in two calves and mildly expanded to other lobes in one calf with 3 to 23% of each lobe considered abnormal (Table 6.5). The affected areas were identified by their increased firmness and redness. Amber to red colored fluid was found in the pleural cavity of each calf and varied in amount from 20 to 750 ml. One calf exhibited a thick layer of fibrin over the surface of the largest lesion on the caudal right lung lobe. The other two calves had no pleural fibrin. These two calves did have widespread subpleural and interstitial petechial and echymotic hemorrhages. The lungs of each calf contained interlobular edema and fibrin. Two calves also had considerable interstitial edema of affected lung lobes.

Group C--The pneumonic lesion was restricted to the right caudal lobe in the area adjacent to the exposure challenge. The mean lesion size is noted in Table 6.5 and comprised 30 to 70% of the lobe in this group. The pneumonic area was readily identified by its increased firmness and darker red color. Mild interlobular edema was present as well as septal infiltration by fibrin. A mild to moderate amount of exudate was seen in airways when pneumonic lesions were incised. The lesion area was very firm in all three calves in the group. The gross pathological lesion score for each calf compared to other groups is shown in Table 6.6.

Group D--The right caudal lobe contained the largest lesion. Most other lobes of the right and left side contained affected areas of pneumonia with 30 to 60% of each lobe abnormal. The affected areas of

lung were identified by their increased firmness and redness. The pleural cavity contained approximately 650 ml of yellow fluid. Marked pleuritis was present in the posterior region of the thorax with a thick (2.5 cm) area of fibrin on the pleural surface of the right caudal lung lobe. Thin layers of fibrin covered the lung lobes. Interlobular septa were plugged with marked deposition of edema and fibrin. Plugging of bronchial lumens by fibrinopurulent exudate was noted when pulmonic lesions were cross sectioned.

Histopathological lesions of lungs--Histopathological lesions between groups of calves were very similar. Inflammation which nearly obliterated normal pulmonary function was present in all groups. Each group had moderate to severe acute inflammation. This was characterized by a mild to moderate amount of neutrophil infiltrates, edema, fibrinous exudate of subpleural and interlobular spaces. Interlobular spaces were also expanded due to greatly dilated lymphatics, some of which contained fibrinous thrombi. In group A calves, these inflammatory changes affected 80 to 90% of tissue in a given slide. In group B calves inflammation was noted in 60 to 100% of tissue in the slides evaluated. In group C and D calves inflammation affected 100% of the tissue examined in a given section. The group C calves also had subacute inflammation as indicated by the presence of coagulation necrosis, increased fibrinous connective tissue, epitheliziation of alveoli and intraalveolar and intrabronchial fibrinosuppurative exudates. The overlying pleura in these areas was thickened due to fibrosis. One calf in group A also had mild chronic inflammation present.

Most alveoli and bronchioles were filled with macrophages, neutrophils, and fibrinous exudate. Many alveoli were hard to discern due to septal necrosis. Perivascular edema and mild to moderate alveolar atelectasis were seen in most calves. Increased numbers of neutrophils were seen in alveolar septal capillaries.

Individual calves had very similar lesions. The primary difference between groups was the presence of chronic inflammation and the percentage of tissue affected in a given slide.

Morphometric evaluation of lesions--The primary lesion size and percentage of each lung lobe affected are shown in Table 6.5. Group D had the greatest percentage of each lobe affected although the primary lesion was not the largest seen. Calves in group B had a reduced percentage of each lung lobe affected and the smallest primary lesion of any group. Calves in group A had lesions confined to the right caudal lung lobe, but the size of the lesion was 3 to 5 times greater than the primary lesion seen in other groups ($p<0.08$). The percentage of each lobe affected was significantly greater in group D than in A, B or C ($p<0.08$).

The summary of the gross and histopathological lesion scores is shown in Table 6.6. The gross lesion score of 18 for group D was 2 to 6 times greater than any other group. Group B had a mean score of 9.3 followed by group C with 7.3 and group A had a mean score of 3.7.

The mean gross lesion score for group D was significantly greater than the mean for group A ($p<0.01$). The mean for group B was significantly different from either A or D ($p<0.01$). Group C was

different from group D and intermediately different from A and B ($p<0.01$) i.e., if group C were grouped with group A, both would be significantly different from group B. But if group C were grouped with group B both would be significantly different from group A ($p<0.01$).

The histopathological lesion scores did not vary greatly between groups. Group B had the largest mean lesion score of 17.7 followed by group C with a score of 15.7 while group D had a lesser score of 15.0. Group A had the smallest mean lesion score of 12.7. Group B was significantly greater than group A score ($p<0.06$) with groups C and D intermediate in significance levels.

Antibody levels--The antibody titers for each group prior to exposure to any antigen and 7 days after the SC inoculation are shown in Table 6.7. Group A calves had the highest serum IgG, serum LNA and pulmonary LNA responses. Group B only had the next highest serum IgG, pulmonmary IgG and pulmonary LNA and the third best serum LNA titers. The group C calves had both a serum IgG and LNA response, but no pulmonary IgG, IgA or LNA response. Group D had no increase in any antibody titer measured. The only significant increase in antibody titer was for an increase in pulmonary LNA in group A following the SC booster ($p<0.05$). Group B also had a significant increase in titer but this also was not different from group A and D ($p<0.05$).

The regression coefficent for antibody levels and primary lesion size, and for antibody levels and gross lesion scores are shown in Table 6.8. The coefficients were derived from the scores of each calf in each group. The general trend indicates negative correlation

Table 6.7. Antibody responses of calves exposed to different regimens of inoculation of P haemolytica.

	Group A		Group B		Group C		Group D*	
	Pre	Post†	Pre	Post	Pre	Post	Pre	Post
Serum IgG§	16	64	32	128	8	32		
	32	128	64	32	8	16	16	8
	16	32	64	16	8	64		
Mean	21.3	74.7	53.3	58.7	8.0	37.3	16	8
std. dev.	9.2	48.9	18.5	60.6	0.0	24.4		
Serum LNA**	250	60	8	48	5	64		
	110	700	128	256	13	250	16	8
	32	250	64	64	35	100		
Mean	130.7	336.7	66.7	122.7	17.7	138.0	16	8
std. dev.	110.5	328.7	60.0	115.8	15.5	98.7		
Pulmonary IgG§	0	4	0	8	0	0		
	0	4	4	4	0	16¶	0	0
	0	4	0	0	0	0		
Mean	0	4.0	1.3	4.0	0	5.3	0	0
std. dev.	0	0	2.3	4.0	0	9.2		
Pulmonary LNA**	3	16	0	6	3	3		
	3	8	8	12	3	4	0	0
	3	7	2	4	3	3		
Mean	3.0	10.3#	3.3#,##	7.3#,##	3.0	3.3	0##	0
std. dev.	0	4.9	4.2	4.2	0	0.6		
Pulmonary IgA§	2	4	2	8	2	2		
	0	4	4	4	0	0	0	0
	2	2	0	0	0	2		
Mean	1.3	3.3	2.0	4.0	0.7	1.3	0	0
std. dev.	1.2	1.2	2.0	4.0	0.9	1.2		

* Group A = Live log phase P haemolytica intraduodenally and formalin killed P haemolytica subcutaneously 14 days later; Group B = Live log phase P haemolytica intraduodenally and phosphate buffered saline solution subcutaneously 14 days later; Group C = Phosphate buffered saline solution intraduodenally and formalin killed P haemolytica subcutaneously 14 days later; Group D = Phosphate buffered saline solution intraduodenally and phosphate buffered saline solution subcutaneously 14 days later.

† Pre = Preexposure titer; Post = Highest titer achieved following subcutaneous inoculation.

§ Titer to somatic antigens of P haemolytica determined by ELISA .

¶ Lavage fluids contaminated with blood.

**Titer determined by neutral red vital stain uptake assay.

Means with different number of # are significantly different ($p<0.05$).

LNA = Leukotoxin neutralizing antibody.

Table 6.8. Correlation coefficients of lesion score vs antibody titers.

Gross Lesion Score	Correlation coefficient
Serum IgG	-.48
Pulmonary IgG	-.21
Serum LNA	-.72
Pulmonary LNA	-.53
Pulmonary IgA	-.41
Histopathology Lesion Score	**Correlation coefficient**
Serum IgG	-.23
Pulmonary IgG	.28
Serum LNA	-.32
Pulmonary LNA	-.40
Pulmonary IgG	.00

LNA = Leukotoxin neutralizing antibody.

between antibody titer and lesion score. The greatest negative correlation was seen with serum LNA titers and gross lesion score (-.72). Pulmonary LNA had a moderate correlation coefficient for gross (-.53) and histopathological (-.46) lesion scores. It is interesting to note that the only positive correlation was with the presence of pulmonary IgG and histopathological scores (.28).

Discussion

Protection from P haemolytica pneumonia has been most closely associated with serum antibodies capable of neutralizing the leukotoxin of the organism.[1,2] In this experiment calves inoculated ID with log phase viable P haemolytica were mucosally primed for a subsequent increase in antibody titers following a SC booster inoculation of a killed P haemolytica bacterin. This was shown by the fact that these calves possessed the highest serum and pulmonary LNA (Table 6.7) of any group of calves. The post SC inoculation pulmonary LNA titers of group A calves were significantly greater than post-SC inoculation titers of calves in groups C or D. The most likely explanation for this difference in pulmonary LNA titer is that antigen (live bacteria) was taken up by the follicle associated epithelium and Peyer's patches in the small intestine, stimulated lymphocytes then entered the general circulation, migrated to the lung where antibodies were produced and detected by assay.

Calves in group A had no spread of lesions to lung lobes other than the site of challenge exposure. The mean primary lesion of this

group of calves was much smaller than group C calves although intermediate in size between group D calves and group B calves. This may be due to conflicting immune mechanisms. The presence of a very large mean primary lesion in group C calves supports the research that killed bacterins induce opsonizing antibodies that can result in increased pulmonary lesions.[7,8] Group C calves did exhibit serum LNA which may explain the decreased pneumonic involvement of other lung lobes. The presence of serum LNA in group C calves may also have played a role in preventing the spread of organisms to other lung lobes and systemically once the mucosal surface barrier was breached by any organisms. Group B calves had the smallest mean primary lesion suggesting that their mucosal immune system was stimulated with the least induction of opsonizing antibodies. The most noticeable increase in antibodies in this group was pulmonary IgG and pulmonary LNA.

The calves in this experiment did not have detectable increases in serum IgG following ID inoculation by live bacteria. Group A calves and previous experiments suggest that this can occur (Chapter 4). Increases in pulmonary LNA were moderate but may have been enough to confer protection. Increases in pulmonary LNA following ID inoculation were not seen in previous experiments (Chapter 4). This may be due to differences in calves which may have had a different history of exposure to P haemolytica. Animals parenterally vaccinated to P haemolytica may have had a suppressed immune response to subsequent GALT exposure to this antigen. Animals previously exposed naturally or possessing mucosal antibody producing cells would have been more likely

to respond with an increase in titer. The presence of immunosuppressive viruses could also have adversely affected the immune response. At present there is no information available on what represents a minimal protective titer of LNA. This is the first study that indicates pulmonary LNA may be important in protection.

Reduced lesions may also be associated with increased cellular immunity. Cell mediated immunity was not evaluated in this study. There has been some evidence of transfer of T cells that may account for increased immune responses at distant mucosal sites.[9] Little is known of the role of GALT transfer of cell mediated immune factors in ruminants.

The intermediate mean lesion size in group B calves may be the result of: cell mediated immunity; the observed increased serum and pulmonary LNA which decreased lesion size by decreasing the number of viable bacteria and amount of leukotoxin; and the likelihood that SC killed bacterins induce opsonizing antibodies which result in increased pulmonary lesion size while at the same time stimulating previously primed mucosal immunity.

Calves in group B had reduced involvement of lung lobes other tnan the challenge site (right caudal lobe) compared to group D calf. The involvement of other lung lobes in spite of the smallest primary lesionof any group is probably due to the overwhelming challenge dose used in this group (Table 6.4). This group received 2.5 times the challenge dose used in group D and 10 times the challenge dose used in groups B and C. Challenge dose has been shown to be correlated with

pulmonary lesions with 2×10^9 CFU the actual target dose in this study.[10]

The number of bacteria isolated from the challenge site lesion was much greater for groups B and C than for groups A and D (Table 6.4). The large number of organisms isolated from all lung lobes as well as other tissues (mesenteric lympn node, liver, heart blood) in groups B and D indicate the presence of a septicemia. The isolation of low numbers of organisms from all organs at post mortem in the group D calf probably reflects the low antibody titers present resulting in little inhibition of spread of the organism with resultant septicemia. Large numbers of organisms were isolated from group B calves in spite of relatively good antibody levels in serum and pulmonary lavage fluids. This reflects the very large challenge dose of bacteria that probably overwhelmed the immune mechanisms. Group A and C calves had organisms cultured only from the primary lesion in the right caudal lung lobe. This was the area of delivery of the challenge inoculum. Each of these groups received a similar challenge dose, but group C had a much larger number of organisms recovered. This may reflect the relatively poor levels of pulmonary IgA, IgG and LNA in these calves compared to group A calves. The lack of spread to other lung lobes may have been due to the presence of serum LNA in these calves.

Evaluation of the severity of gross lesions indicated that group D calves had the greatest lesion score and group A had the lowest lesion score at a significant level ($p<0.05$). Group B calves had a slightly greater lesion score than group C calves, but this probably reflects

the overwhelming challenge dose received by this group of calves. These differences may have been due to reduced fibrin deposition and interlobular edema as a result of reduced endothelial damage. This could have been achieved by neutralization of the toxic effects of endotoxin and leukotoxin of P haemolytica. Direct damage to endothelial cells by endotoxin and by peroxidases, oxygen radicals and enzymes released by leukocytes lysed by leukotoxin leads to leakage of serum from vessels into the parenchyma, and subsequent stimulation of fibrinogenesis to repair damaged vessels.

Histopathological lesion scores appear to reflect the gross lesion damage although with smaller differences between groups. This indicates that the type of damage is similar between groups. Protection mostly reduced the amount of lung affected with only subtle differences histopathologically in the degree of alveolar fibrin and cellular deposition. However, the degree of damage did vary at a significant level ($p<0.06$) with group A less affected than group B, and group C and D affected at an intermediate level of significance between groups A and B. The greatest difference was between groups A and B and not A and D as expected. This probably reflects the greater challenge dose group B received.

The relationship between antibody titers and lesion scores is shown in Table 6.8. These values suggest that the greatest negative correlation existed between serum and pulmonary LNA titers and smaller lesion scores. Negative coefficients were present for most titers in gross and histopathological lesion scores except for pulmonary IgG.

This can be attributed to several factors. First, group C had one calf with a relatively high pulmonary IgG titer. This lavage sample was grossly contaminated with blood so this may in fact be serum IgG. The value could also be positive due to the presence of opsonizing IgG to somatic antigens that has been associated with increased pulmonary lesions.[8,9] There was no notable correlation between lesion size and antibody titer. All three correlation coefficients are depressed to a degree due to the larger lesions in group B calves. These animals had a very large challenge dose of pathogens and, therefore, the lesions in this group are probably larger than expected.

These challenge studies suggest that intraduodenal presentation of actively growing P haemolytica can reduce the severity of pulmonary lesions following challenge. The reduced lesions appear to be associated with the presence of LNA in pulmonary lavage fluids and perhaps increased CMI activity. Protection is indicated by reduction in gross lesion size, gross lesion score and moderation of histopathological lesion score evidenced by decreased alveolar infiltrates.

The reduced lesions present in group A and group B calves suggest that the oral route of administration can be used to effectively immunize calves against P haemolytica. Usage of P haemolytica antigenic factors or identification of (recombinant) technology to deliver live bacteria to the lower intestine would be necessary to fulfill this potential of an oral vaccine.

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CHAPTER 7

CHANGES IN COAGULATION FACTORS AND LEUKOCYTE NUMBERS IN CALVES INDUCED BY EXPOSURE TO *PASTEURELLA HAEMOLYTICA* ENDOTOXIN AND LEUKOTOXIN

The investigation of the pathogenesis of *Pasteurella haemolytica* in pneumonic pasteurellosis has centered on the role of its leukocyte-specific cytotoxin. Partial characterization of this toxin has been performed with preparations of culture supernatants.[1-3] Research has determined that this toxin is lethal to bovine leukocytes. However, it does not affect other ruminant tissues (spleen, kidney, or erythrocytes), nor leukocytes of other species (pig, horse or man).[4,5] The role of *P haemolytica* leukotoxin as a virulence factor has been deduced from various observations: (1) exposure of alveolar macrophages, peripheral neutrophils or bovine lymphocytes to this toxin results in observable damage and death within a very short period of time (20 minutes);[2,3,5] (2) animals immunized experimentally by injection of live bacteria produced antibodies to this toxin and have decreased pulmonary lesions,[6] (3) animals entering feedlots without antibodies capable of neutralizing this toxin in vitro are more prone to *Pasteurella* pneumonia.[7]

Investigation of the role of leukotoxin in pasteurellosis is relatively recent. Prior to discovery of this exotoxin, interest was focused on the role of the endotoxin of *P haemolytica*. Work by Keiss et al.[8] demonstrated that lipopolysaccaride (LPS) composed about 12 to 25% of the content of the bacteria. This endotoxin possesses traits similar to endotoxin of other gram negative organisms. Rabbits

injected with P haemolytica endotoxin exhibited signs which confirmed its ability to cause dermonecrocity, pyrogenicity, and neutropenia. Injection of P haemolytica endotoxin into sheep caused an early increase in pulmonary pressure, followed by decreased cardiac output and late phase systemic hypotension.[9] Further investigations by Brodgen et al[10] suggest P haemolytica LPS binds to pulmonary surfactant increasing its specific gravity. This decreases the surface tension in the lung contributing to pulmonary edema, hemorrhage and atelectasis.

Although many properties of endotoxins are shared by a wide spectrum of gram negative organisms, others differ. For example, the ability of neutrophils (PMN) or peripheral blood leukocytes (PBL) to migrate differs when exposed to endotoxin of Escherichia coli or P haemolytica. Olson et al.[11] found bovine PMNs increased migration while PBL decreased migration when exposed to E coli endotoxin. Confer et al.[12] found that P haemolytica endotoxin had no effect on migration by PMN or mononuclear leukocytes (MNL) whereas PBL had marked increased migration. Confer et al.[12] observed no effect on the viability of PMNs by P haemolytica endotoxin. Observations made at the University of Tennessee suggest that the LPS of P haemolytica has little cytocidal effect on BL-3 bovine lymphocytes as determined by vital stain uptake. However, PMNs and macrophages have been shown to have increased phagocytosis as measured by nitroblue tetrazolium reduction (NBTR) function following exposure to P haemolytica endotoxin.[12]

Polymorphonuclear neutrophils are among the first cells drawn to the lung following exposure to P haemolytica. Macrophages become the

predominant cell in the lung several days after the onset of infection.[13] Endotoxin apparently stimulates metabolic activity by PMNs and macrophages making them more susceptible to action by the leukotoxin. Receptor sites may also be altered by LPS to make these cells more susceptible to the effects of leukotoxin.

Endotoxins of other gram negative organisms exert wide ranging physiological effects. Fever, disseminated intravascular coagulopathy, thrombocytopenia and neutropenia, pulmonary hypertension, systemic hypotension, and lethal shock have all been associated with circulatory failure and endotoxic shock. These effects are mediated largely by biological substances such as histamine,[14] serotonin,[15] catecholamines,[16] prostaglandins,[17] thromboxanes,[18] and other cyclic nucleotides.[19] P haemolytica endotoxin induces production of prostaglandins E and F, thromboxane B_2 and serotonin when injected into sheep.[20]

The pulmonary lesions associated with pneumonic pasteurellosis have some of the properties that comprise other endotoxin associated respiratory lesions eg., alveolar edema, thrombi, and fibrin which are also seen in adult respiratory disease complex.[21] However, attempts to induce such lesions by instillation of P haemolytica LPS or killed bacteria into the lung have had mixed success. Transtracheal inoculation of purified LPS had no effect on calf lungs.[a] Heat killed

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[a] Slocombe RF, Derksen FJ, Robinson NE. Responses of calves to intrabronchial challenge with P haemolytica and endotoxin (abst.) 20th Annual Meeting of the American College of Veterinary Pathologists, No. 36, 1985.

P haemolytica instilled into the lungs of goats resulted in typical lesions of pasteurellosis.[22]

Challenge experiments resulted in recovery of bacteria from numerous organs indicating septicemia in these calves (Chapter 6). If the challenge dose of bacteria was especially high (>10^{10} CFU/ml), the calves often died acutely with lesions typical of disseminated intravascular coagulopathy (DIC). Clinical signs in calves described in experiments in Chapter 5 consisted of fever, increased respiratory effort and rate, diarrhea, depression and anorexia. These clinical and pathological changes suggest the presence of endotoxemia.

The presence of DIC lesions in calves inoculated with a subcutaneous injection of crude leukotoxin plus endotoxin raised further questions regarding the role of endotoxin and leukotoxin in the pathogenesis of P haemolytica infection. The purposes of this study were: (1) to determine whether endotoxin and leukotoxin of P haemolytica interact to produce pathophysiology; (2) to determine if oral priming of leukotoxin and endotoxin have any effect on subsequent parenteral exposure to these toxins and resultant pathophysiology, and (3) to determine what changes in parameters commonly used to measure DIC can be attributed to the leukotoxin and the endotoxin of P haemolytica.

Materials and Methods

Calves--Sixteen calves were purchased as previously described (Chapter 3). Ages varied from 3 to 11 months. Surgery to insert

intraduodenal catheters in each animal was performed as previously described (Chapter 2).

Bacterial antigens--Concentrated leukotoxin supernatants were prepared as described previously (Chapter 3). Total toxin preparation was pooled and allotted so that each animal received an identical preparation of toxin. Each inoculation contained 1 ug/kg body weight endotoxin and 3000 toxic units/kg body weight of leukotoxin activity in each injection.

Evaluation of coagulation factors, leukocyte and platelet numbers--Blood was collected as indicated in Table 7.1 for the determination of total leukocyte and differential counts, platelet count, fibrinogen and fibrin degradation product levels, one stage prothrombin and activated partial thromboplastin times.

Complete blood cell counts were performed on an automated cell counter.[b] Differential counts were performed on blood smears prepared by Wright-Giemsa stain in an automated slide stainer.[c] A total of 100 cells were counted twice and averaged. Samples with too few cells were determined using 50 cells.

Platelet number was determined by a microcollection system.[d] Counts were performed on a hemocytometer using phase contrast microscopy. Two counts were performed per sample. Counts that varied by more than 20% were repeated using a fresh reagent capsule.

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[b] Model 770 Coulter Counter Company, Hialeah, FL.

[c] Ames Hema-Tek, Miles Laboratories, Inc., Elkhart, IN.

[d] Unopette®, Becton-Dickenson and Company, Rutherford, NJ.

Table 7.1. Blood samples collected and assays performed.

Volume	Anticoagulant	Assays Performed
3.0 cc	4.5 mg EDTA	CBC and differential platelet count
7.0 cc	3.2% Sodium citrate	APTT OSPT
5.0 cc	Thrombin	Fibrinogen FDP
5.0 cc	None	IgG (ELISA)

CBC = Complete blood count; APTT = Activated partial thromboplastin time; OSPT = One stage prothrombin time; FDP = Fibrin(ogen) degradation products.

Total fibrinogen was determined using a quantitative assay.[e] Kit directions were followed to perform this test. Briefly, a 1:10 dilution of test plasma and several dilutions of known reference plasmas were made. Each sample was added to a receiving cup in a warmer to which (human) thrombin was added. The time to clot was determined by the use of an automated fibrometer.[f] A standard curve of clotting time versus dilution of standards was plotted. From this curve the quantity of fibrinogen in each test sample could be determined by measuring clotting time.

Fibrin(ogen) degradation products were measured using a staphylococcal clumping test[g] for serum. Serum was collected as indicated in Table 7.1. Directions were followed as indicated in the kit. Briefly, serial dilutions of test serum were made and 0.05 ml placed on a black plate. Known standard dilutions of FDP were also diluted serially and 0.05 ml of each placed on another plate. To each drop of serum and a known standard amount of FDP was added 0.05 ml of staphylococcal cells. A wooden applicator stick was used to mix each sample. Once mixed, the plate was rotated for 2 minutes. At that time clumping of cells in serum samples was compared to clumping of a known standard dilution of FDP. To calculate the quantitity of FDP present the inverse of the last dilution of sample with clumping was multiplied

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[e] Ortho Diagnostic Systems, Inc., Raritan, NJ.

[f] Fibrosystem fibrometer, BBL, Inc., Cockeysville, MD.

[g] Sigma Diagnostics, St. Louis, MO.

by the amount of reference FDP in the standard which had clumping most comparable to that sample.

Activated partial thromboplastin time (APTT) was determined using activated Thrombofax®[e] and measuring the time by an automated fibrometer.[f] Directions were followed as described by Ortho Diagnostic[e] reagent insert with one exception as noted. Briefly, 0.1 ml test plasma was mixed with 0.1 ml of Thrombofax®[e], a mixture of bovine brain cephalin and ellagic acid. This mixture was incubated at 37 C for exactly 2 minutes rather than 5 minutes recommended by the insert. This time was found to be optimal for determination of bovine APTT by Greene et al.[23] After this incubation 0.1 ml of calcium chloride was added, mixed briefly, and the time for a clot to form was recorded by the fibrometer.

One stage prothrombin time (OSPT) was determined using rabbit brain tissue thromboplastin acquired in a commercial kit.[e] The procedure for this test was followed as described in the reagent insert. Briefly, 0.1 ml of test plasma was brought to 37 C for 5 minutes. To the plasma was added 0.2 ml of brain thromboplastin.[e] The tube was inserted into an automated fibrometer[f] and the time for a clot to form was measured.

All plasma and serum used for these tests were frozen at -70 C immediately following collection. Tests to measure APPT and OSPT were performed on plasma which was allowed to thaw for no more than 30 minutes prior to testing.

Statistical analysis--The means of each parameter were compared for each time point within each group and between each group by the Waller-Duncan analysis of variance test.

Procedure

Calves were divided into four groups consisting of four calves each. Each calf had an intraduodenal (ID) catheter surgically implanted and was allowed to recover for 10 days prior to inoculation. One group received tissue culture media ID and two groups were primed ID by a concentrated leukotoxin and endotoxin preparation prepared as described in Chapter 3. Two weeks later each calf received a subcutaneous (SC) injection of either tissue culture medium, leukotoxin and endotoxin, or heat treated leukotoxin and endotoxin as outlined in Table 7.2. Immediately following injection of the SC dose of inoculum blood samples were collected at 0, 2, 4, 6, 12, and 24 hours as described in Table 7.1. These samples were assayed for activated partial thromboplastin time (APTT), one stage prothrombin time (OSPT), fibrinogen, fibrin degradation products (FDP), platelets, total number of leukocytes (WBC), number of polymorphonuclear cells (PMN), body temperature, and clinical evaluation score. At each time point calves were clinically evaluated as described by Potgieter et al.[24] based on a scale of 0 to 10 with 0 as normal. Factors evaluated were respiratory rate, respiratory effort, attitude, diarrhea, coughing, and nasal/salivary discharge making the maximum possible abnormal score 60. At 24 hours each calf was euthanized and a complete gross and histopathological examination was performed.

Table 7.2. Schedule of inoculation of calves with 100X concentrated leukotoxin and endotoxin.

Group	Intraduodenal Prime (Day 0)	Subcutaneous Booster (Day 14)	Blood Sample (0,2,4,6,12, and 24 hours)	Post Mortem Exam
A	Leukotoxin*	Leukotoxin	X	X
B	Leukotoxin	Heat treated† Leukotoxin	X	X
C	RPMI-1640	Leukotoxin	X	X
D	RPMI-1640	RPMI-1640	ND	X

* Each dose of leukotoxin contained 3000 toxic units/kg body weight of calf of leukotoxin as determined by neutral red uptake assay and 1 ug/kg body weight endotoxin as determined by limulus lysate assay.

† Same preparation of leukotoxin used to inoculate other calves but placed in water bath at 56 C for 30 minutes prior to inoculation.

ND = Not done

Results

The means and standard deviations of clinical scores and body temperature were recorded for groups A through C (Table 7.3). The mean natural logarithm (ln) was computed for fibrin degradation products for each time period for each group. The amount decreased from time 0 was recorded for WBC, PMN, platelets, and fibrinogen. A negative number indicates that the value increased by that amount compared to time 0. The increase in time compared to time 0 was recorded for OSPT and APTT. Values within each group that varied at a significantly different level between time points are indicated by a different letter.

Each group had significantly higher clinical scores from 2 hours through 24 hours ($p<0.05$). Body temperature did not change significantly over time for group B. Groups A and C had biphasic (but significant) increases in body temperature at 2 and 12 hours. Total WBC counts decreased significantly from 2 to 24 hours in groups A and B with a return to normal in group C by 24 hours ($p<0.05$). Total PMN decreased to a significantly different level in each group from 2 through 12 hours with group C returning to pre-exposure level by 24 hours ($p<0.05$). Platelets decreased to a significantly different level in groups A and B by 4 hours post-SC inoculation and remained abnormal the duration of the experiment ($p<0.05$). Group C calves did not attain a significantly different change in platelet number until 12 hours, but was still decreasing at 24 hours.

Changes in APTT were significantly increased in group A and B from

TABLE 7.3. Physiological changes in calves following subcutaneous injection of leukotoxin and endotoxin.

TIME	GROUP*	WBC†	PMN†	PLATELETS† X1000	OSPT† SECONDS	APTT† SECONDS	FIBRINOGEN† ul/mg	LN FDP ug/ml	BODY TEMP. FAHRENHEIT	CLINICAL SCORE
0 HOURS	A	0±0	0±0	0±0	0±0	0±0	0±0	-0.22±0.98^{b}	102.3±0.5^{b}	0±0^{c}
	B	0±0	0±0	0±0	0±0	0±0	0±0	-0.22±0.0^{b}	102.3±0.7^{a}	0.50±1.0^{d}
	C	0±0	0±0	0±0	0±0	0±0	0±0	-0.40±0.35^{b}	103.0±0.6^{b}	0.50±1.0^{b}
2 HOURS	A	8625±1466^{c}§	3540±550^{a}	153±134^{b,c}	1.5±1.0^{d}	6.9±8.3^{d,e}	21±33^{c}	0.64±0.66^{b}	103.0±0.7^{a}	25.0±10.00^{b}
	B	7400±2515^{c}	2560±869^{a}	65±91^{b,c}	2.5±1.2^{b,c}	15.5±8.0^{c,d}	19±11^{c}	0.225±0.8^{b}	102.8±0.5^{a}	33.75±1.26^{b}
	C	6250±1323^{b}	3271±543^{a}	45±111^{c}	1.4±1.1^{d}	5.7±3.6^{d}	31±21^{a}	0.125±0.40^{b}	102.9±0.7^{b}	26.50±5.74^{a}
4 HOURS	A	9050±1240^{c}	3279±1035^{a}	216±170^{b}	3.9±2.6^{c}	15.0±10.6^{c,d}	130±44^{b}	2.38±0.34^{b}	102.3±1.8^{b}	32.75±6.18^{a}
	B	7950±2457^{c}	2607±833^{a}	167±44^{b}	3.8±0.9^{b,c}	30.5±13.0^{c}	80±50^{a,b}	1.34±0.35^{b}	101.7±1.6^{a}	36.00±0.82^{a}
	C	7250±1800^{b}	3034±918^{a}	164±142^{b,c}	5.4±2.2^{c}	10.0±3.8^{d}	211±134^{a,b}	2.03±0.87^{a,b}	102.6±0.8^{b}	31.50±3.32^{a}
6 HOURS	A	8975±1417^{c}	3235±687^{a}	261±117^{b}	4.8±3.3^{c}	24.0±4.5^{b,c}	194±82^{b}	3.4±0.35^{b}	102.2±1.8^{b}	36.00±3.37^{a}
	B	7875±2490^{c}	2546±866^{a}	159±61^{b}	4.1±0.8^{b}	56.0±18.0^{b}	109±71^{a}	2.38±0.66^{a,b}	101.9±1.3^{a}	34.00±0.82^{b}
	C	7175±1646^{b}	3319±566^{a}	110±48^{b,c}	7.0±3.3^{c}	34.0±18.0^{c}	286±153^{a}	3.24±1.5^{a}	102.4±0.6^{b}	32.25±4.99^{a}
12 HOURS	A	7400±1791^{b}	3432±573^{a}	475±187^{a}	8.3±2.4^{b}	46.5±13.6^{a}	297±108^{a}	4.45±0.66^{a}	103.6±1.6^{a}	36.00±3.37^{a}
	B	7225±2370^{c}	2330±1056^{a}	318±79^{a}	5.5±2.1^{a,b}	95.0±11.0^{a}	131±74^{a}	3.24±0.57^{a}	103.7±0.9^{a}	33.75±1.26^{b}
	C	5367±1531^{b}	2530±325^{a,b}	278±174^{a,b}	8.9±1.4^{b}	58.0±19.0^{a}	285±74^{a}	4.17±0.40^{a}	104.1±0.6^{a}	31.33±5.51^{a}
24 HOURS	A	4500±2858^{a}	2107±631^{b}	452±149^{a}	12.6±3.4^{a}	34.6±5.1^{b}	280±88^{a}	4.4±0.4^{a}	102.8±0.4^{a}	36.00±1.73^{a}
	B	3633±4067^{b}	-176±2835^{b}	349±113^{a}	7.8±1.1^{a}	87.0±38.0^{a}	107±58^{a}	3.0±1.0^{a}	102.7±0.6^{a}	23.33±1.53^{c}
	C	1767±3885^{a}	757±2875^{b,c}	382±161^{a}	14.3±2.5^{a}	45.0±20.0^{b}	265±65^{a}	4.17±0.40^{a}	102.4±0.3^{b}	28.67±13.65^{a}

* Group A = Leukotoxin intraduodenally followed 2 weeks later by leukotoxin subcutaneously; Group B = leukotoxin intraduodenally followed 2 weeks later by heat treated leukotoxin subcutaneously; Group C = RPMI-1640 culture medium intraduodenally followed 2 weeks later by leukotoxin subcutaneously.

† Mean ± standard deviation decrease in WBC, PMN, platelets, and fibrinogen and increase in OSPT and APTT compared to time 0.

§ Means with different superscript letters are significantly different at ($p<0.05$) except for ln FDP group C where ($p<0.10$).

4 hours to the end of the experiment, and from 6 hours to the end of the experiment for group C ($p<0.05$). There was a significant increase in OSPT from 4 hours to the end of the experiment in groups A and C and from 6 hours to the end of the experiment in group B ($p<0.05$). The ln FDP increased significantly at 12 and 24 hours for groups A and B and did not show a significant increase for group C over time. Total fibrinogen levels decreased significantly in each group from 6 hours through the end of the experiment.

For clinical score and body temperature there were no significant differences for any time point between any group (Fig. 7.1 & 7.2). For WBC there was no significant difference between groups for any time point. Figure 7.3 shows the mean decrease in WBC compared to time 0 for each time point for each group. Groups A and C had greater decreases in WBC than group B at 2 and 4 hours. Group A had a greater decrease than group B which had a greater decrease than group C from 6 through 24 hours. Figure 7.4 depicts decreases in the PMN over time for each group. Group A has a greater decrease than group B which had a greater decrease than group C for most time points. There were no significant differences between groups at any time point. Group A had a greater decrease than group C which had a greater decrease than group B for all time points. Figure 7.5 depicts decreases in platelets over time for each group. Group A had a significantly greater decrease in platelets than group C at 6 hours ($p<0.05$).

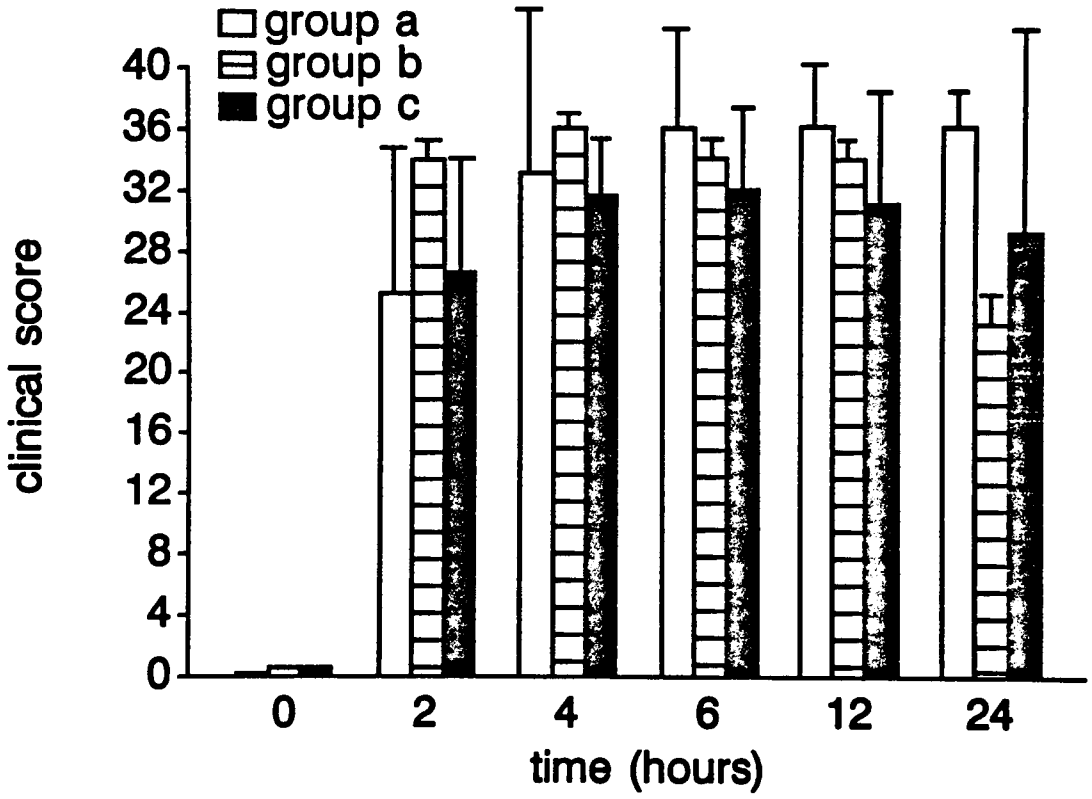

Figure 7.1. Mean clinical score of calves. Bars indicate standard deviation. Group A = leukotoxin intraduodenally followed 2 weeks later by leukotoxin subcutaneously; group B = leukotoxin intraduodenally followed 2 weeks later by heat treated leukotoxin subcutaneously; group C = RPMI-1640 culture medium intraduodenally followed 2 weeks later by leukotoxin subcutaneously.

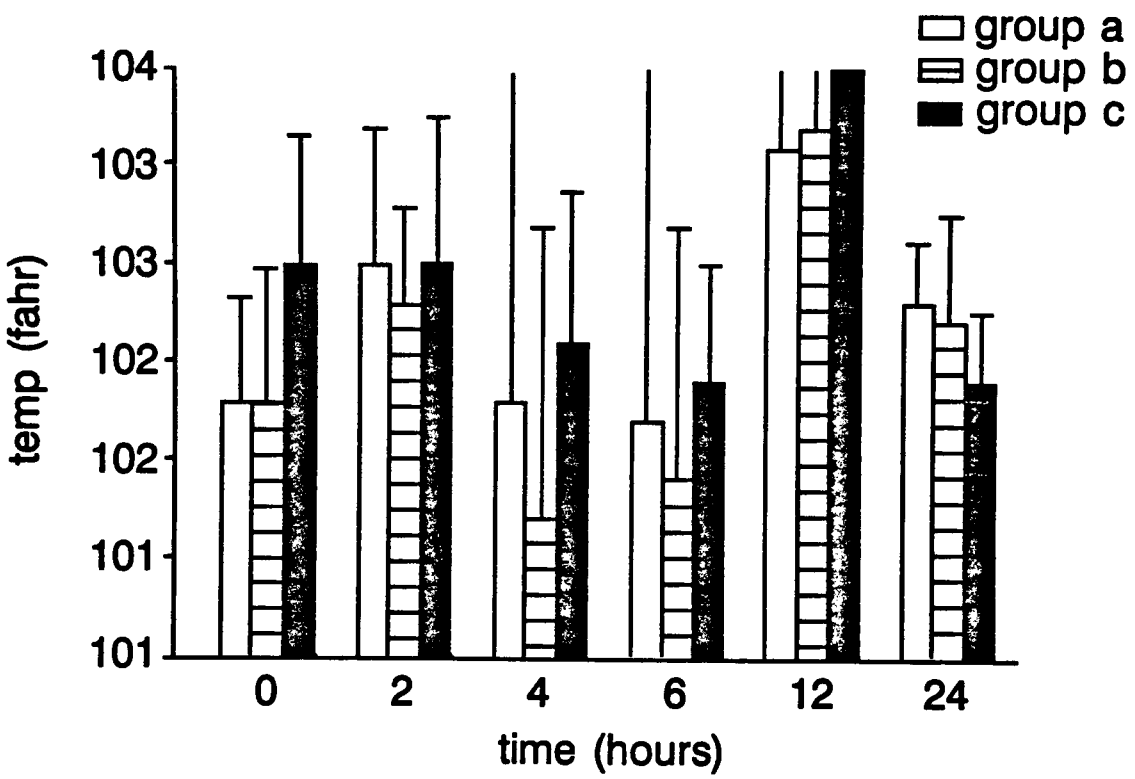

Figure 7.2. Mean body temperature of calves. Bars indicate standard deviation. Group A = leukotoxin intraduodenally followed 2 weeks later by leukotoxin subcutaneously; group B = leukotoxin intraduodenally followed 2 weeks later by heat treated leukotoxin subcutaneously; group C = RPMI-1640 culture medium intraduodenally followed 2 weeks later by leukotoxin subcutaneously.

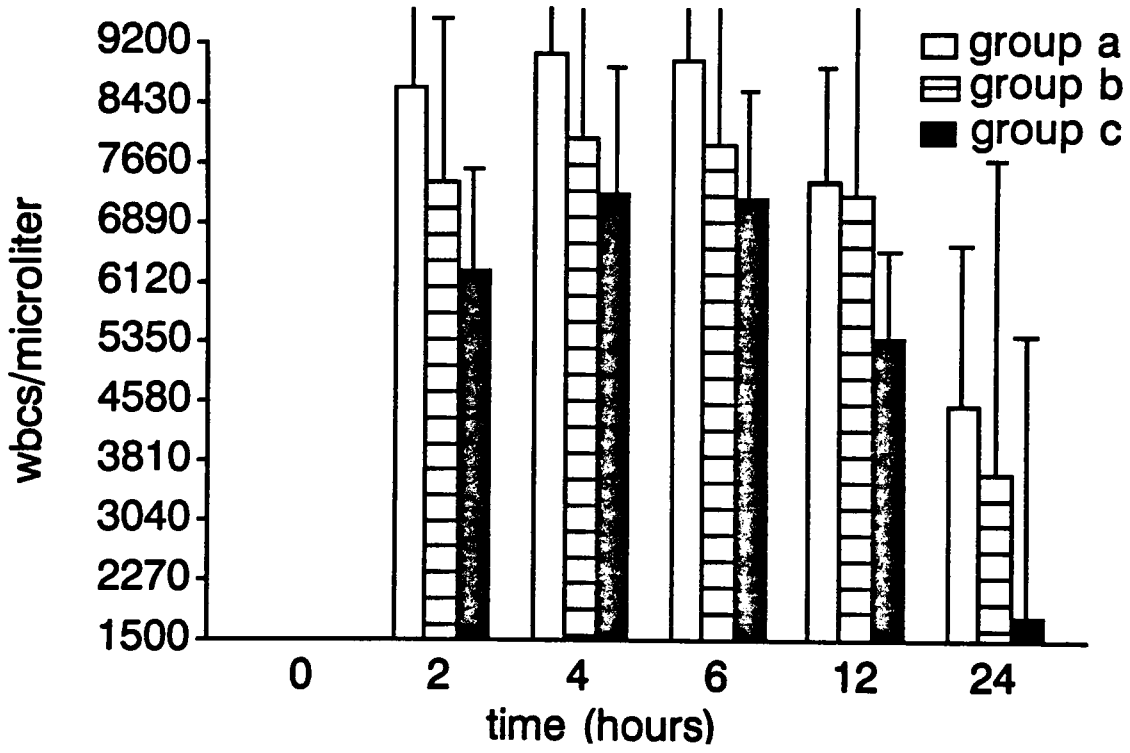

Figure 7.3. Mean decrease in the total number of leukocytes compared to time 0. Bars indicate standard deviation. Group A = leukotoxin intraduodenally followed 2 weeks later by leukotoxin subcutaneously; group B = leukotoxin intraduodenally followed 2 weeks later by heat treated leukotoxin subcutaneously; group C = RPMI-1640 culture medium intraduodenally followed 2 weeks later by leukotoxin subcutaneously.

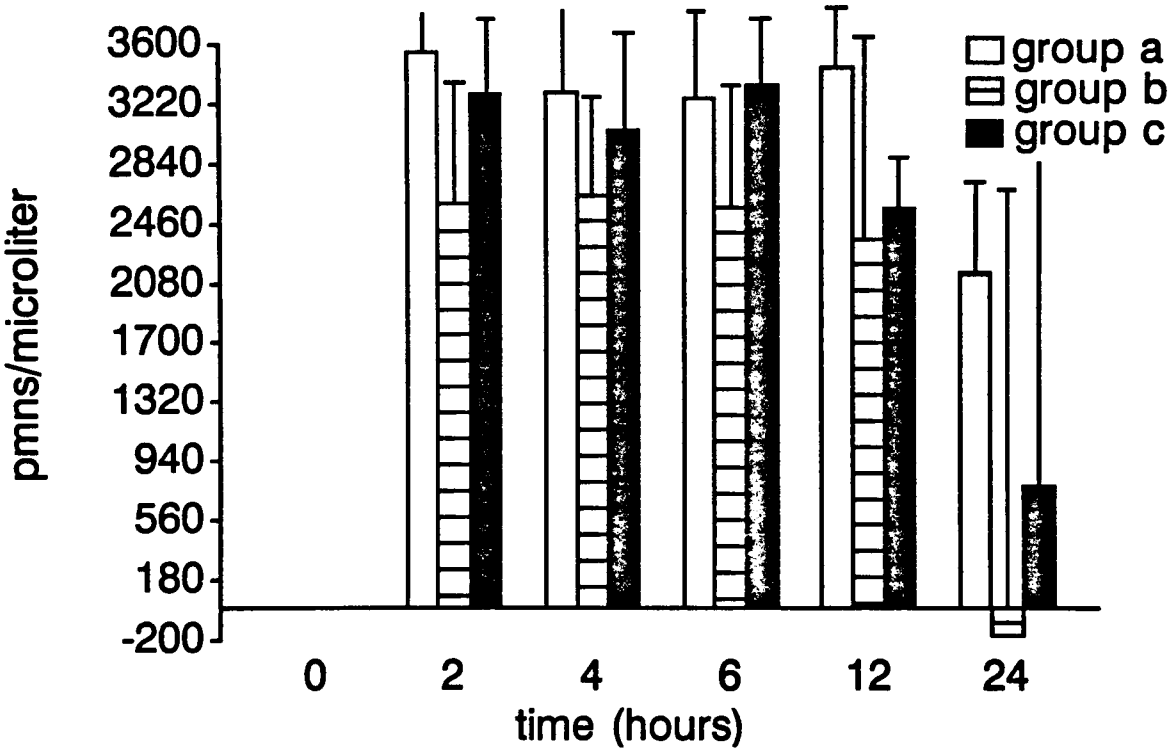

Figure 7.4. Mean decrease in the total number of polymorphonuclear cells compared to time 0. Bars indicate standard deviation. Group A = leukotoxin intraduodenally followed 2 weeks later by leukotoxin subcutaneously; group B = leukotoxin intraduodenally followed 2 weeks later by heat treated leukotoxin subcutaneously; group C = RPMI-1640 culture medium intraduodenally followed 2 weeks later by leukotoxin subcutaneously.

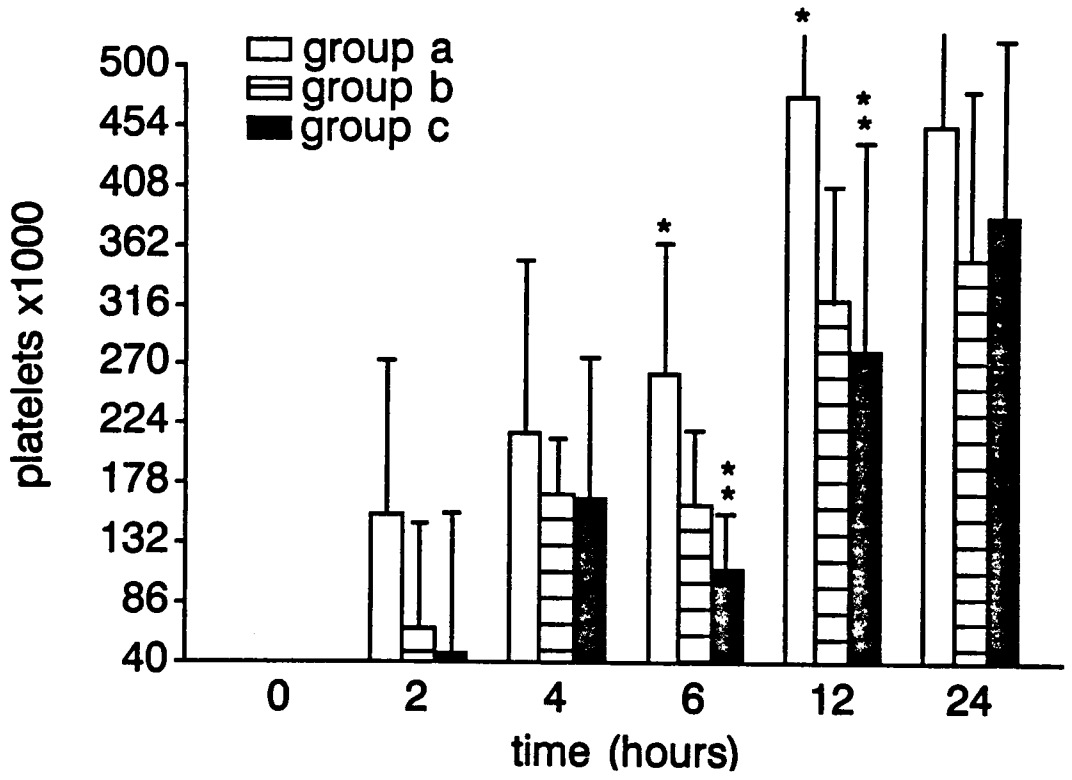

Figure 7.5. Mean decrease in the total number of platelets compared to time 0. Bars indicate standard deviation. Group A = leukotoxin intraduodenally followed 2 weeks later by leukotoxin subcutaneously; group B = leukotoxin intraduodenally followed 2 weeks later by heat treated leukotoxin subcutaneously; group C = RPMI-1640 culture medium intraduodenally followed 2 weeks later by leukotoxin subcutaneously. Means with a different number of asterisks are significantly different ($p<0.07$ at 6 hours, $p<0.10$ at 12 hours).

Figure 7.6 demonstrates the increase in APTT over time for each experimental group. Group B was significantly greater than group A and C at all times points except time 0 ($p<0.001$). Figure 7.7 demonstrates increases in OSPT. Group C was longer than group A which was longer than group B for all time points. Groups A and C were both significantly longer than group B at 12 and 24 hours ($p<0.05$). Figure 7.8 depicts the ln FDP for each group. From 6 hours on, group A was greater than C which was greater than group B. Group A had significantly greater ln FDP than group B ($p<0.05$) at 4 hours and groups A and C were significantly greater than group B at 12 hours ($p<0.10$). Figure 7.9 depicts decreases in total fibrinogen. Group C had a greater decrease than group A which was greater than B for most time points. Groups A and C had significantly greater decreases than group B at 12 and 24 hours ($p<0.05$).

Discussion

The decreases in WBC, PMN, platelets, and fibrinogen with concomitant increases in FDP, OSPT, and APTT seen in this experiment are consistent with those seen in calves exposed to endotoxin of *Escherichia coli* and *Pseudomonas* sp.[25,26]

Two groups of calves in this experiment also demonstrated a biphasic increase in body temperature which agrees with classical pyrogenicity seen in *E coli* endotoxin exposure.[25] The clinical evaluation scores in this experiment are consistent with clinical signs of endotoxemia--diarrhea, tachypnea, dyspnea, salivation, coughing. These changes were seen in this experiment with 1 ug/kg LPS, a

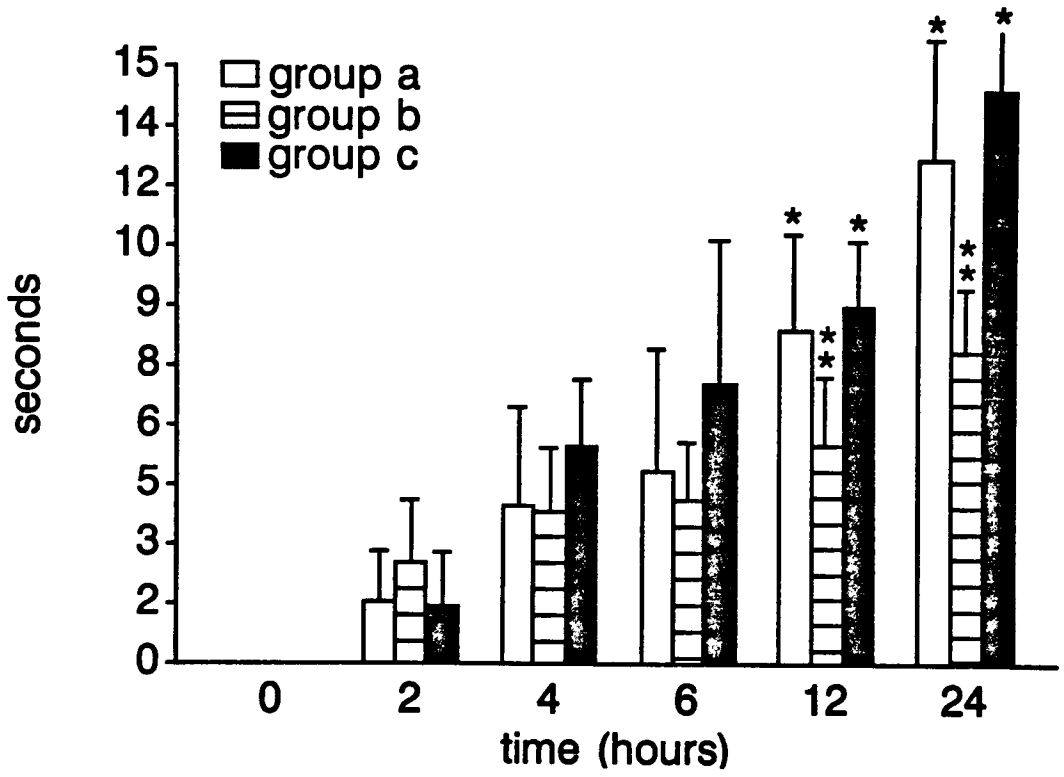

Figure 7.6. Mean increase in activated partial thromboplastin time compared to time 0. Bars indicate standard deviation. Group A = leukotoxin intraduodenally followed 2 weeks later by leukotoxin subcutaneously; group B = leukotoxin intraduodenally followed 2 weeks later by heat treated leukotoxin subcutaneously; group C = RPMI-1640 culture medium intraduodenally followed 2 weeks later by leukotoxin subcutaneously. Means with a different number of asterisks are significantly different ($p<0.01$).

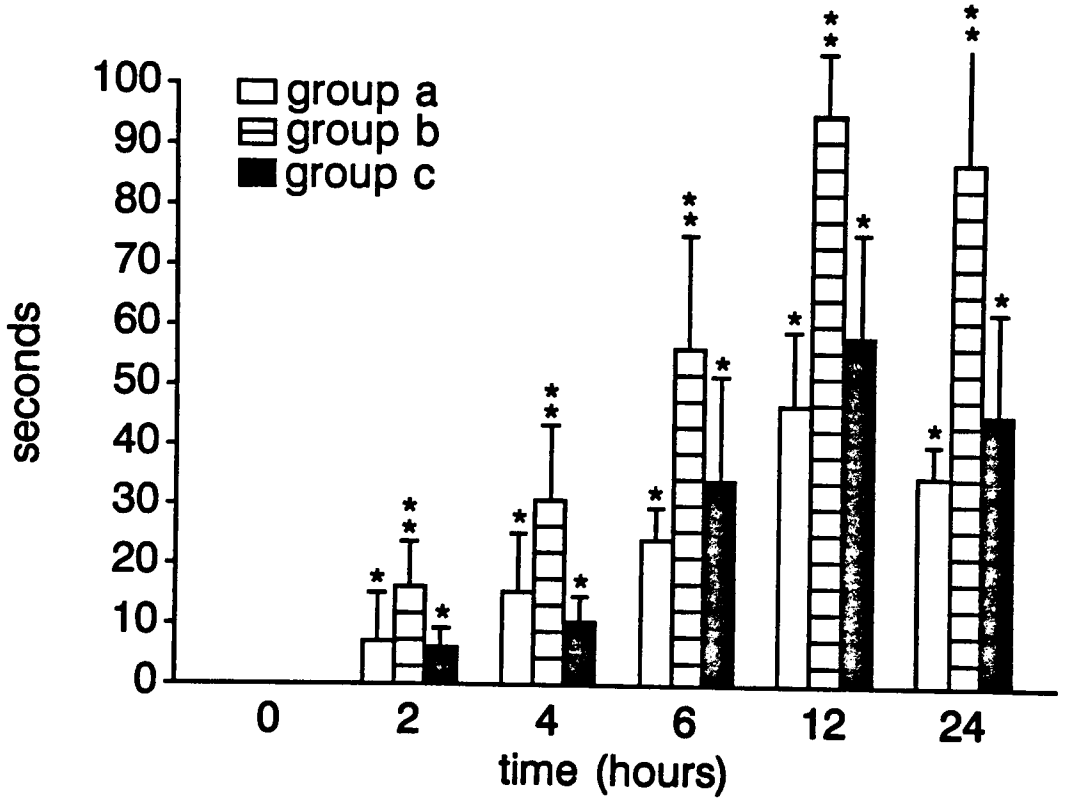

Figure 7.7. Mean increase in one step prothrombin time compared to time 0. Bars indicate standard deviation. Group A = leukotoxin intraduodenally followed 2 weeks later by leukotoxin subcutaneously; group B = leukotoxin intraduodenally followed 2 weeks later by heat treated leukotoxin subcutaneously; group C = RPMI-1640 culture medium intraduodenally followed 2 weeks later by leukotoxin subcutaneously. Means with a different number of asterisks are significantly different ($p<0.01$).

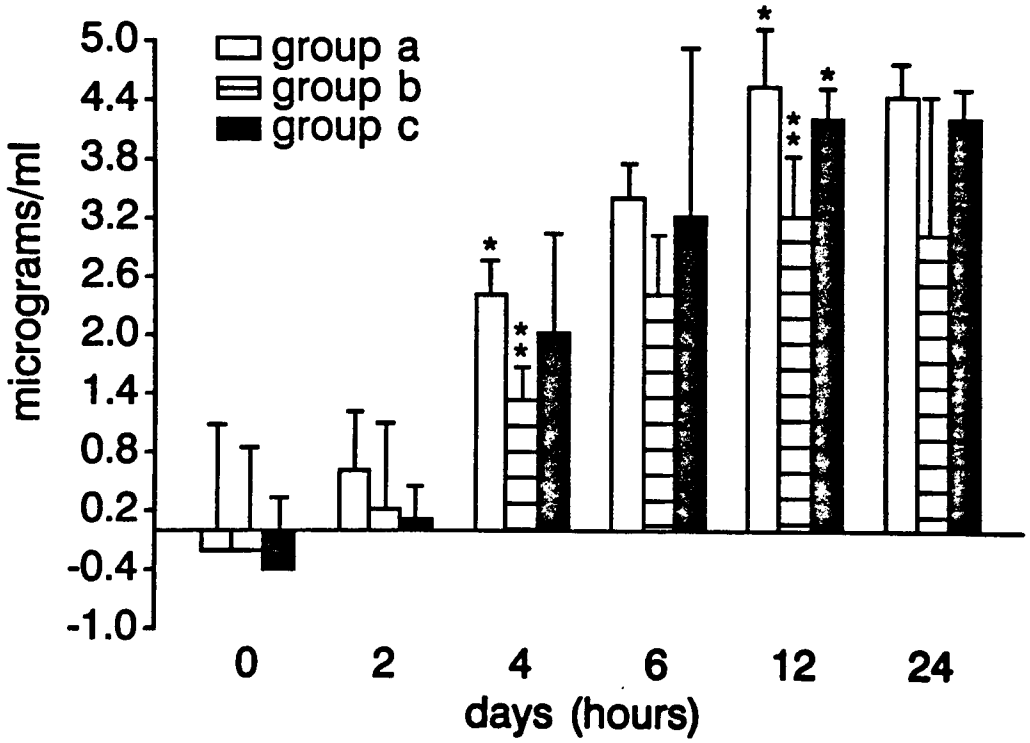

Figure 7.8. Mean natural logarithm of fibrin/fibrinogen degradation products. Bars indicate standard deviation. Group A = leukotoxin intraduodenally followed 2 weeks later by leukotoxin subcutaneously; group B = leukotoxin intraduodenally followed 2 weeks later by heat treated leukotoxin subcutaneously; group C = RPMI-1640 culture medium intraduodenally followed 2 weeks later by leukotoxin subcutaneously. Means with a different number of asterisks are significantly different ($p<0.08$ at 4 hours, $p<0.05$ at 12 hours).

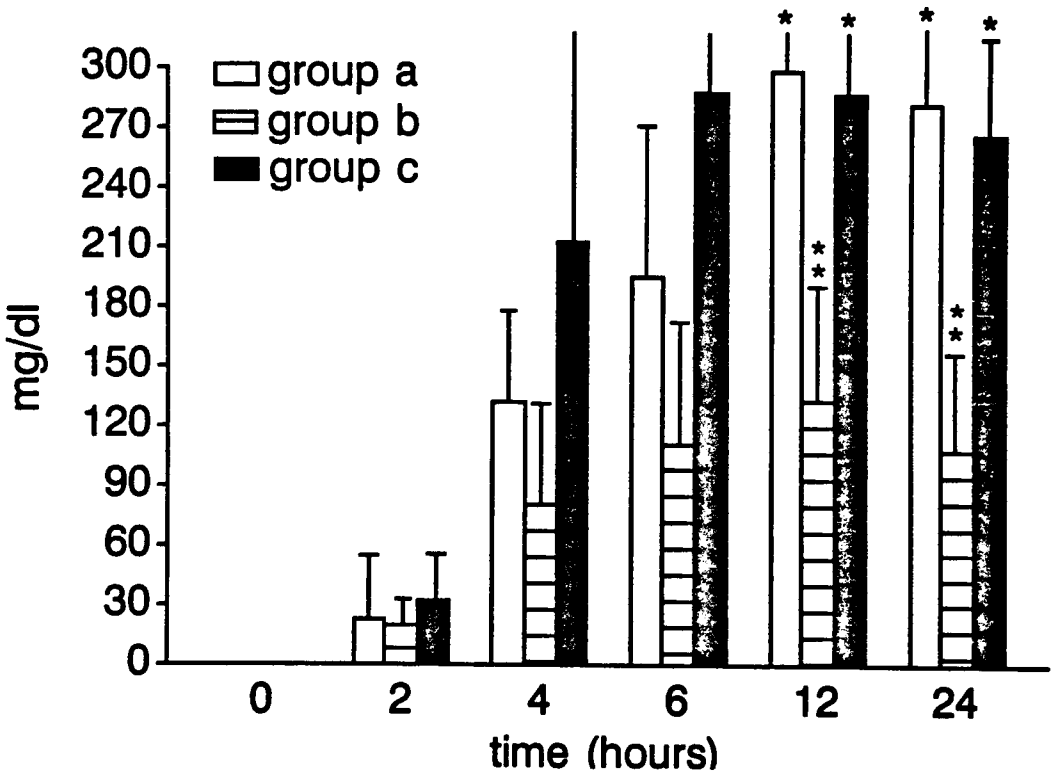

Figure 7.9. Mean decrease in fibrinogen level compared to time 0. Bars indicate standard deviation. Group A = leukotoxin intraduodenally followed 2 weeks later by leukotoxin subcutaneously; group B = leukotoxin intraduodenally followed 2 weeks later by heat treated leukotoxin subcutaneously; group C = RPMI-1640 culture medium intraduodenally followed 2 weeks later by leukotoxin subcutaneously. Means with a different number of asterisks are significantly different ($p<0.05$).

comparatively low dose of endotoxin compared to amounts used in other experiments where the dosage varied from 2 ug/kg to 20 ug/kg with similar results. This may indicate a sensitivity of the calves used in this experiment to the LPS of P haemolytica or suggest that the LPS of P haemolytica is relatively more potent compared to E coli or other gram negative organisms.

According to Bick[27] the relative sensitivity of parameters to measure DIC in order of relative importance are: (1) fibrin/fibrinogen degradation products; (2) platelet number; (3) prothrombin time, and 4) partial thromboplastin time. Each experimental group of calves demonstrated changes in these parameters to abnormal levels associated with the onset of lesions commonly associated with DIC (Chapter 4). The pattern of change indicated that changes in group A exceeded changes seen in group C which exceeded changes seen in group B except for APTT. In this case the changes seen in group B were highly significant compared to those in group A or C. Perhaps the relative insensitivity of this test[27] accounts for the difference in the pattern of response.

Fibrinogen and FDP are well known inhibitors of blood clot formation. The fibrinogen and ln FDP changed the least in this group. Therefore it is unlikely that the APTT was prolonged due to interference in clot formation by these products. The OSPT and APTT are usually prolonged when fibrinogen decreases to less than 100 mg/dl. It is felt that increased plasmin activity inhibits factors V, VIII, IX and lyses fibrinogen and fibrin.[19] The resultant increased

fibrin degradation products act as anticoagulants by inhibiting fibrinogen cross-linking, thus increasing OSPT. Calves in groups A and C met this criteria. Calves in group B did not have as low as fibrinogen but still had a significant change in OSPT and APTT. This has been found to be typical of horses and cows and supports previous studies in this regard.[25]

The total WBC and platelet numbers changed more in group A than group B than group C. The total PMNs decreased more in group A than group C than group B. These differences were not significantly different but suggest that peripheral blood leukocytes (PBL) other than PMNs and platelets are affected differently by leukotoxin and endotoxin. The effect on WBC and platelets could be attributed to the total dose of leukotoxin and endotoxin each calf was exposed to. Group A calves received 2 full doses of the toxins; group B received 1 full dose with 1 dose heat treated to inactivate the leukotoxin; and group C received only 1 dose of toxin. PMNs could have been more susceptible to leukotoxin than other PBLs since group A was greater than group C than group B in the decrease in the number of PMNs. Part of the change in WBCs in group A could be due to GALT stimulation increasing the homing of lymphocytes to mucosal tissues or activation of T suppressor cells. Endotoxin could also have caused margination of WBCs to endothelial surfaces reducing the number of circulating WBCs. Either mechanism would decrease the pool of circulating lymphocytes. A decrease in WBCs may also be attributed to (opsonizing) antibodies directed to the endotoxin that would increase the recognition and

phagocytosis of toxin, and subsequent lysis of the cells. Parenteral stimulation of individuals previously exposed naturally increases mucosal antibody production.[28] The changes in WBCs could also be partly due to a decrease in lymphocytes number caused by increased serum cortisol. Endotoxin has been shown to increase adenocorticotrophic hormone production which is correlated with lymphopenia.[27]

In DIC platelet counts typically decrease to less than 10^5/ul. Only group A reached this level, but at 24 hours the platelet count was still decreasing in each group. This suggests that platelets were still being removed from the circulation faster than they were being produced. Usually in endotoxin induced thrombocytopenia, platelet counts will eventually increase dramatically due to a potent stimulating effect of endotoxin on megakaryocyte metabolism. However, this rebound effect does not usually occur until after 30 hours post-exposure.[25] The calves in this study were followed for a total of 24 hours so it was probably too soon for this effect to be seen when the experiment ended. The endotoxin induced decrease in platelets can be attributed to several factors: (1) endotoxin binds directly to platelets to cause aggregation; (2) damage to endothelial lining exposes collagen which is a potent activator of platelet aggregation; (3) direct stimulation of factor VII of the intrinsic clotting mechanism which activates platelet aggregation; (4) activation of complement which damages platelets and causes aggregation,[27] and (5) sequestration of platelets in spleen, lung, and liver (due to damage of

endothelial macrophages and binding of platelets to these cells as well as endothelium).[h]

The decrease in PMNs can be explained by several mechanisms. Endotoxin induces margination of neutrophils along blood vessels. At the same time endotoxin increases the lability of lysosomes which leads to degeneration of neutrophils. These are then destroyed in the microcirculation of the capillary beds and in liver, kidney, spleen and lung. Neutrophils have been implicated in mediating the pathology associated with endotoxic shock presumably due to release of their lysosomal enzymes.[29] Recent investigations by Fenwick et al[i] suggest that PMNs actually may modulate endotoxin induced pathology by enzymatic deacylation of the lipopolysaccharide. Deacylation would reduce the toxicity of LPS without affecting the immunological or inflammatory activity of the endotoxin. This activity is attributed to acyloacyl hydrolase (AOHA) activity contained in lysosomes of the PMNs. The end result is that calves that had been neutrophil depleted had lower serum levels of the enzyme and greater toxic damage than neutrophil competent calves.

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[h] Molina RM, Warner AD, Brain JD. Pulmonary intravascular macrophages are an important part of the mononuclear phagocyte system in ruminants and cats. (abst.) 6th Veterinary Respiratory Symposium, p. 20, 1987.

[i] Fenwick BW, Cullor JS. Neutrophils mediate rather than contribute to the severity of endotoxic shock (abst.) 68th Annual Meeting of the Conference of Research Workers in Animal Disease, No. 239, 1987.

Calves with the greatest reduction in PMNs in this experiment had the greatest pathological lesions (Chapter 8). Groups A and C had greater changes than group B in most parameters (PMN, fibrinogen, ln FDP and OSPT) suggesting that leukotoxin exerts an effect on coagulation factors beyond the changes attributed to endotoxin. The effects of both leukotoxin and LPS must be considered as virulence mechanisms of P haemolytica affecting not only pulmonary tissues, but seriously affecting clotting homeostasis and leukocyte numbers. Any methods used to control this disease should consider the role these factors play in the pathogenesis of the disease. The potent effects of parenterally administered toxins suggest that toxic activity must be altered or reduced in the preparation of any immunizing toxoid product.

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CHAPTER 8

PATHOLOGICAL CHANGES IN CALVES INDUCED BY EXPOSURE TO *PASTEURELLA HAEMOLYTICA* ENDOTOXIN AND LEUKOTOXIN

Experiments have demonstrated that calves inoculated intraduodenally (ID) with crude supernatant of logarithmic phase *Pasteurella haemolytica* cultures followed 2 weeks later by a subcutaneous (SC) booster injection reacted adversely with clinical signs of respiratory distress, fever, diarrhea, coughing, salivation and death (Chapter 5). Subsequent experiments to evaluate the role of endotoxin, leukotoxin, and gut priming in inducing these changes evaluated physiological parameters affected (Chapter 7). Other experiments have shown that ID stimulation by *P haemolytica* crude supernatant endotoxin and leukotoxin does not result in any adverse effects yet can result in increases in pulmonary and humoral antibodies capable of neutralizing the leukotoxin of *P haemolytica* (Chapter 4,5). However, this method of stimulation has been shown also to increase pulmonary and systemic IgE titers.

The physiological effects of *P haemolytica*'s endotoxin are fairly well understood. Less well characterized is the physiological effect of *P haemolytica*'s leukotoxin. It is known to decrease phagocytosis by alveolar macrophages and neutrophils and to kill these cells as well as lymphocytes of ruminants.[1-3] Strathdee et al.[4] have suggested it also contains alpha-hemolysin-like properties. However, the leukotoxin appears to be specific for ruminant leukocytes exclusively. This toxin has no effect on ruminant spleen cells, erythrocytes, kidney tissue cells or leukocytes of other species (pig, horse, human).[5] Pulmonary

and systemic hypertension 30 minutes following intravenous (IV) administration of crude leukotoxin has been suggested by Klernstiver et al.[a] Effects seen were quite similar to those induced by endotoxin.

The role of the endotoxin and leukotoxin of P haemolytica in pathological changes in the lung and other organs has been controversial. It is known that P haemolytica contains significant amounts of endotoxin.[6] However, very little is known about the specific role of this endotoxin in the pathogenesis of P haemolytica pneumonia. Attempts to evaluate the role of endotoxin in ruminant pneumonia have used E coli endotoxin as a model.[7,8] However, Confer et al.[9] has shown that the endotoxin of P haemolytica produces very different effects on calf leukocyte function than E coli endotoxin.

The purpose of this experiment was to evaluate the role of P haemolytica endotoxin and leukotoxin administered subcutaneously in inducing pathological changes in calves following ID administration of leukotoxin or endotoxin.

In this way the role of leukotoxin, endotoxin, intraduodenal priming, and parenteral administration in causing changes could each be evaluated.

Materials and Methods

Calves--Crossbred beef calves were used as previously described (Chapter 2).

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[a]Klernstiver P, Black W, Eyre P. Pulmonary vascular changes to P haemolytica exotoxin infusion in calves (abst.) 6th Annual Meeting Research Workers in Animal Disease, No. 150, 1986.

P haemolytica leukotoxin and endotoxin preparation--Concentration of crude leukotoxin supernatants were prepared as previously described (Chapter 3). Supernatants were concentrated 100-fold and contained endotoxin activity as determined by the limulus lysate[b] assay. Leukotoxin activity for BL-3 cells was determined by neutral red uptake assay as previously described.

Procedure

Calves were divided into four groups with four calves in each group. Intraduodenal catheters were inserted surgically and calves were allowed to recover 10 days. Each calf then received 2 doses of inoculum by the protocol shown in Table 8.1. Each dose of concentrated leukotoxin and endotoxin contained 1 ug/kg body weight of lipopolysaccharide and 3000 toxic units/kg body weight of leukotoxin activity. The first dose was administered intraduodenally with the second dose given subcutaneously 2 weeks later. Following administration of the second dose of inoculum, blood was collected for evaluation of coagulation factors, leukocyte and platelet counts (Chapter 7). Immediately following the collection of blood at 24 hours each calf was euthanized by injection of T-61 euthanasia solution.[c] A complete necropsy was performed and gross lesions scored on a basis of 0-3 with 3 = severe, 2 = moderate, 1 = mild and 0 = no change. The pathologist

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[b]Sigma Chemical Company, St. Louis, MO.

[c]American Hoechst Corp., Somerville, NJ.

Table 8.1. Experimental groups of calves and timing of exposure to toxin preparations.

Injection	Group A	Group B	Group C	Group D
Intraduodenal injection Day 0	Leukotoxin/ endotoxin*	Leukotoxin/ endotoxin	RPMI-1640	RPMI-1640
Subcutaneous injection Day 14	Leukotoxin/ endotoxin	Heat-treated leukotoxin†	Leukotoxin/ endotoxin	RPMI-1640

* Leukotoxin and endotoxin = 100-fold concentrated supernatants prepared from live log phase P haemolytica grown for 90 min in RPMI-1640 supplemented with 1% fetal calf serum.

† Leukotoxin and endotoxin (concentrated 100-fold) incubated in 56 C waterbath for 30 minutes prior to injection in order to inactivate any leukotoxin present.

had no knowledge of the group identity of any calf. Histopathological lesions were scored using the same scale by a pathologist with no knowledge of the group identity of any calf.

Gross lesions of organs were scored based on parameters shown in Table 8.2. The maximum score possible was 24. Histopathological lesions were graded based on parameters shown in Table 8.3. The maximum score possible for respiratory lesions was 24 and for other tissues was 27. The mean scores of each group were compared using the Waller-Duncan analysis of variance test.

Results

Gross Lesion Scores

Group A--Widespread ecchymotic hemorrhages were present in the parietal and costal pleura and the serosal surface of most peritoneal viscera. Severe hemorrhage and edema were present in the masseter muscle and at the cervical subcutaneous injection site extending into the underlying muscle. Severe congestion was present in tracheal, bronchial and internal iliac lymph nodes, and also in the abomasal mucosa. Approximately one liter of clear red fluid was present in the peritoneal cavity. Lungs were uniformly dark red on cut surface, firm, with severe interlobular edema and multiple ecchymotic hemorrhages on the serosal surface. Thrombosed vessels were present subcutaneously in the injection area.

Group B--Numerous ecchymotic hemorrhages were present on the costal pleura, the serosal surfaces of most peritoneal viscera, and the

Table 8.2 Gross post mortem lesion parameters evaluated.

Cardiopulmonary Organs	Other Organs
Lung - hemorrhage	Lymph nodes
edema	Gastrointestinal tract
Heart	Muscle
Vessel thrombi	Inoculation site (skin)

Table 8.3 Respiratory parameters and other tissues evaluated for histopathological lesions.

Respiratory	Other Tissues
Septal edema	Tracheal bronchial lymph node
Septal infiltrates	Spleen
Congestion	Heart
Hemorrhage	Liver
Alveolar exudate	Adrenal gland
Alveolar fibrin	Kidney
Alveolar edema	Duodenum
Alveolar thrombi	Skin (injection site)
	Cerebrum

epicardium with frank hemorrhage into the cerebellum and large intestine. A few petechia were present at the inoculation site subcutaneously. The lungs were congested with isolated areas of hemorrhage present. The total lesions present, and especially lesions of the lung, were noticably less severe in group B than group A or C. However, serosal hemorrhages were greater for this group than any other.

Group C--Widespread hemorrhages were present subcutaneously. The serosal surfaces of most abdominal viscera were hemorrhagic with intraluminal hemorrhage into the cecum, spiral colon and descending colon. Hemorrhages were also seen in the prescapular lymph node, epicardium, and numerous skeletal muscles with large muscles noticeably pale in color. Hemorrhage extended into underlying muscle at the subcutaneous cervical inoculation site. There was pericardial edema and subcutaneous edema. Lungs were moderately congested with increased firmness; there was marked interlobular edema, but no exudate was present. Petechiae were noted diffusely over the surface of all lobes. Overall severity of lesions were similar in appearance to group A.

Group D--In one animal there was edema and an area of slight hemorrhage at the inoculation site. The lung of one calf had an isolated 3 x 4 cm area that was plum colored and firm, and congested on cross section. No organisms were cultured from this area nor from the pulmonary lesions of any other experimental group.

The scores for each group of calves were compared using the

Waller-Duncan analysis of variance test. The overall and respiratory lesion scores are shown in Tables 8.4. Scores with superscripts of different letters were significantly different. For both scores groups A and C differed from group D the control calves, group B differed from group D but was not significantly different from group D nor from groups A and C. These results correlate well with the clinical impression of the calves and the coagulation scores of calves previously described (Chapter 7).

Histopathological Lesion Scores

Group A--The cortex of the tracheal bronchial lymph node was well populated by lymphocytes. The medullary sinuses contained moderate numbers of macrophages and neutrophils. In the spleen the red pulp was moderately congested with increased numbers of neutrophils present. The white pulp contained periarterial lymphoid sheets with moderate to severe lymphoid necrosis. In the adrenal glands there was congestion of sinusoids in the area of the zona reticularis with an increased number of neutrophils. The kidney had marked congestion of the vasculature, fibrinous thrombi in the glomeruli, and focal hemorrhages at the corticomedullary junction. The heart had extensive interstitial hemorrhages separating muscle fibers of the myocardium with some hemorrhages extending into the epicardium. The liver had mild to moderate infiltrates of lymphocytes and neutrophils into portal areas. The injection site in the cervical region contained extensive intramuscular hemorrhage that separated muscle fibers. These areas were infiltrated with lymphocytes and neutrophils. The brain had small

Table 8.4. Gross post mortem lesion scores in calves following parenteral exposure to P haemolytica leukotoxin and endotoxin.

	Group A*	Group B	Group C	Group D
Mean of overall lesion score	8.25±4.50†	4.75±3.34†,††	7.00±4.83†	0±0††
Mean of respiratory lesion score	3.75±2.50†	0.75±1.30†,††	3.00±2.12†	0±0††

* Group A = intraduodenal inoculation with leukotoxin and endotoxin (LE) followed 2 weeks later by a subcutaneous inoculation with LE; Group B = intraduodenal inoculation with LE followed 2 weeks later by a subcutaneous inoculation with LE heated to 56 C for 30 minutes; Group C = intraduodenal inoculation with RPMI-1640 culture medium followed 2 weeks later by a subcutaneous inoculation with LE; Group D = intraduodenal inoculation with RPMI-1640 followed in 2 weeks by a subcutaneous inoculation with RPMI-1640.

† Means with different number † are significantly different ($p<0.10$).

focal hemorrhages associated with vessels in the cerebral cortical grey and white matter.

The lesions of the spleen, adrenal gland, kidney and injection site were the most severe of any group.

In the lungs interlobular septa contained moderate to severe edema, hemorrhage and congestion of blood vessels. Moderate to severe infiltrates of mononuclear cells and neutrophils were also present. There was diffuse congestion of the pulmonary vasculature. Submucosal aggregates of lymphoid follicles were associated with bronchi. Alveoli contained mild amounts of exudate and moderate to severe amounts of hemorrhage and edema. There was severe edema of the alveolar septa which also contained fibrin and many erythrocytes along with some macrophages and neutrophils. Perivascular hemorrhage of small arterioles was present in alveolar septa. Moderate amounts of fibrinous thrombi were also present in alveolar septa.

Alveolar edema and congestion along with changes in the interlobular septa were the most severe of any group.

Group B--The cortex of the tracheal bronchial lymph node was well populated by moderate numbers of macrophages and a few neutrophils. The spleen was characterized by congested red pulp. The white pulp was well populated with periarteriolar lymphoid sheets of primary and secondary follicles with focal areas of acute splenic lymphoid necrosis and accumulations of neutrophils. The liver had mild infiltrates of neutrophils and lymphocytes around portal areas with some congestion present. The injection site in the cervical region

contained diffuse infiltrates of neutrophils and occasional hemorrhagic foci around small vessels. All meningeal vessels in the brain with focal areas of hemorrhage in the cerebral cortex were congested.

The lesions noted for group B were the least severe of any group for these organs (except brain) with no lesions noted in heart, kidney or adrenal glands.

In the lung there was moderate interlobular septal edema with mild infiltrates of neutrophils. Occasional submucosal lymphoid aggregates were noted in the bronchi. Bronchioles contained some erythrocytes, occasional neutrophils and moderate amounts of fibrin and edema. There was only slight neutrophilic exudate in alveoli with no fibrin present. Alveolar septa were thickened and mildly congested, but free of infiltrates. Mild to moderate intraalveolar edema was noted with moderate amounts of hemorrhage. No fibrinous thrombi were noted.

Group B contained the smallest lesions of any experimental group concerning interlobular septal infiltrates, the presence of alveolar fibrin, hemorrhage or fibrinous thrombi.

Group C--The cortex of the tracheal bronchial lymph node was well populated by lymphocytes. The medullary and cortical sinuses contained moderate numbers of macrophages and neutrophils. The red pulp of the spleen had widespread severe congestion with increased numbers of neutrophils present. The white pulp had marked lymphoid necrosis with neutrophil infiltrates and lymphoid depletion. The kidneys had mild perivascular infiltrates of lymphocytes with moderate widespread congestion of the corticomedullary interstitium. The heart

contained marked diffuse hemorrhage of the myocardium with separation of muscle fibers. These hemorrhagic areas were infiltrated by lymphocytes and neutrophils. The liver had mild infiltrates of lymphocytes and neutrophils in the portal area and in the parenchyma.

The injection site in the cervical region had extensive hemorrhage into the subcutaneous tissue extending into muscle fascia and muscle with infiltrates of neutrophils. The brain contained occasional small foci of hemorrhage in the cerebral white and grey parenchyma. The lesions of the injection site, spleen, and heart were equal to or the most severe of any experimental group.

In the lung interlobular septa were mildly edematous with moderate infiltrates of neutrophils. There were moderate amounts of submucosal lymphoid follicles associated with bronchi. The alveoli contained moderate amounts of cellular infiltrates composed of macrophages, neutrophils, erythrocytes and fibrin. Alveoli had moderate to marked edema, mild to marked congestion and marked hemorrhage. Some alveoli contained fibrinous thrombi.

The interlobular septal infiltrates and alveolar hemorrhage were equal to or the most severe lesions seen in the lungs of any experimental group.

Group D--The tracheal bronchial lymph node contained moderate infiltrates of neutrophils and macrophages into cortical sinuses. Medullary sinuses had similar infiltrates with occasional erythrocytes also present. No other lesions were noted in this group.

The group means of overall and respiratory lesion scores are shown

in Table 8.5. These were compared using two-way the Waller-Duncan analysis of variance. Groups with different superscript letters had significant differences. For overall and respiratory lesion scores groups A and C differed significantly from group D whereas group B differed but not at a significant level. For overall lesion scores this difference was significant ($p < 0.005$); for the respiratory lesion score ($p < 0.01$).

Discussion

Each group of calves exhibited clinical signs of biphasic fever, leukopenia, neutropenia, thrombocytopenia, as well as decreased fibrinogen, increased fibrin degradation products, prolonged one step prothrombin time (OSPT) and prolonged activated partial thromboplastin time (APTT). The changes in these parameters were consistent with endotoxemia. The gross post mortem lesions were also consistent with those expected of animals with disseminated intravascular coagulopathy (DIC). Numerous organs had ecchymotic or frank hemorrhages of the serosal and/or mucosal surfaces. The lungs were particularly affected. Since the lung has been shown to be the shock organ in the bovine,[10] this further supports the presence of endotoxic shock syndrome.

Each experimental group (A, B and C) contained gross pathologic lesions that were obviously different from control animals (group D). Groups A and C were significantly different from controls with group B not significantly different from any other group. These data indicate that calves that received a subcutaneous injection of leukotoxin and endotoxin had greater overall lesions and greater respiratory lesions

Table 8.5. Histopathological lesion scores in calves following parenteral exposure to P haemolytica leukotoxin and endotoxin.

	Group A*	Group B	Group C	Group D
Mean of other tissues lesion score	19.25±6.76†	13.00±6.75†,††	18.50±4.03†	0.50±0.87††
Mean of respiratory lesion score	11.00±3.50§	7.50±6.00§,§§	10.75±4.20§	0±0§§

* Group A = intraduodenal inoculation with leukotoxin and endotoxin (LE) followed 2 weeks later by a subcutaneous inoculation with LE; Group B = intraduodenal inoculation with LE followed 2 weeks later by a subcutaneous inoculation with LE heated to 56 C for 30 minutes; Group C = intraduodenal inoculation with RPMI-1640 culture medium followed 2 weeks later by a subcutaneous inoculation with LE; Group D = intraduodenal inoculation with RPMI-1640 followed in 2 weeks by a subcutaneous inoculation with RPMI-1640.

† Means with different number † are significantly different ($p<0.005$).

§ Means with different number § are significantly different ($p<0.01$).

than calves that received the heat treated toxin preparation subcutaneously. This suggests that the difference in pathology is due to the destruction of P haemolytica's leukotoxin. Other toxic properties have been attributed to these crude supernatants of P haemolytica including neuraminidase, protease, and alpha-hemolysin-like properties.[11] Although not identified in the preparations here, these enzymes would be expected to be susceptible to heat treatment also. Therefore, part of the reductions of lesions may be attributed to the loss of activity of neuraminidase or other toxic factors.

The gross respiratory post mortem lesions demonstrate that group A and C were significantly different from group D with group A significantly different from group B and C. Group B was not significantly different from group D due largely to the large standard deviation for this group of few animals. The wide variance between animals makes statistical evaluation difficult. Banks et al.[12] noted this in cattle. He further recognized that variation in results could also change day to day within the same animal. These data suggest that each experimental treatment had an effect on the pulmonary tract but was greatest in the group receiving the toxin preparation both ID and SC. The group that received a heat treated toxin preparation subcutaneously had the least lesions. This supports the overall trend seen in lesion scores, but also reflects the sensitivity of the respiratory tract to these toxins. The greater respiratory lesion score for group A could be attributed to the fact these calves received the greatest total dose of toxin when intraduodenal and subcutaneous

doses were combined. It may also reflect mucosal boostering of preexisting serum antibodies to endotoxin or somatic cell products resulting in increased serum opsonizing antibodies. These have been demonstrated in the past to result in increased pathology in P haemolytica pneumonia.[13,14]

Overall and respiratory histopathological lesion scores were similar to the pattern of significance established for overall gross lesion scores, i.e. group A and C were significantly different from group D with group B differing from neither A and C nor D. This trend supports the pattern seen in changes in coagulation parameters (Chapter 7). Group B animals were the least affected experimental group. This appears to be due to the decreased leukotoxin injected into these calves.

Previous studies have suggested that purified endotoxin administered directly to the lungs of cattle does not result in lesions typical of P haemolytica pneumonia[d] due to the inconsistent presence of vascular thrombosis which usually accompanies pasteurellosis. Although not present in every animal, animals in group A and B exhibited signs of vascular thrombosis. This is presumed to be due to the endotoxin since group B calves had received heat treated toxin preparation subcutaneously. This suggests that endotoxin may be the trigger for the onset of pneumonic lesions in pasteurellosis.

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[d]Slocombe RF, Derksen FJ, Robinson NE. Responses of calves to intrabroncheal challenge with P haemolytica and endotoxin (abst.) 20th Annual Meeting of the American College of Veterinary Pathologist, No. 36, 1985.

Previous studies investigating endotoxin effects on calves have used E coli purified endotoxin.[8] Confer et al.[9] has shown that P haemolytica endotoxin does not behave the same as E coli endotoxin in affecting leukocyte function. This suggests that P haemolytica's endotoxin may affect other tissues somewhat differently than other gram negative endotoxin preparations. Although coagulation parameters may be affected in a similar manner by endotoxins of different gram negative bacteria including P haemolytica, one cannot assume that all other lesions or malfunctions associated with E coli endotoxemia will necessarily be seen in pasteurellosis.

These data also suggest that parenterally administered leukotoxin has the capability of inducing pneumonic lesions with or without introduodenal priming by P haemolytica toxin preparation. This suggests P haemolytica leukotoxins may have a predilection for pulmonary tissue much like parenterally administered P multocida type D exotoxin has the ability to cause turbinate atrophy in swine.[e] Calves in group B had the least cellular infiltrates of interlobular septa, decreased fibrin, hemorrhage and fibrinous thrombi associated with alveoli. Although no calf in this study had the typical severe fibrinous pneumonia normally seen in P haemolytica pneumonia, the histopathological presence of fibrin and cellular exudates were similar to pasteurellosis. Therefore, the observation that group B had less

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[e]Williams PP, Hall MR, Rimber RB. Host response to Pasteurella multocida toxin in pseudorabies-infected swine. (abst.) 67th Annual Meeting Research Workers in Animal Disease, No. 70, 1986.

pulmonary changes suggests that the lack of leukotoxin inoculated subcutaneously resulted in this moderation of lesions.

Lesions seen in the lungs of these calves were similar to those induced by transtracheal inoculation of leukotoxin and endotoxin.[f] Therefore this experiment suggests that P haemolytica may adversely affect the lungs by both these virulence factors. Endotoxin may exert its effect in two ways both of which were seen in these calves. Initially, pulmonary edema is seen due to pulmonary hypertension. Later a second phase edema is seen that is related to vascular permeability which is associated with leukocyte invasion of the lung.[15]

The pathological changes caused by leukotoxin are presumably due to damage inflicted on neutrophils which release enzymes or oxygen radicals that in turn damage pulmonary endothelium and parenchyma. Breider et al. (unpublished, 1988) has shown that crude leukotoxin may directly damge endothelial cells so the leukotoxin may actually work in two ways to damage pulmonary tissue. The decreased lesions in group B in this experiment indicated that each of these factors may be occurring simultaneously in an additive manner since inactivation of leukotoxin resulted in less pulmonary lesions.

This experiment supports the hypothesis that the endotoxin of P haemolytica is an important virulence factor in pasteurellosis. It not

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[f]Slocombe RF, Derksen FJ, Babiuk L, Robinson NE. A comparison of structural and functional derangements of calf lungs exposed to Pasteurella haemolytica or P haemolytica-derived cytotoxin. (abst.) 14th World Congress on Disease of Cattle. World Association for Buitatrics, pp. 573-578, 1986.

only can induce pulmonary lesions but is capable of causing lesions associated with disseminated intravascular coagulopathy. The leukotoxin of P haemolytica has been suggested to be of primary importance in inducing pneumonia.[d,f,13,14] This experiment supports that proposition and suggests that the effects of leukotoxin are additive to those of endotoxin in inducing not only pulmonary lesions but lesions of DIC also. The observation that parenterally administered endotoxin and leukotoxin can exert these effects suggests that caution must be used when using these preparations in immunization products. The effects of endotoxin and leukotoxin may be reduced by removing endotoxin, or by altering the leukotoxin to eliminate its toxic effects without affecting its immunogenicity. Investigations performed in rabbits (Chapter 3) suggest that this can be done successfully.

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CHAPTER 9

SUMMARY

The leukotoxin of Pasteurella haemolytica is a potent virulence factor in the pathogenesis of this organism. Humoral antibodies capable of neutralizing its activity LNA have been associated with a decrease in pulmonary lesions. The best means of inducing these antibodies has been inoculations containing live organisms. This has implicit disadvantages in immunization protocols to control infection. The relative importance of pulmonary versus serum LNA in preventing pneumonia is unknown. Recent evidence that serum LNA may not always be present in protected animals further raises the question of the role of pulmonary mucosal immunity in reducing pneumonia.

An alternative method of inducing pulmonary mucosal immunity is by stimulation of the common mucosal associated lymphatic tissue (MALT) by the introduction of antigens to the gut-associated lymphatic tissue (GALT). Demonstration of the migration of specific antibody-producing lymphocytes from GALT to bronchial associated lymphatic tissue (BALT) would offer the possibility of an oral vaccine to prevent pneumonic pasteurellosis as well as to prevent other infectious diseases that involve mucosal surfaces.

Stimulation of GALT in calves with live P haemolytica through surgically implanted catheters in the duodenum did not stimulate a direct serum or pulmonary LNA response. When these calves were later inoculated with a subcutaneous injection of killed bacterin of P haemolytica, there was a marked immune response of serum and pulmonary

LNA. These results suggest that live P haemolytica primed memory lymphocytes to respond to common antigens, including leukotoxin, contained in the killed bacterin as well as in the live bacteria.

Killed bacterins have not been previously shown to induce serum LNA responses. Their usage has induced opsonizing antibodies to somatic antigens that actually increase the pathology of pneumonic pasteurellosis. In this study parenteral inoculations of killed bacterins did increase serum LNA, and were effective as a second signal to increase pulmonary and serum LNA following ID priming by live bacteria. This may be due to the preparation of the bacterin. The preparation used in this experiment consisted of unwashed log phase organisms treated with formalin. It is very likely that there was considerable leukotoxin in this preparation at the time of treatment with formalin. Killed bacterin was a much better second signal following ID stimulation by live bacteria than following ID stimulation by leukotoxin. This may reflect the different levels of leukotoxin in the priming antigen (live bacteria versus leukotoxin), or may reflect the presence of antibodies to other surface antigens (cell wall proteins, capsule, etc) that are present in the whole cell preparation in greater quantity than in the leukotoxin preparation.

Once it was demonstrated that live bacteria could be used to prime the mucosal immune response to leukotoxin, it was felt that the immunogenicity of crude leukotoxin should be explored by this route of administration. This was done for several reasons. First, any practical oral vaccine using a subunit vaccine would be more acceptable

if it did not require live organisms. Second, the chances of bypassing the rumen digestive processes would be better if an antigen were used that could be processed to bypass the rumen. This would favor subunit components over live organisms. Finally, one of the advantages of stimulating GALT is that a less pure antigen mixture is required. This would make vaccine production less expensive and more commercially appealing.

Crude leukotoxin administered ID stimulated a pulmonary leukotoxin neutralizing antibody response. A subsequent SC injection of killed bacterin did not produce an improved immune response. However, these results were misleading due to possible immune suppression by a BVD virus infection in these calves or due to the difference in antigen preparation. A SC injection of crude leukotoxin following an ID inoculation with crude leukotoxin further increased serum and pulmonary antibody responses in calves, but also resulted in severe physiological responses suggestive of endotoxic shock or hypersensitivity. Evaluation of pulmonary lavage fluids and serum indicated elevation of IgE antibodies in the serum and pulmonary lavage fluids of these calves. Post mortem examination indicated the presence of lesions consistent with disseminated intravascular coagulapathy (DIC) associated with endotoxemia.

Dosages of concentrated leukotoxin supernatants used to inoculate calves were based on inoculation studies in rabbits. Rabbits were used to determine the antigenicity of concentrated supernatant leukotoxin, reconstituted lyophilized leukotoxin, and lipopolysaccharide. Leukotoxin preparations treated by heating to 56 C for 30 minutes (to

inactivate the biological activity of the leukotoxin) or with buffered neutral formalin were injected into rabbits to determine the effect of these manipulations on the antigenicity of the toxin. The results indicated that rabbits responded well to heat treated, formalin treated and untreated lyophilized leukotoxin. The LNA response was slightly better in rabbits injected with the formalin or untreated leukotoxin preparations. These results suggest that manufacturing or packaging of leukotoxin that involves heat or chemical alteration would not adversely affect its antigenicity.

Although treatment of the leukotoxin had little adverse effect on its antigenicity, the biologic activity was reduced. There was very little reduction in the killing of BL-3 cells when they were exposed to preparations of reconstituted lyophilized leukotoxin altered by treatment with formalin or by dialysis. However, when the lyophilized leukotoxin was placed at 56 C for 30 minutes, most of the biologic activity was lost. These results varied from those obtained when crude supernatant leukotoxin preparations were treated with heat, formalin or dialysis and placed on BL-3 cells. Dialysis reduced biologic activity as much as formalin treatment. This suggests that considerable leukotoxic activity is lost by dialysis. Heat treatment of supernatant leukotoxin eliminated almost all leukotoxic activity. The greater reduction in the biologic activity by treatment of the supernatant leukotoxin compared to reconstituted lyophilized leukotoxin suggests that much of the reduction in activity may be attributed to the dialysis. The lyophilized leukotoxin was exhaustively dialyzed prior

to freezing. Methods to reduce the biologic activity of leukotoxin while retaining immunogenicity are very important considerations for vaccine production.

The use of antigens of P haemolytica administered ID to stimulate pulmonary antibodies suggests that ruminant as well as monogastric animal species can have mucosal immunity manipulated through stimulation of GALT. Challenge studies in cattle in these experiments suggest that stimulation of GALT by live bacteria may reduce pulmonary lesions. Calves inoculated ID with live P haemolytica had less gross and histopathological pulmonary lesions than calves inoculated ID with PBSS or SC with a killed bacterin of P haemolytica. The presence of serum LNA appeared to be most closely correlated with protection although there was evidence that protection may be associated with pulmonary LNA. Other factors, including antibodies against capsular or other cell wall antigens, and the immunomodulatory effect of cellular immunity may also contribute to the immune response to prevent pneumonic pasteurellosis.

The role of lipopolysaccharide (LPS) in the pathogenesis of pasteurellosis has not been well studied. These studies suggest that the severe pulmonary lesions as well as pathological changes in coagulation factors and leukocyte numbers can be attributed to LPS and leukotoxin acting together. Calves were inoculated with a preparation of leukotoxin and endotoxin intraduodenally and boostered two weeks later with either leukotoxin and endotoxin, or heat-treated leukotoxin and endotoxin. These calves were compared to a third group of calves

that received RPMI-1640 intraduodenally followed 2 weeks later by leukotoxin and endotoxin subcutaneously. The intraduodenal inoculation of leukotoxin and endotoxin did not appear to affect the responses of the calves as much as heat-treatment of the leukotoxin and endotoxin. Calves that received the heat-treated leukotoxin and endotoxin subcutaneously had less pulmonary pathological changes and less change in their coagulation factor profile than calves in the other 2 groups.

Stimulation of GALT-induced antibodies appears to be one way to avoid the potent physiological effects of these toxins while inducing LNA and reducing pulmonary pathology. Results reported here suggest that development of oral vaccines for the prevention of P haemolytica infection, and possibly other pathogenic organisms in ruminants, is feasible and worthy of serious consideration.

VITA

Terry Lynn Bowersock was born in Lima, Ohio December 1, 1953. He attended public schools in Wapakoneta, Ohio and graduated from Wapakoneta Senior High School in 1972. He received his B.A. in Biology-Psychology from Lawrence University in Appleton, Wisconsin in 1976. A Doctor of Veterinary Medicine degree was achieved at The Ohio State University in 1981. Terry entered private veterinary practice in Wisconsin where he worked for three years specializing in dairy preventative medicine. He entered the University of Tennessee in the fall of 1984 and received his Doctor of Philosophy degree in Comparative and Experimental Medicine in 1988. Terry is a member of Phi Chapter of Phi Zeta, Phi Kappa Phi, and an associate member of Sigma Xi. He is a member of The American Veterinary Medical Association, American Association of Bovine Practitioners, American Society for Microbiologists, American Society for Veterinary Microbiologists, and The Comparative Respiratory Society.