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PASTEURELLA HAEMOLYTICA CYTOTOXIN:

PURIFICATION, CHARACTERIZATION,

AND AN EVALUATION OF ITS

IMMUNOGENICITY

By

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CHAPTER I

INTRODUCTION AND REVIEW OF THE LITERATURE

Of all diseases in cattle, 40% to 80% have been reported to involve the respiratory system (Lillie, 1974). The economic importance of respiratory diseases to the beef cattle industry, particularly feedlot operations is extremely significant. Losses due to fatalities, loss of conditioning, inefficient feed conversion, and expensive treatments run into the millions of dollars each year (Rehmtulla and Thomson, 1981). The major cause of these losses in feedlot cattle is the shipping fever complex. In a feedlot survey, 75% of the clinical diagnoses and 64% of the necropsy diagnoses were respiratory tract diseases. Of the fatalities from respiratory disease, 75% were due to shipping fever pneumonia (Jensen et al., 1976). The exact nature and etiology of shipping fever in cattle have long been sources of debate and confusion (Lillie, 1974; Rehmtulla and Thomson, 1981; Thomson, 1980b). Though the term shipping fever encompasses numerous etiological factors, such as stress and viral infections, it has also come to be used to refer specifically to the disease pneumonic pasteurellosis.

Pathogenesis

The pathogenesis of pneumonic pasteurellosis is a complex, multifactorial process. Management practices, stress, multiple

infectious agents, local pulmonary defense mechanisms, and immunological responses combine to produce the disease syndrome. The precise role and significance of each of these factors remain poorly understood. The one common feature shared by most cases of shipping fever is the involvement of Pasteurella haemolytica. The importance of this organism in the disease has become well established (Carter, 1964; Hamdy and Trapp, 1967; Thomson et al., 1969), and it is generally regarded as the ultimate cause of the clinical and pathological characteristics commonly associated with shipping fever.

Pasteurella haemolytica is part of the normal, basal microflora present in the bovine nasal cavity (Magwood et al., 1969). In normal cattle, cultures of nasal swabs are frequently negative for the organism (Hoerlein et al., 1961). However, if several areas of the nasal mucosa are cultured, P. haemolytica can often be isolated from animals which had been negative on single nasal swabs (Pass et al., 1971). Normally, the organisms are present in small numbers and localized to the upper respiratory tract, especially the nasopharyngeal region and the tonsillar crypts. They can remain there for long periods of time without producing any signs of clinical illness (Babiuk and Acres, 1984). Transient flucuations in both the type and quantity of nasal bacterial flora occur. P. haemolytica has been found to be capable of dominating the nasal flora for periods of several days, at which time colonization and active shedding of the organism are possible (Magwood et al., 1969). Rapid proliferation of P. haemolytica in the nasal cavity also occurs in cattle that have been recently congregated and shipped (Thomson, 1980a,b; Thomson et al., 1969; Frank and Smith, 1983). The presence of greater numbers of organisms in the

nasal cavity increase the chance of aerosolization and deposition of organisms into deeper portions of the airways or into the lungs themselves (Thomson et al., 1969; Grey and Thomson, 1971). In some studies, the frequency of isolation of P. haemolytica from the nasal cavity was much greater in sick cattle than in normal cattle (Thomson et al., 1975; Hoerlein et al., 1961). In other instances, however, no significant difference in isolation frequency could be detected between healthy and diseased calves (Frank and Smith, 1983).

A potentially important factor that determines the incidence of disease in calves with increased numbers of intranasal P. haemolytica is the ability of the organism to adhere and to colonize on the respiratory mucosa. Bacterial adherence to host tissues appears to be a primary step in the colonization and subsequent infection of mucosal surfaces of the gastrointestinal, genitourinary, and respiratory tracts (Beachey, 1981; Ramphal et al., 1980). Bacterial adherence depends upon the presence of both specific receptors on mucosal epithelium as well as surface structures on bacteria which have adhesive characteristics (adhesins) (Beachey, 1981). Certain strains of P. multocida Type A will adhere to rabbit pharyngeal mucosa while other types will not. Adherent strains, but not non-adherent types, possess fimbriae which acted as adhesins. Fimbriae were able to attach to N-acetyl glucosamine-containing receptors on the epithelium and promote colonization both in vivo and in vitro (Glorioso et al., 1982). In humans, a high degree of specificity is involved in the attachment of certain types of bacteria to nasal mucosa. The adherence of Staphylococcus aureus was found to be significantly higher in people who were carriers of the organism as normal flora compared to people

who were non-carriers. The enhanced staphylococcal adherence of carriers may be due to a difference in either the surface characteristics of the mucosal epithelium or an altered local mucosal environment (Aly et al., 1977). Bacterial factors do not appear to effect staphylococcal adherence. Adherence and colonization of Gram negative bacteria on respiratory mucosa is enhanced in humans who are ill (Johanson et al., 1980). Viral infection both in vivo and in vitro and mechanical mucosal damage also result in increased bacterial adherence when compared to non-infected or undamaged mucosa (Sanford et al., 1978; Ramphal et al., 1980). The mechanism of this enhanced adherence may be related to an alteration of the mucosal surfaces which promotes adherence or a reduction in fibronectin levels resulting from destruction of fibronectin-producing mucosa (Aly et al., 1977; Babiuk and Acres, 1984). Enhanced adherence and subsequent replication can result in microcolonies which increase the potential of exposure of the lower respiratory tract to bacteria. Adhesion has not been described for P. haemolytica.

Under normal conditions, bacteria gaining access to the respiratory tract are quickly cleared. In normal calves, 75% of inhaled P. haemolytica were cleared by 2 hours, with 92% clearance being accomplished by 8 hours (Lillie and Thomson, 1972). Mechanisms responsible for clearance include the cough reflex, physical filtration of the bacteria in the nasopharyngeal region, the upper respiratory mucociliary apparatus, the alveolar macrophage system, and cellular and humoral defense mechanisms (Thomson, 1980b). Aerosols containing large numbers of P. haemolytica have occasionally been successful in causing clinical and pathologic evidence of disease (Duncan and

Thomson, 1970; Thomson, 1980a). In most cases, however, attempts to cause clinical infection with P. haemolytica alone have been largely unsuccessful and resulted in no change or only mild transient fever and illness (Thomson, 1980b; Baldwin et al., 1967; Jericho and Langford, 1978). In most situations, additional factors which either inhibit some segment of the pulmonary defense system, or otherwise alter the host response to the bacteria are necessary for significant disease to occur (Thomson, 1980a). Many of these predisposing factors are related to management or physical conditions and are best categorized as stress. Other factors include either the direct or indirect results of concurrent infection with another organism, most often a virus. Stress factors can encompass a wide variety of conditions. These include transport, overcrowding, food and water deprivation, wet or cold weather, and overzealous processing of cattle at the feedlot. Decreased pulmonary clearance of bacteria resulted from exposure of mice to cold, wet conditions (Green and Kass, 1965), or physical stress in rabbits (Lockard et al., 1973). Administration of hydrocortisone to calves caused reduced pulmonary clearance. This effect, however, was suspected to be mediated by an induced pulmonary edema rather than any immunosuppressive effects (Gilka et al., 1974). Cold stress, as well as transportation and handling have been found to increase serum cortisol levels (Filion et al., 1984; Kelley, 1984). High cortisol levels can possibly act to increase susceptibility of calves to pneumonic pasteurellosis by reducing immune responsiveness and leukocyte function, as well as by enhancing replication of any predisposing virus which may be present in the upper respiratory tract (Filion et al., 1984). Cold stress caused an initial decrease in serum IgM and enhanced

cell-mediated immune functions (Kelley, 1984). In contrast, transportation and handling caused decreased lymphocyte blastogenic response to phytohemagglutinin (Filion et al., 1984). These results indicate the multiple and complex factors involved in the response of calves to various types of stress.

A large percentage of all bacterial pneumonias in the bovine are estimated to be secondary to a viral infection or some other form of debility (Lillie, 1974). Viral predisposition of the lung to bacterial infection could be caused by either direct tissue damage or by inhibition of pulmonary defense mechanisms. Many viruses replicate in the ciliated and nonciliated epithelium of the upper respiratory tract. Degeneration and desquamation of these cells creates regions with enhanced susceptibility to bacterial adherence and colonization. In addition, normal functioning of the mucociliary apparatus is impaired (Lopez et al., 1976). Certain viruses can also destroy type II pneumocytes with a resultant reduction in intraalveolar surfactant production (Jakab, 1984). Surfactant has been shown to enhance phagocytosis of certain bacteria by alveolar macrophages (LaForce, 1976; Coonrod and Yoneda, 1983). In addition, it contains substances, probably lysophospholipids, which have direct lytic effects on certain bacteria (Coonrod and Yoneda, 1983). Pulmonary edema, commonly produced by viral respiratory infections involving lung parenchyma, consistently results in prolonged pulmonary clearance times of bacteria (Gilka et al., 1974; Lopez et al., 1976).

Viral infections may play a vital role in the pathogenesis of shipping fever. Exposure to bovine herpesvirus-1 or parainfluenza virus-3 followed by aerosol exposure to P. haemolytica several days

later will consistently produce lesions resembling shipping fever (Jericho and Langford, 1978; Stockdale et al., 1979a,b; Thomson, 1980a; Baldwin et al., 1967; Jakab, 1974). This synergism appears to be due to the ability of viruses to inhibit the clearance of Pasteurella sp. from the lung (Jakab, 1974; Lopez et al., 1976; Yates, 1982). Increased clearance time does not seem to be associated with decreased mucociliary apparatus function, but rather is caused by a transient suppression of pulmonary bactericidal activity caused by dysfunction of the alveolar macrophage system (Jakab, 1974; Couch, 1981).

Many viral-induced defects in macrophage function have been described. Parainfluenza virus inhibited both non-specific and Fc receptor-mediated phagocytosis by mouse macrophages (Jakab and Warr, 1981; Warr and Jakab, 1979). Ingestion and intracellular killing of Staphylococci or Candida sp. by influenza virus infected macrophages were also suppressed (Warr and Jakab, 1979; Warshauer et al., 1977; Jakab and Green, 1976). The defect in intracellular killing appears to be partially due to inhibition of phagosome-lysosome fusion (Jakab et al., 1980; Hesse and Toth, 1983). In addition, viral infection inhibits chemotaxis and aggregation of macrophages and monocytes (Kleinerman et al., 1974, 1975, 1976). Neutrophils also have defects in chemotaxis, phagocytosis, chemiluminescence, and bactericidal activities when exposed to influenza virus (Abramson et al., 1981; Larson and Blades, 1976; Sawyer, 1969). Maximum suppression of the alveolar macrophage system appears to occur 1 week following the initial viral infection (Abramson et al., 1981; Jakab, 1974; Jakab et al., 1980; Lopez et al., 1976). This period is associated with rapidly declining viral titers, an increased expression of local antiviral

immune responses, and increased susceptibility of the lungs to a secondary bacterial infection (Lopez et al., 1976; Wells et al., 1981; Jakab, 1982). The time frame for maximum suppression suggests that alveolar macrophage dysfunction may be secondary to the humoral and cell-mediated antiviral immune responses of the host rather than due to a direct effect of the virus (Jakab, 1982). This suggestion is supported by studies which showed that viral infected immunodeficient and immunosuppressed mice developed less cellular infiltration and milder histopathological lung lesions than normal, immunocompetent mice (Wells et al., 1981; Hurd and Heath, 1975). In addition, fatalities in immunosuppressed mice occurred later than in normal mice and were due to extrapulmonary dissemination of the virus rather than pulmonary infections as were seen in the normal mice (Hurd and Heath, 1975). Possible explanations for this secondary viral inhibition of macrophages include the generation of cytotoxic immune responses against viral-infected macrophages and a decrease in Fc receptor-mediated phagocytosis due to increased levels of immune complexes (Jakab, 1982).

Once colonies of P. haemolytica have become established in the pulmonary parenchyma, many characteristics of the bacteria itself become prominent factors in pathogenesis. Early logarithmic-phase growths of P. haemolytica are encapsulated (Corstvet et al., 1982). The presence of capsules has been shown to enhance the pathogenicity of a variety of bacterial diseases (Smith, 1977; Densen and Mandell, 1980). Encapsulation can function in several ways to make the bacteria more resistant to host defense mechanisms. Certain components of the capsule may be able to enhance bacterial adherence to respiratory mucosa (Smith, 1977; Glorioso et al., 1982). An important function for

bacterial capsules is to prevent phagocytosis. The mechanism for this antiphagocytic effect is unclear, but may be related to alterations in cell membrane-bacterial interactions or to the inhibition of binding of opsonins to the surface of the encapsulated bacteria (Densen and Mandell, 1980). The presence of a capsule may be the reason for the low rate of phagocytosis of P. haemolytica by bovine alveolar macrophages in vitro in the absence of opsonins (Maheswaran et al., 1980). Immunization with extracts of capular material from P. haemolytica and P. multocida have produced variable degrees of protection in mice, sheep, and cattle (Mukkur, 1977; Tadayon and Lauerman, 1981; Gilmour et al., 1979; Nagy and Penn, 1976; Yates et al., 1983). These studies suggest a possible role for capsular material in the pathogenesis of shipping fever. Encapsulation of P. haemolytica does appear to enhance resistance against the bactericidal activity of bovine serum (Gentry, 1984). Antiphagocytic effects or other depression of pulmonary defense mechanisms by P. haemolytica capsular material, however, have not been firmly established.

Endotoxins are part of the outer membrane of Gram negative bacteria and can play important roles in many bacterial infections (Cluff, 1971). These are complex lipopolysaccharides released from dying bacteria. Lipopolysaccharides consist of a core polysaccharide, polysaccharide side chains, and a toxic lipid A portion. Endotoxins cause a transient decrease in resistance to bacterial infections due to a wide variety of mechanisms. Included among these are consumption of complement components, transient depression of reticuloendothelial function, decreased leukocyte chemotaxis, shifts in distribution of granulocytes, variation of blood coagulability, alterations of cellular

metabolism, fever, and vasoactive effects. Well defined changes seem to occur in the lungs due to endotoxin exposure (Snell, 1966). An aerosol of Eschericia coli endotoxin was rapidly absorbed causing an immediate transient leukopenia followed in 2 hours by leukocytosis and fever. By 24 hours, focal capillary hemorrhage, alveolar edema, and alveolar infiltrates of macrophages, neutrophils, and eosinophils were present. Proliferation of alveolar macrophages associated with a reduction in edema occurred by 48 hours. Aerosols of P. multocida have been found to produce similar pulmonary edema and capillary hemorrhage (Rhoades et al., 1967). The endotoxin of P. haemolytica demonstrates typical properties of endotoxins and exerts its major physiologic effect in the bovine by increasing pulmonary vascular resistance (Keiss et al., 1964). It has been hypothesized that many of the clinical and pathological alterations associated with pneumonic pasteurellosis may be attributed to endotoxin (Lillie, 1974; Keiss et al., 1964; Jensen et al., 1976).

An exotoxin is produced by logarithmic-phase P. haemolytica (Benson et al., 1978). This toxin (cytotoxin), can interfere with the function of bovine leukocytes (Benson et al., 1978; Markham and Wilkie, 1980; Berggren et al., 1981). Inhibition of phagocytosis when low numbers of organisms are exposed to bovine alveolar macrophages or neutrophils has been attributed to the toxin (Benson et al., 1978; Markham and Wilkie, 1980; Walker et al., 1984; Maheswaran et al., 1980). At higher bacterial concentrations or with bacteria-free supernatants, cytotoxin is toxic to bovine alveloar macrophages, neutrophils, lymphocytes, and monocytes (Benson et al., 1978; Markham and Wilkie, 1980; Shewen and Wilkie, 1982; Berggren et al., 1981;

Kaehler et al., 1981). Cytotoxicity occurs rapidly *in vitro* and is highly specific for bovine or ovine leukocytes (Shewen and Wilkie, 1982). The exotoxin has been partially purified and characterized, but the range of its biological activity, biochemical parameters, and mechanism of action are still unclear (Baluyut et al., 1981; Himmel et al., 1982).

Studies on the cytotoxin neutralizing capabilities of bovine sera have demonstrated that cattle dying of fibrinous pneumonia have lower anticytotoxin activity than non-pneumonic cattle (Shewen and Wilkie, 1983; Cho et al., 1984; Gentry et al., 1985). Anticytotoxin activity has been demonstrated to be antibody in nature and is present in nasal secretions and lung washings as well as sera (Cho et al., 1984). These findings implicate cytotoxin as a possible factor involved in the pathogenesis of pneumonic pasteurellosis.

Several serotypes of *P. haemolytica* possess neuraminidase activity (Frank and Tabatabai, 1981; Otulakowski et al., 1983). Neuraminidase production by several species of bacteria has been associated with virulence (Milligan et al., 1978; Ray, 1977; Smith, 1977). In one study, *P. haemolytica* serotype 1 had higher neuraminidase activity than *P. haemolytica* serotype 2. This suggested a possible relationship between the higher neuraminidase levels and the greater incidence of disease due to serotype 1 (Frank and Tabatabai, 1981). It has been suggested that neuraminidase may play a role in disease by altering leukocyte membranes with a resultant unmasking of receptor sites for cytotoxin (Otulakowski et al., 1983). Overall, however, neuraminidase probably plays a minor role, if any, in pneumonic pasteurellosis.

Protease activity has also been detected in supernatants from several P. haemolytica serotypes, including serotype 1. The enzyme was characterized as a probable metal-dependent neutral protease with specificity for sialoglycoproteins. Possible virulence enhancing mechanisms proposed for this protease include membrane damage with resultant enhanced cytotoxin susceptibility, unmasking of receptor sites for cytotoxin in a similar manner to that proposed for neuraminidase, or cleavage of cytotoxin into active components if it is secreted as a prototoxin (Otulakowski et al., 1983).

Besides the possible direct effects of P. haemolytica or its products on producing the lesions of pneumonic pasteurellosis, the host immune response to these bacterial antigens may also provide a significant contribution to the pathogenesis of disease. Numerous components of the inflammatory response are capable of initiating pulmonary damage. Alveolar macrophages can play an active role in many forms of pulmonary injury by both their phagocytic and secretory functions (Nathan et al., 1980). Activated and stimulated macrophages can secrete hydrolytic enzymes and proteases in response to lymphokines, immune complexes, foreign material such as bacteria, endotoxin, complement, and numerous others (Slauson, 1982). The major leukocytic enzymes involved in pulmonary damage are the neutral proteases collagenase, elastase, and cathepsin G, as well as the acid protease cathepsin D. These enzymes produce damage by degrading connective tissue substrates in vessel walls, perivascular tissues, and interalveolar septa (Davies and Bonney, 1980). Phagocytic and secretory functions of macrophages are enhanced as the degree of their activation increases and has been demonstrated by a positive correlation between

macrophage activation and the extent of histological lesions in hypersensitivity pneumonitis (Stankus et al., 1978). Macrophages can affect other aspects of inflammation which have potential to cause pulmonary injury by their secretion of complement components, endogenous pyrogens, reactive oxygen metabolites, bioactive lipids (prostaglandins and leukotrienes), and regulatory monokines (Nathan et al., 1980; Slauson, 1982). This ability to secrete damaging enzymes and to interact with other components of the inflammatory response give the alveolar macrophage the ability to not only defend the lung but also to damage it.

Neutrophils can also cause significant pulmonary damage by their response to infection. During influx to the lungs, neutrophils aggregate in pulmonary capillaries and subsequently migrate into the interalveolar septa. Neutrophil sequestration in vessels has been shown to cause endothelial damage both *in vivo* and *in vitro* (Hammerschmidt, 1983). Like macrophages, neutrophils have a potent array of lysosomal enzymes which can be released extracellularly to cause tissue damage (Baggiolini, 1980). Enzyme release can occur either by death and lysis of the leukocyte, leakage of enzymes due to premature phagosome-lysosome fusion, or release of enzymes against a non-phagocytosible object (Slauson, 1982). In addition, toxic oxygen free radicals produced by neutrophils, and to a lesser extent by macrophages during phagocytosis, can diffuse extracellularly and damage pulmonary tissues as well as the leukocytes themselves (Fridovich, 1978). Neutrophil enzyme products can also modify other aspects of inflammation by liberation of chemotactic factors, kinin generating activity, stimulation of B- and T-lymphocytes, complement cleavage,

and interaction with the fibrinolytic system (Slauson, 1982).

The lung contains many vasoactive substances including histamine, serotonin, kinins, and prostaglandins. All of these are liberated during an inflammatory response and can increase vascular permeability (Slauson, 1982). Permeability changes can cause leakage of edema fluid, fibrinogen, and other plasma proteins from the vessels into alveolar spaces. Leakage of these substances, particularly fibrinogen, appears to be an important mechanism in the development of pneumonic pasteurellosis. Fibrinogen can polymerize to form fibrin which fills alveoli, plugs airways, and provides a medium which enhances bacterial growth and inhibits clearance. Fibrinogen can also inactivate surfactant causing an increase in surface tension and greater tendency for alveolar collapse. Bactericidal activities of surfactant are also reduced by the presence of fibrinogen (Slauson, 1982).

All of the proteins of the complement system are present in the lung and can be activated by a wide variety of immunologic and non-immunologic stimuli. Consequences of complement activation include generation of anaphylatoxins and chemotaxins, lytic reactions, and opsonization of antigens. Complement has been shown to cause sequestration of neutrophils in terminal pulmonary vasculature (Henson et al., 1979), and produces variable degrees of pulmonary inflammation following instillation of C3 or C5 fragments into the lung (Larsen et al., 1980; Henson et al., 1979). These reactions and the association of complement with leukocytes and bacteria during inflammation can result in substantial pulmonary injury.

The effect of the humoral immune response to P. haemolytica on the pathogenesis of pneumonia is variable. As previously mentioned,

cytotoxin neutralizing antibody titers appear to correlate positively with resistance to disease (Shewen and Wilkie, 1983; Cho et al., 1983; Gentry et al., 1985), while anticapsular antibody effects are variable (Tadayon and Lauerman, 1981; Yates et al., 1983; Matsumoto et al., 1984). In some studies, a strong antibody response against somatic antigens seems to enhance the disease (Bennett, 1982; Friend et al., 1977; Wilkie et al., 1980). This adverse effect is incompletely understood, but may be related to increased opsonization and phagocytosis of bacteria with a resultant magnification of cytotoxic effects on alveolar macrophages (Wilkie et al., 1980).

A well characterized cell-mediated immune response occurs against some of the viruses which commonly predispose to pneumonic pasteurellosis (Campos et al., 1982; Davies and Carmichael, 1973; Gerber et al., 1978). The cell-mediated response to P. haemolytica, however, has not been adequately evaluated. The possible significance of cell-mediated immunity in pneumonic pasteurellosis has been occasionally mentioned (Markham and Wilkie, 1980; Yates et al., 1983; Confer et al., 1984), but association of this immunity with resistance to the disease has not been shown.

Gross and Microscopic Lesions

Failure to develop adequate resistance to the various factors involved in the pathogenesis of shipping fever results in an acute fibrinous pleuropneumonia. Clinically, there is fever, anorexia, depression, nasal discharge, and bronchial and pleuritic sounds in the anteroventral portion of the thorax (Jensen et al., 1976). These signs can be present over a period of several days and generally terminate

fatally if left untreated. In cases caused by P. haemolytica, there are bilaterally extensive sheets and strands of fibrin present on the visceral and parietal pleura (Schiefer et al., 1978). Fibrinous pericarditis and variable amounts of yellow, fibrinous thoracic fluid are sometimes present. The anteroventral two-thirds of the lung are usually pneumonic and appear enlarged, firm, and heavy. The cardiac lobe is usually the most severely affected and can be 10 times its normal weight due to filling with fluid and exudate. Lobules vary in color from red to gray to red-black and are separated by widened interlobular septa which contain edema, fibrin, and air bubbles. Interlobular lymphatics are sometimes dilated and may contain fibrin clots. On cross section, lobules are firm and airless. Occasionally, regions of infarction and coagulative necrosis characterized by pale brown to black tissue surrounded by a gray zone are present. Thrombi can sometimes be detected in associated vasculature. Bronchioles and affected bronchi have reddened mucosa and are partly filled with blood, fibrin, mucus, and pus (Jensen et al., 1976; Friend et al., 1977; Rehmtulla and Thomson, 1981; Scheifer et al., 1978).

Histologically, subpleural connective tissue and interlobular septa are widened and often contain dilated, thrombosed lymphatics. Approximately 98% of the thickened septa contain fibrin, 83% contain edema fluid, and 56% are emphysematous (Graham, 1953). Bronchi and bronchioles frequently contain variable amounts of fibrin, mucus, neutrophils, cellular debris, erythrocytes, and macrophages. Mucosal epithelium is sometimes desquamated and contains inflammatory cells, but in most cases the mucosa is intact and there is little evidence of bronchitis or bronchiolitis (Scheifer et al., 1978). In recently

infected lobules, alveoli are filled with fibrin. In the well-developed lesion, alveoli contain fibrin, neutrophils, alveolar macrophages, and scattered colonies of bacteria. Macrophages are sometimes large, round, and contain pale eosinophilic cytoplasm, but in many cases they are dark and fusiform. In some areas, these fusiform macrophages dominate the lesion and fill many alveoli. Variable amounts of hemorrhage are present in alveoli. Pulmonary veins and capillaries are frequently thrombosed or contain immature clots. Thrombosed veins are often surrounded by fusiform macrophages and sometimes are associated with regions of coagulative necrosis. Necrosis is seen occasionally in association with thrombosed lymphatic vessels as well. Necrotic areas frequently extend across interlobular septa and are surrounded by a variable sized zone containing numerous neutrophils, fusiform macrophages, and bacteria (Jensen et al., 1976; Scheifer et al., 1978).

In experimentally induced disease, lesions at 18 hours consist primarily of variable degrees of atelectasis with alveoli containing fibrin, neutrophils, and macrophages. By 3 days, the lesion is fully developed and compatible with the changes previously described. In animals surviving the acute phase of the disease, organization of fibrin begins by 7 days. The lesion at this point is characterized by early obliterative bronchiolitis, lymphoid hyperplasia around airways, granulation of necrotic tissue, and occasionally abscess formation (Friend et al., 1977).

In contrast to the fibrinous pleuropneumonia commonly associated with P. haemolytica, P. multocida often causes a bronchopneumonia (Scheifer et al., 1978). The lesion is characterized by airways which are filled with purulent material, fairly uniform dark red lobules,

and a peribronchiolar distribution of alveoli containing neutrophils and macrophages. Fibrin is not an important component of the lesion. Interlobular septal lymphatic thrombosis and necrosis are not commonly present (Scheifer et al., 1978).

Based upon the nature of the pulmonary lesion caused by P. haemolytica, endotoxin has been considered to be a major pathogenetic mechanism of tissue damage once colonization by bacteria has occurred (Jensen et al., 1976). Endotoxin has been blamed for the clotting and thrombosis of lymphatics, veins, and capillaries and the resulting hemorrhagic infarction and necrosis. Endotoxin can cause increased vascular permeability with large amounts of exuded fibrin which are characteristic of the disease. The role of cytotoxin, capsular antigens, viral infections, the host immune response, or other factors are difficult to determine on the basis of morphological evaluation of the in vivo lesion. Fatalities are generally attributed to hypoxia, endotoxemia, pulmonary necrosis, shock, and heart failure (Jensen et al., 1976).

Vaccination

Because of numerous factors involved in the etiology and pathogenesis of pneumonic pasteurellosis, prevention of the disease has proven difficult. Factors such as stress and exposure to various infectious agents are difficult to control. The major emphasis in management of the disease has been to devise methods to enhance the hosts° immunological resistance to commonly incriminated infectious agents. Several experimental approaches to this problem have been taken. These are to vaccinate cattle with P. haemolytica, P.

multocida, or components of these bacteria, to vaccinate against the viruses which commonly predispose to pneumonic pasteurellosis, or to use the bacterial and viral vaccines in combination (Collier, 1968; Anonymous, 1968; Engelbrecht, 1968).

Pasteurellae are frequently considered to be the sole cause of the advanced clinical signs and lesions of shipping fever, while other factors act only to predispose the calf to this bacterial infection (Collier, 1968; Lillie, 1974). Due to the apparent central role of Pasteurellae in pneumonic pasteurellosis, much effort has been devoted to examination of various P. haemolytica and P. multocida bacterins. The use of bacterins to prevent the disease, however, has proven largely ineffective (Engelbrecht, 1968; Hoerlein, 1957). The questionable efficacy of bacterins may be due to failure to incorporate the correct antigenic serotypes into the vaccines, too few numbers of bacteria, inadequate adjuvants, or the use of the wrong growth phase of the organisms (Collier, 1968; Anonymous, 1968). Early reports on the use of bacterins were mainly from uncontrolled field trials and the results were uninterpretable. A bivalent P. haemolytica/P. multocida bacterin was used with apparent success (Carter, 1957). In later studies, the use of hemorrhagic septicemia bacterins (P. multocida) and antisera as preventatives seemed to result in some reduction in the incidence of shipping fever, but management practices seemed to have a greater influence than either of these bacterial products (Schipper et al., 1962). Palotay et al. (1963) evaluated several bacterins in cattle. Agents used for vaccination included commercially available hemorrhagic septicemia bacterin, a formalinized vaccine produced by injection of P. haemolytica into the yolk sac

of chick embryos, and a bivalent P. haemolytica/P. multocida bacterin. Vaccination with any of these agents prior to shipment was reported to result in a significant reduction in the incidence of respiratory disease when compared to unvaccinated controls. It was noted, however, that there was still a fairly high incidence of respiratory disease even in vaccinated animals, indicating that factors other than the bacteria may be involved in shipping fever. Contrasting results have been obtained in more recent studies. Use of a bivalent P. haemolytica/P. multocida bacterin was found to have no effect on reduction of clinical and pathological signs compared to controls (Amstutz et al., 1981). In a later study, use of a similar bacterin resulted in a significant reduction in the incidence of bovine respiratory disease and improved response to treatment. The difference between these two trials was suggested to be due to extraneous factors such as prior exposure, stress during shipment, or bacterial virulence (Morter et al., 1982). In many field studies, the effect of bacterins was either minimally positive or had no effect (Martin, 1983). In contrast, several reports have indicated that bacterins may cause adverse effects and enhance the disease. Friend et al. (1977), vaccinated calves with P. haemolytica bacterin both subcutaneously and by aerosolization, then exposed the calves intratracheally with live P. haemolytica. The degree of pneumonia correlated positively with antibody reponse to P. haemolytica as measured by either the whole cell agglutination or indirect bacterial agglutination tests. This adverse response was suggested to be due to an immune-mediated hypersensitivity pneumonitis. Using the same bacterin and challenge method, Wilkie et al. (1980) obtained similar results. They found that

subcutaneous vaccination resulted in adverse clinical and pathological effects, whereas intrabronchial vaccination may have induced minor protection. The reason for the adverse effect of systemic vaccination may have been due to the stimulation of IgG opsonizing antibody which enhanced phagocytosis and cytotoxin-mediated destruction of leukocytes. These results demonstrated the questionable value of bacterins, and indicated that other factors must be considered to obtain consistent protection against the disease.

The association of parainfluenza-3 (PI-3) virus with Pasteurellae in many cases of shipping fever prompted the inclusion of PI-3 vaccines with bacterins. In early studies, inactivated PI-3 was used either alone, or in combination with P. haemolytica and P. multocida. Hemagglutination inhibition titers to PI-3 generally increased in vaccinated animals, but no obvious reductions in the incidence of clinical signs or respiratory disease were detected between vaccinated and non-vaccinated groups following shipment (King et al., 1963; Gale et al., 1963). Calves given an inactivated PI-3, P. haemolytica, and P. multocida vaccine followed by an experimental challenge consisting of stress and an aerosol of PI-3, P. haemolytica, and P. multocida were found to have an increased resistance to disease (Matsuoka et al., 1966; Hamdy and Trapp, 1964). These results suggested that this vaccine combination stimulated a good immune response against experimental challenge. Vaccination with various combinations of formalinized PI-3, P. haemolytica, and P. multocida, and modified live infectious bovine rhinotracheitis (IBR) virus produced substantial protection against experimental challenge exposure by either PI-3, P. haemolytica, and P. multocida, PI-3 and IBR, or IBR alone (Hamdy

and Trapp, 1964; Matsuoka et al., 1972).. In studies using the same combinations under field conditions, however, the vaccines had no appreciable effects in protecting against clinically evident shipping fever (Hamdy et al., 1965). In another experiment, inactivated PI-3, P. haemolytica, and P. multocida vaccines caused an increase in the number of cases of shipping fever following weaning. PI-3 titers were higher in vaccinates than in controls, but this was not associated with any difference in the incidence of disease (Woods et al., 1974). Under feedlot conditions, one group of calves was given an IBR/PI-3 vaccine, whereas another received IBR/PI-3 plus a P. haemolytica bacterin. Respiratory disease morbidity and mortality rates were consistently higher in the group receiving the bacterin than in the group receiving the viral vaccine alone (Bennett, 1982). Results of these last two studies suggest that the occasional adverse effects observed with the use of bacterins may also occur in vaccines containing viral- bacterial combinations.

Viral vaccines have been used alone in attempts to reduce the incidence of pneumonic pasteurellosis. The rationale for this approach is that by preventing the predisposing viral infection, subsequent secondary Pasteurella sp. infections will not occur. Vaccination with PI-3 caused significant rises in hemagglutination-inhibition titers, but had only minimal effects on reducing the incidence of respiratory disease. It was found that the time of administration relative to weaning and the number of vaccinations given were important factors in determining the potential success of the vaccine (Woods et al., 1962). In several experiments, IBR-vaccinated or IBR/PI-3 vaccinated calves had higher survival rates and less severe lesions

than unvaccinated controls following experimental challenge by either an aerosol of IBR, a combination of aerosol with IBR followed by P. haemolytica, or stress and transportation (Jericho et al., 1976; Stockdale et al., 1979ab). Some of the viral vaccine-bacterin combinations have resulted in enhanced resistance to experimental challenge with the homologous organism. Under field conditions, however, viral vaccine-bacterin combinations still do not provide consistent and reliable protection (Collier, 1968; Anonymous, 1968; Engelbrecht, 1968).

Live P. haemolytica vaccines have been used to try to stimulate a protective immunity. In calves vaccinated with live P. haemolytica by either aerosol or parenteral routes, transthoracic challenge lesions were less severe than in control animals (Corstvet et al., 1978; Panciera et al., 1984). Calves vaccinated with live P. haemolytica by either route or calves which had been previously exposed naturally to the organism had high serum antibody titers to somatic antigens. These titers correlated with minimal experimental lesions (Panciera et al., 1984; Confer et al., 1984a,b). When logarithmic cultures of P. haemolytica were used as the immunogen, slightly greater resistance to challenge exposure occurred compared to when stationary phase organisms were used (Confer et al., 1984a). Vaccination with a chemically altered strain of P. haemolytica also provided various degrees of resistance to subsequent challenge by intranasal IBR and intratracheal P. haemolytica (Kucera et al., 1983).

The more promising results obtained with live vaccines compared to bacterins could be due to several factors. In bacterins, bacterial surface membrane antigens are the primary stimuli for the immune

resulted in variable amounts of protection following an IBR, P. haemolytica challenge (Yates et al., 1983). Parenteral routes appeared to provide much more protection than the aerosol route. These results suggested that protection against pneumonic pasteurellosis may be possible with a subcellular extract of P. haemolytica. It is still unclear, however, which bacterial antigenic determinants are necessary for protection.

In cattle which were vaccinated with a potassium thiocyanate extract of P. haemolytica, but subsequently developed pneumonia following experimental challenge, serum cytotoxin neutralizing activity was much lower than in similarly treated animals which did not develop pneumonia (Cho et al., 1984). High levels of cytotoxin neutralizing activity also have been detected in serum from cattle which have been either experimentally or naturally exposed to P. haemolytica, but did not develop evidence of disease. In contrast, low levels of serum anticytotoxin activity are commonly present in animals dying of fibrinous pneumonia (Cho et al., 1984; Shewen and Wilkie, 1983; Gentry et al., 1985). This evidence suggests a significant role for antibody against cytotoxin in protection from pneumonic pasteurellosis. The protective effects of immunization with antigens derived from cytotoxin, however, have yet to be determined.

Exotoxins

The production of extracellular enzymes that break down large molecules into smaller, usable units was an important step in the evolution of parasitism by certain microorganisms. A side effect of these enzymes and other products of bacterial metabolism is damage to

system. With live organisms, however, capsular material, cytotoxin, and other bacterial products are produced and may also serve as antigens (Confer et al., 1984a,b; Panciera et al., 1984). The greater resistance to experimental challenge obtained when using logarithmic phase versus stationary phase organisms as the immunogen suggests possible roles for these bacterial components in protection against pneumonic pasteurellosis (Confer et al., 1984a).

Several studies on the protective effect of vaccines comprised of capsular extracts of Pasteurella have been undertaken in mice, hamsters, sheep, and cattle (Tadayon and Lauerman, 1981; Gilmour et al., 1979; Matsumoto et al., 1984; Yates et al., 1983). In mice and hamsters, a potassium thiocyanate extract of P. haemolytica yielded 55% to 100% protection when compared to unvaccinated controls (Tadayon and Lauerman, 1981). In the same study, a saline capsular extract gave 30% to 40% protection, whereas bacterins produced 20% to 60% protection. Testing of these extracts along with some other bacterial components suggested that bacterial immunogenic factors may be able to stimulate better immunity when freed from the bacteria than when the bacteria is still intact (Tadayon and Lauerman, 1981). In sheep, sodium salicylate extracted capsular material from P. haemolytica was antigenic and protective upon challenge exposure with aerosol PI-3 and P. haemolytica (Gilmour et al., 1979). Results of vaccination of cattle with capsular material have been variable. With a saline extract of P. haemolytica no clear difference between vaccinated and non-vaccinated cattle could be detected following challenge exposure (Matsumoto et al.,1984). A potassium thiocyanate extract of P. haemolytica given subcutaneously, intramuscularly, and by aerosol

the tissues and cells of the host. Since the first experiment demonstrating a detrimental effect of these enzymes (toxins) by Roux and Yersin in 1888, a wide variety of bacterial toxins have been isolated and described. In contrast to endotoxins, bacterial exotoxins are predominately protein, are produced by the bacteria during the logarithmic or declining phases of growth, are neutralized by homologous antibody, are heat-labile, and each have their own particular pharmacologic effects. Some of these have been purified and have well defined biological effects. Many others have less well understood mechanisms of action and it is unclear what role, if any, they play in the pathogenesis of disease (van Heyningen, 1970). Some of the more important exotoxins and general exotoxin classifications are summarized in Table I.

A number of bacterial exotoxins have been reported to affect leukocytes. These affects include depression of chemotaxis and random mobility, inhibition of phagocytosis and intracellular killing, and complete cell lysis. The most extensively studied of these leukocyte toxins are the leukocidin of Pseudomonas aeruginosa and the Panton-Valentine leukocidin of Staphylococcus aureus.

Pseudomonal leukocidin is produced by most strains of pathogenic P. aeruginosa. It is secreted as an inactive precursor which undergoes activation following protease (elastase) treatment (Scharmann, 1976). The toxin is cytotoxic to neutrophils and lymphocytes of a wide variety of animal species, but has no effect on erythrocytes (Scharmann, 1976b). A substance believed to be identical to leukocidin has been shown to inhibit phagocytosis and intracellular killing by neutrophils as well (Nonoyama et al., 1979). The mechanism of action of leukocidin

TABLE I

GENERAL CLASSIFICATIONS OF EXOTOXINS

Category	Representative Species	Major Biological Properties
Protein synthesis inhibitors	Corynebacterium diptheriae Pseudomonas aeruginosa	Inhibition of eukaryotic protein synthesis via ADP ribosylation of elongation factor-2
Enterotoxins	Vibrio cholerae Escherichia coli	Stimulation of adenylate cyclase with resultant hypersecretion of water and electrolytes
	Staphylococcus aureus	Unknown mechanism
Neurotoxins	Clostridium tetani	Block release of inhibitory neurotransmitters at central reflex arcs
	Clostridium botulinum	Block release of acetylcholine at cholinergic neuromuscular junctions
Phospholipases	Clostridium perfringens Bacillus cereus Staphylococcus aureus Pseudomonas aeruginosa	Degradation of specific phospholipids of cell membranes
Oxygen-labile (thiol-activated) Cytolysins	Listeria monocytogenes Streptococci Groups A,B,C,G Clostridium perfringens Clostridium chauvoei	Alteration of membrane permeability through binding, aggregation, and removal of cholesterol from cell membranes
Proteases	Escherichia coli Pasteurella haemolytica Serratia marcesens	Proteolytic cleavage of structural or functional proteins and immunoglobulins

involves an initial binding of the toxin to a single population of specific, but poorly characterized sites on the leukocyte membranes (Hirayama et al., 1984). These sites are most likely integral proteins of the plasma membrane and not lipid in nature (Scharmann, 1976c). The original hypothesis as to the mechanism of cytotoxicity was that membrane binding resulted in an increased permeability to low molecular weight substances based on the release of various intracellular markers from neutrophils exposed to leukocidin (Scharmann, 1976d). Subsequent studies found that the toxin stimulated a rapid turnover of phosphatidylinositol which resulted in increased levels of free cytoplasmic calcium. Increased calcium levels activated several membrane associated, calcium-dependent enzymes which may have been involved in the leukocidal process (Hirayama and Kato, 1983). The precise mechanism of toxicity remains unclear.

Morphologic changes in neutrophils exposed to Pseudomonal leukocidin occur in three stages. Initially, there is a rapid loss of cell motility and withdrawal of pseudopodia. Cytoplasmic protrusions containing granules appear on the surface of the cell. By 5 minutes post exposure, there is swelling and rounding of the cell; and by 30 minutes, the nucleus is swollen and spherical and cytoplasmic architecture is poorly defined (Scharmann et al., 1976).

Staphylococcal leucocidin is produced by several strains of Staphylococcus aureus. It consists of two components, F (Fast), and S (Slow), as determined by their migration on carboxymethylcellulose columns. The two components of leukocidin have been purified, crystallized, and partially characterized (Noda et al., 1980). Individually, these components are inactive, but when combined act

synergistically causing lysis of rabbit and human neutrophils and macrophages. A dermonecrotic effect on rabbit skin has also been attributed to the toxin, but otherwise the toxic complex appears to be highly specific for leukocytes (Ward and Turner, 1980).

Extensive systematic studies into the mode of action of leukocidin were undertaken by Woodin and Wienke during the 1960°s (Woodin, 1970). The result of these studies led to a hypothesis that the major event in the mode of action of leukocidin is an altered membrane permeability to cations. Briefly, component F was postulated to interact with the hydrophobic portions of the cell membrane inducing expansion and a conformational change in F. Adsorption of the S component then occurs to the altered surface of the F component. Interaction of S with exposed hydrophilic portions of triphosphoinositide induces the creation of a membrane channel which is permeable to electrolytes. Potassium efflux and calcium influx increase the ionic strength of the region causing deformation of the F-membrane complex and the subsequent release and dissociation of the S and F components into two inactive proteins. Membrane triphosphoinositide then reverts to its natural conformation and is capable of repeating the reaction with fresh leukocidin molecules (Woodin, 1970). More recent studies showed that pretreatment of rabbit neutrophils with the S component followed by exposure to the F component resulted in rapid lysis of the cells. In contrast, pretreatment with F followed by the addition of S was accompanied by a lag phase before cytotoxicity occurred. This suggested that the S component may be more responsible for the initial interaction with the cell membrane than the F component. Specific inactivation of the S component by ganglioside Gm1 and competitive inhibition of the binding of S to

leukocyte membranes by subunit B of cholera toxin, which utilizes a ganglioside Gm1 membrane receptor (van Heyningen,1971), indicates that ganglioside Gm1 may resemble or be part of the receptor site for component S (Noda et al., 1980). Binding studies have confirmed that S binds to a single type of membrane site without cooperative interaction. Component F, however, requires the presence of S before the full number of binding sites for it are expressed (Noda et al., 1981). The examination of enzyme functions in leukocytes exposed to leukocidin showed high levels of membrane-associated component S induced phospholipase A2 activity. Upon exposure to component F, there was inhibition of binding of cyclic AMP to cyclic AMP-dependent protein kinase as well. Therefore the original hypothesis regarding the mode of action of leukocidin has been modified to reverse the roles of F and S. It was suggested that the binding of S component to ganglioside Gm1 receptors on the leukocyte membrane stimulates phospholipase A induced alterations in membrane phospholipids, thus allowing F to bind and induce an increased permeability to cations (Noda et al., 1982).

Morphologic studies of rabbit leukocytes exposed to Staphylococcal leukocidin indicate that the sequence of events leading to leukolysis occur rapidly. Two minutes after exposure to the toxin, leukocytes are swollen, rounded, and have spherical instead of lobulated nuclei. Terminal events are characterized by cytoplasmic degranulation, nuclear rupture, appearance of empty intracellular vacuoles, and complete cell lysis. All of these effects were reported to be fully developed after only 10 minutes of incubation (Noda et al., 1980).

Several of the bacterial-derived thiol-activated cytolysins affect leukocyte function and viability. The oxygen-labile hemolysin of

beta-hemolytic Streptococci, streptolysin O, has been examined most thoroughly. At low concentrations, streptolysin O markedly depressed chemotaxis and random mobility of human neutrophils. Neutrophils of several other species are affected also, but to a lesser extent (van Epps and Andersen, 1974; Andersen and van Epps, 1972). At higher concentrations, streptolysin O causes rapid degranulation and lysis of neutrophils and peritoneal macrophages (Hirsch et al., 1963; Ofek et al., 1972; Fauve et al., 1966). The mechanisms of these effects have been hypothesized to be due to either an alteration of leukocyte membrane permeability by toxin-cholesterol complexes, or a toxin induced configurational change in a membrane protein causing premature activation of the respiratory burst mechanism. In the latter, there would be subsequent formation of toxic oxygen intermediates ultimately resulting in cellular dysfunction and death (Andersen and Duncan, 1980).

A cell bound hemolysin of Group A Streptococci, streptolysin S, has been shown to produce similar effects on leukocytes. This toxin is distinct from streptolysin O and has different biochemical properties. The toxicity of streptolysin S is much less than streptolysin O and is dependent upon the toxin being either cell associated or bound to an extracellular carrier protein. The toxic mechanism is unclear, but may involve penetration of the cell membrane and damage to the subcellular organelles (Ofek et al., 1970; Hirsch et al., 1963; Ofek et al., 1972).

Pneumolysin is the sulfhydryl-activated cytolysin of Streptococcus pneumoniae. Like streptolysin O, low doses of pneumolysin inhibit chemotaxis and random migration while higher doses cause cell death

and lysis of human neutrophils (Johnson et al., 1981; Paton and Ferrante, 1983). Immunization of mice with purified pneumolysin followed by aerosol challenge with virulent S. pneumoniae resulted in significantly longer survival times in immunized mice compared to non-immunized controls. High circulating antibody titers to pneumolysin correlated positively with increased resistance against S. pneumoniae challenge. These findings suggest that pneumolysin may play a role in the pathogenesis of S. pneumoniae in mice, most likely by its effects on neutrophils (Paton et al., 1983).

Leukocidal effects on macrophages have also been detected with the sulfhydryl-activated cytolysin of Listeria monocytogenes. These effects were not present when macrophages were exposed to non-hemolytic Listeria strains. This toxin remains poorly characterized, however, and its role in listerial infections is unclear (Kingdon and Sword, 1970; Watson and Lavizzo, 1973).

Several reports of leukocidal activity have also been made on some unrelated toxins. An alpha hemolysin of E. coli has been shown to depress phagocytosis and chemotaxis by human leukocytes (Cavalieri and Snyder, 1982). At higher concentrations, this toxin is cytolytic for these cells (Cavalieri and Snyder, 1982b). Staphylococcal alpha toxin depresses phagocytosis by and decreases viability of human neutrophils (Gemmell et al., 1982) and rabbit alveolar macrophages (McGee et al., 1983). A delta hemolysin of Staphylococcus sp. caused complete lysis of neutrophils, monocytes, and lymphocytes from a variety of different species (Gladstone and Yoshida, 1967). Certain strains of Actinobacillus actinomycetemocomitans, a Gram negative organism which plays an important role in certain types of periodontal disease,

produces a leukotoxin which is cytocidal for human neutrophils and monocytes (Tsai et al., 1979; Taichman et al., 1980). The toxin appears to act at the cell membrane, but the mechanism of toxicity does not appear to be due to osmotic lysis caused by extensive permeability changes (Taichman et al., 1980). The isolation of a significantly higher number of leukotoxin producing strains of bacteria compared to non-leukotoxin producing strains of bacteria from patients with periodontal disease supports the hypothesis that leukotoxin may be an important virulence factor in the pathogenesis of certain forms of periodontal disease (Zambon et al., 1983; Tsai et al., 1979).

There have been several reports of a substance produced by Fusobacterium necrophorum which is toxic for neutrophils, lymphocytes, and peritoneal macrophages (Fales et al., 1977; Roberts, 1967; Coyle-Dennis and Lauerman, 1978). Original reports were of a heat stable toxin, which may have been endotoxin. The most recent isolate, however, was heat labile and had characteristics typical of an exotoxin (Coyle-Dennis and Lauerman, 1978). Using a mouse model, it was found that leukotoxin producing strains of Fusobacterium necrophorum produced substantially more liver abscesses following intraperitoneal injection than non-leukotoxin producing strains. Those studies suggested a possible role for the leukotoxin in the pathogenesis of the lesions produced in this model system (Coyle-Dennis and Lauerman, 1978).

Haemophilus pleuropneumoniae, the cause of acute to chronic pleuropneumonia in swine, has been found to be toxic to porcine alveolar macrophages and blood monocytes. Both live bacteria and sterile culture supernatants were cytotoxic, whereas heat-killed organisms had no effect

on these cells. The toxin was heat-stable and could be neutralized by the sera of pigs with chronic Haemophilus pneumonia (Bendixen, 1981). Previously, it had been shown that nonviable, sonicated H. pleuropneumoniae or sterile culture supernatants could induce localized pleuropneumonia when instilled into the lung (Rosendal et al., 1980). Together, these findings support a potential role for this toxin in the pathogenesis of porcine pleuropneumonia.

Cytotoxin

The first report of a P. haemolytica-associated cytotoxin was made by Benson et al. (1978). Live cultures of P. haemolytica serotypes 1, 2, 7, and 12 were found to induce morphologic cytotoxic changes in and inhibit phagocytosis by alveolar macrophages in vitro. With heat-killed organisms, however, only mild cytotoxic changes and no inhibition of phagocytosis were noted. The toxic factor was also found in bacteria free filtrates of serotype 1. It was suggested that this toxic factor was either a product of metabolically active bacteria or a heat-labile substance of capsular origin. Markham and Wilkie (1980) measured the rate of phagocytosis by the uptake of formalin-killed, radiolabeled P. haemolytica. They found that macrophages phagocytized Yersinia enterocolitica more rapidly than macrophages which were incubated with P. haemolytica. The rate of phagocytosis of both organisms was enhanced by the presence of immune serum, but as the number of Pasteurella haemolytica organisms increased, this rate decreased. It was suggested that the increased uptake of opsonized bacteria occurring at high bacterial concentration was detrimental to alveolar macrophages. High bacterial numbers caused

an increased detachment of macrophages from coverslips which was suggestive of cell death. Chromium release assays, however, failed to demonstrate cytotoxixity in these macrophages exposed to whole formalin-killed bacteria. Low concentrations of bacteria free supernatents from P. haemolytica cultures were found to inhibit phagocytosis, while higher concentrations were found to cause cytotoxicity as determined by ^{51}Cr release. It was not determined whether the toxic material present in the culture supernatants was also present in association with the formalin-killed bacteria. Phagocytosis of live [^{3}H]-thymidine labelled P. haemolytica by cultured alveolar macrophages also has been examined (Maheswaran et al., 1980). P. haemolytica were poorly phagocytosed in the absence of opsonins from normal adult bovine serum or P. haemolytica-specific antiserum. Incubation of cultured macrophages with low alveolar macrophage:bacteria ratios (1:10) resulted in complete phagocytosis and intracellular killing of bacteria with no evidence of cytotoxicity as determined by transmission electron microscopy. At a 1:20 ratio, however, some bacteria were not phagocytized and changes were noted in macrophages between 30 and 90 minutes of initial exposure. These changes were characterized by loss of cytoplasmic ground substance, decreased electron density of the cytoplasm, dispersion of nuclear chromatin, and eventually plasma membrane rupture. Changes were present in less than 10% of the alveolar macrophages at 30 minutes, but by 90 minutes almost all cells showed severe cytotoxic changes. It was suggested that the cytotoxic effect was due to a substance released by the excess unphagocytized bacteria (Maheswaran et al., 1980). Live P. haemolytica cultures were found to be cytotoxic to blood mononuclear

leukocytes by trypan blue exclusion (Kaehler et al., 1980a). In contrast, cytotoxicity was not detected when the cells were exposed to either heat-killed or x-irradiated P. haemolytica. Cytotoxicity was dose related as indicated by an increase in cell death as bacterial numbers were increased. Enriched monocyte cultures had a higher percentage cell death than lymphocyte cultures. It was speculated that phagocytosis of the bacteria by monocytes may speed their death, whereas lymphocytes were killed only after a certain concentration of bacteria was reached. The suggestion was made that the toxic factor was a property or product of metabolically active bacteria (Kaehler et al., 1980a). P. haemolytica was also found to exert cytotoxic effects on sheep and goat blood mononuclear leukocytes (Kaehler et al., 1980b). Horse, swine, and human mononuclear leukocytes, however, were not susceptible. The specificity of the cytotoxin towards ruminant leukocytes was postulated to be due to a specific membrane receptor not present on non-ruminant leukocytes or due to differences in certain metabolic pathways in the cells of these species (Kaehler et al., 1980b). Subsequent studies using sterile, bacterial culture supernatants, further delineated cell and species specificity of P. haemolytica cytotoxin (Shewen and Wilkie, 1982). Significant ^{51}Cr release occurred with bovine alveolar macrophages, lymphocytes, neutrophils, and blood monocytes exposed to the toxic culture supernatant. Slight release was detected from cultured bovine kidney cells, porcine alveolar macrophages, lymphocytes, neutrophils, or monocytes. No release was present from bovine spleen cell cultures, or bovine or porcine erythrocytes. Because leukocytes are necessary to mount an immune response to bacterial infection, it was suggested

that neutralization of this cytotoxic component may be essential in prevention of pneumonic pasteurellosis (Shewen and Wilkie, 1982).

A difference between the interaction of bovine neutrophils with logarithmic-phase versus stationary-phase P. haemolytica cultures has been determined. Berggren et al. (1981) found that logarithmic-phase organisms opsonized with normal bovine sera or anti-P. haemolytica sera were poorly phagocytized and caused release of ^{51}Cr from the neutrophils. Under the same conditions, stationary-phase organisms were nearly all phagocytized and cytotoxicity was not detected. At higher bacteria to neutrophil ratios (100:1), a smaller percentage of stationary-phase organisms were phagocytized and cytotoxicity occurred following prolonged incubation. This cytotoxicity was attributed to continued growth and elaboration of a toxic substance by unphagocytized bacteria. Transmission electron microscopy showed that at 10:1 bacteria : neutrophil ratios stationary phase organisms were completely phagocytized and degraded. In contrast, neutrophils exposed to the same number of logarithmic-phase bacteria showed marked cytotoxic changes characterized by cell membrane rupture, aggregated cytoplasmic matrices, and numerous cytoplasmic vesicles (Berggren et al., 1981). The same workers demonstrated that maximum ^{51}Cr release occurred when neutrophils were incubated with 6 hour bacteria cultures. Steadily declining amounts of ^{51}Cr release were obtained from neutrophils incubated with 8-18 hour bacterial cultures. Adult bovine serum and antisera from calves inoculatated with logarithmic-phase organisms neutralized the cytotoxin. Bovine fetal serum and serum from neonatal calves, however, had no effect on cytotoxic activity. Cytotoxicity was confirmed by transmission electron

microscopy of neutrophils exposed to cytotoxin for 20 minutes. Morphologic changes included ruptured cell membranes, aggregation of cytoplasmic matrices, cytoplasmic vesicles, pyknotic nuclei, and evidence of lysosomolysis (Baluyut et al., 1981). Similar results were obtained using alveolar macrophages lavaged from calves at various intervals following aerosol exposure to P. haemolytica (Walker et al., 1984). Regardless of when the macrophages were obtained, logarithmic-phase P.haemolytica were poorly phagocytized and induced cytotoxicity. Stationary (declining)-phase organisms were phagocytized. Toxic changes were characterized by blunting of cytoplasmic processes, debris filled cytoplasmic vesicles, and abundant extracellular debris.

Purification and characterization studies of the cytotoxin have yielded variable results. In supernatant obtained from growth of P. haemolytica in RPMI-1640 medium without other growth additives, it was found that cytotoxic activity was retained by a 300,000 molecular weight cutoff membrane during ultrafiltration. This toxic supernatant was heat-labile, oxygen-stable, susceptible to pH extremes, inactivated by trypsin, and contained no detectable endotoxin. It was cytotoxic to both bovine neutrophils and monocytes and was not hemolytic to bovine or ovine erythrocytes (Baluyut et al., 1981). In another study, cytotoxin produced by P. haemolytica grown in brain heart infusion broth containing fetal calf serum in a dialysis culture system was isolated by ultrafiltration, and high pressure size exclusion gel chromatography. Supernatant subjected to gel filtration yielded three major peaks of interest. These were a void volume peak, a 50K protein, and a 150K protein. All 3 peaks reacted with polyvalent antisera to

P. haemolytica in double immunodiffusion tests. The highly purified 150K antigen showed little to no cytotoxicity. Earlier less purified fractions which also contained this antigen along with growth factor components resulted in high cell mortality. Increased cell mortality could also be obtained by addition of the cationic detergent cetyltrimethylammonium bromide to the purified 150K antigen. Double immunodiffusion studies of the 150K and 50K antigens revealed convergent lines of identity suggesting that all antigenic determinants present in the 150K antigen were also present in the 50K antigen. Dithiothreitol reduction and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the 150K antigen resulted in two major components of 50K and 20K. It was speculated that these were subunits of the 150K antigen and that the 50K component may be identical to the 50K antigen obtained from gel filtration chromatography. Further double immunodiffusion studies with the 150K antigen showed lines of identity against 12 serotypes of P. haemolytica. This suggested that the 150K antigen could be used as a protective antigen against all major serotypes of P. haemolytica involved in bovine respiratory diseases (Himmel et al., 1982).

Research Objectives

Current evidence suggests an important role for P. haemolytica cytotoxin in the pathogenesis of pneumonic pasteurellosis. Major studies incriminating cytotoxin, however, have been made using crude bacterial supernatants. Two studies on the purification and characterization of cytotoxin have been reported and contain some conflicting and incomplete conclusions. The purpose of this study was

to purify cytotoxin for use in immunologic assays in order to more adequately determine the role of cytotoxin in pneumonic pasteurellosis.

Chapter II

PURIFICATION AND CHARACTERIZATION OF CYTOTOXIN

Introduction

Numerous predisposing factors have been implicated in the pathogenesis of shipping fever (pneumonic pasteurellosis) of cattle (Thomson, 1974; Thomson et al., 1975; Thomson, 1980; Lillie, 1974). The ultimate cause of clinical disease and death, however, is a severe fibrinous pneumonia caused by Pasteurella haemolytica serotype 1 (Lillie, 1974; Jensen et al., 1976). Despite various approaches used to control the disease, pneumonic pasteurellosis continues to be a major economic problem to the feedlot industry (Jensen et al, 1976; Martin et al., 1980).

P. haemolytica produces a toxin (cytotoxin) that is lethal for bovine leukocytes in vitro (Benson et al., 1978; Baluyut et al., 1981; Shewen and Wilkie, 1982). It has been suggested that cytotoxin participates in the pathogenesis of pneumonic pasteurellosis by either directly impairing lung defenses through its effect on alveolar macrophages and neutrophils, or indirectly by causing an inflammatory response mediated by lysosomal enzymes released from lysed leukocytes (Otulakowski et al., 1983). Serologic studies have demonstrated that high cytotoxin neutralizing antibody titers in both experimentally and naturally infected cattle are associated with increased resistance to pneumonic pasteurellosis (Shewen and Wilkie, 1983; Cho et al., 1984;

Gentry et al., 1985). These findings suggest an important role for cytotoxin in the pathogenesis of this disease.

Initial characterization of cytotoxin demonstrated that it was a high molecular weight substance optimally produced by P. haemolytica during their logarithmic growth phase (Baluyut et al., 1981). Cytotoxin was lethal to both bovine neutrophils and mononuclear leukocytes. It was also heat-labile, oxygen-stable, susceptible to pH extremes, and inactivated by trypsin (Baluyut et al., 1981). The toxic effect of this substance was neutralized by either adult bovine serum or serum from steers immunized with live P. haemolytica. Subsequent chromatographic studies identified a 150 kilodalton (kDa) protein which was toxic to alveolar macrophages (Himmel et al., 1982). The protein migrated as a single band on polyacrylamide gel electrophoresis (PAGE) and was separated into 50 kDa and 20 kDa bands after complete denaturation and reduction on sodium dodecyl sulfate (SDS)-PAGE. Immunodiffusion tests demonstrated that the separated cytotoxic antigen cross reacted with antisera from 12 different P. haemolytica serotypes. The purpose of the current study was to purify cytotoxin for more extensive examination of its biochemical, cytotoxic, and antigenic characteristics.

Materials and Methods

Crude Cytotoxin Preparation

The most efficient production of crude cytotoxin was by the method of Shewen and Wilkie (1982). Lyophilized cultures of P. haemolytica biotype A, serotype 1 isolated from the trachea of a feedlot calf (Corstvet et al., 1973) were reconstituted in phosphate buffered saline

(PBS, 0.01M $NaPO_4$, 0.15M NaCl, pH 7.4) and grown for 18 hours at 37° C on modified brain heart infusion agar . Isolated bacterial colonies were incubated in brain heart infusion broth for 4.5 hours, then transfered into RPMI-1640 medium (Gibco Laboratories, Grand Island, New York) containing 7% fetal bovine serum. Following a 1 hour incubation, bacteria were removed by centrifugation and the supernatant was filtered through a 0.2 um membrane (Millipore Corporation, Bedford, Mass.). The filtered supernatant was then concentrated in a hollow fiber concentration system with a Model H1P-10-43 10,000 molecular weight exclusion hollow fiber cartridge (Amicon Corporation, Danvers, Ma.). Concentrated cytotoxin was dialysed against 0.01M Bis tris propane-Cl (BTP-Cl), pH 7.4, and stored at -60° C.

Chromatography

All chromatographic separations were conducted at 5° C. Concentrated crude cytotoxin equivalent to a volume of 20 liters of bacterial supernatant was applied to a 5 x 60 cm column packed to a height of 43 cm with DEAE Sephacel (Pharmacia Fine Chemicals, Uppsala, Sweden), which was equilibrated with 0.01M BTP-Cl, pH 7.4. The sample was followed by 600 ml of 0.01M BTP-Cl buffer, and the column was then eluted with a 1500 ml linear gradient from 0 to 0.1M NaCl in 0.01M BTP-Cl. Subsequent elution was by steps using NaCl concentrations of 0.1M, 0.25M, and 0.5M in 0.01M BTP-Cl. The NaCl concentration of selected fractions was determined by conductivity.

Fractions from ion exchange experiments which contained cytotoxic activity were pooled, concentrated and dialyzed against PBS. These samples were applied to a 2.6 x 100 cm column packed to a height of 82

cm with Ultrogel AcA-34 (LKB Biochrom Ltd., Gaithersburg, Maryland), and eluted with PBS. The column was calibrated for molecular weight determination (Andrews, 1965) with Blue dextran and proteins ranging in molecular weight from 13.7 kDa (ribonuclease) to 669 kDa (thyroglobulin) (Sigma Chemical Company, St. Louis, Mo.). Also included were catalase, albumin, chymotrypsin A, ferritin, aldolase, and ovalbumin.

Fractions from gel filtration experiments which contained cytotoxic activity were pooled, concentrated, and dialyzed against 10 mM Tris, pH 8.3. Samples were applied to a 1.6 x 30 cm column packed to a height of 24 cm with Polybuffer Exchanger 94 (Pharmacia Fine Chemicals, Uppsala, Sweden) which had been equilibrated with 10 mM Tris, pH 8.3. Elution was performed with 250 ml of polybuffer 74, adjusted to pH 4.0 with HCl followed by 10 mM Tris plus 0.5M NaCl, pH 4.5.

Cytotoxicity Assay

Cytotoxicity of individual chromatographic fractions was determined by ^{51}Cr release from neutrophils. Bovine neutrophils were separated on Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) with osmotic lysis of erythrocytes. Following three washes in PBS, neutrophils were suspended in RPMI-1640 medium to a concentration of $1x10^7$ cells/ml. ^{51}Cr, as sodium chromate (ICN Radiochemicals, Irvine, Ca.) was added to the neutrophil suspension at a concentration of 50 uCi/ml and the suspension was incubated for 1 hour at 37° in a shaking water bath. Neutrophils were washed three times in PBS and then resuspended in RPMI-1640 medium to a concentration of $8x10^6$ cells/ml. A

100 ul portion of the ^{51}Cr-labelled neutrophil suspension was added to each well of a 96-well round bottom cell culture plate (Corning Glass Works, Corning, N.Y.). Two hundred ul of the preparation to be assayed was added to triplicate wells and incubated for 1 hour at 37° C. The plates were centrifuged at 150 xg for 7 minutes and 200 ul of supernatant was transferred from each well to plastic tubes and counted for 4 minutes in an automatic gamma counter (Searle Analytical Inc., Des Plaines, Ill.). Mean counts and standard deviations were calculated for each fraction tested.

For a more quantitative assay of cytotoxic activity, two-fold dilutions of pooled and concentrated fractions from various steps in the purification procedure were tested using ^{51}Cr release. Mean counts were plotted against dilution, and cytotoxin activity was defined as the inverse of the dilution required to produce 50% ^{51}Cr release. Background counts and 100% release were determined with RPMI-1640 medium containing 7% fetal bovine serum (FBS) and 4M NaOH respectively. The total cytotoxin activity was expressed either on a volume basis, or for specific activity adjusted according to the protein concentration.

Immunologic Assays

The reaction of individual chromatographic fractions with antisera to P. haemolytica was determined by an enzyme-linked immunosorbent assay (ELISA). The general procedure and preparation of bovine anti-P. haemolytica IgG and horseradish peroxidase (HRPO) conjugated anti-P. haemolytica IgG used in the assay have been previously described (Lessley et al., 1985). In the current study, plates were coated using

30 ug/ml of bovine anti-P. haemolytica IgG. HRPO-conjugated anti-P. haemolytica IgG was diluted 1:25 in PBS containing 0.05% Tween 20 and 1% BSA. Absorbance was read at 490 nm (A_{490}) on a manual ELISA reader (Bio-Tek, Burlington, Vermont). Results were reported as the average A_{490} for fractions minus the average A_{490} for PBS controls.

Antibody response to purified cytotoxin was measured in rabbits and mice by ELISA (Voller et al., 1979). Doubling dilutions of serum from 1:100 to 1:1600 were tested in both species. For mice, plates were coated with 1 ug/ml cytotoxin in carbonate buffer. Alkaline phosphatase labelled anti-mouse IgG (Sigma Chemical Company, St. Louis) was used at a 1:200 dilution in PBS-Tween containing 1% BSA. For rabbits, plates were coated at 10 ug/ml cytotoxin in carbonate buffer and alkaline phosphatase labelled anti-rabbit IgG (Sigma Chemical Company, St. Louis, Mo.) was used at a dilution of 1:400 in PBS-Tween containing 1% BSA. The substrate consisted of 1 mg/ml p-nitrophenyl phosphate in 10% diethanolamine buffer. Absorbance was read at 405 nm (A_{405}) on a manual ELISA reader.

Rabbit and mouse serum were also reacted with cytotoxin in Ouchterlony immunodiffusion disks. The center wells of 0.9% agarose disks were charged with 10 ul of serum and the outer wells were filled with 10 ul of cytotoxin which was either undiluted or diluted 1:2, 1:5, or 1:10. The disks were incubated for 24 hours at 28° C in a humidified chamber.

Anticytotoxin globulin and serum preparation

Serum was obtained from a steer which was hyperimmunized with live P. haemolytica and possessed a high anti-P. haemolytica titer as

determined by a quantitative fluorometric assay (Confer et al., 1983). Serum was adsorbed with live encapsulated P. haemolytica with stirring at 4° C for 3 hours to remove antibodies to somatic and capsular antigens. Following centrifugation and filtration, the adsorbed serum was fractionated by gel filtration on Ultrogel AcA-34. Fractions from each major protein peak were tested for their ability to neutralize crude cytotoxin in a ^{51}Cr-release assay. Cytotoxic neutralizing capacity was demonstrated in the IgG fractions. These fractions were designated adsorbed sera and pooled, concentrated, and stored at -60 C.

Antibody to cytotoxin was produced in 2 rabbits and 5 mice by injection of 100 ug purified cytotoxin in 1 ml Freund's incomplete adjuvant per animal. Three injections at 2 week intervals were given intradermally in rabbits and intraperitoneally in mice.

Electrophoresis and Immunoblotting

Pooled and concentrated samples from various stages of the purification procedure were subjected to SDS-PAGE, PAGE, and immunoblotting. Samples were adjusted to 0.5 mg protein per ml and denatured by heating in the presence of SDS and B-mercaptoethanol. Samples of 150 ul were applied to a 2% acrylamide stacking gel and a 10% acrylamide resolving gel and separated by discontinuous SDS-PAGE (Laemmli, 1970). Some samples were also analyzed without denaturation in a 7.5% resolving gel by PAGE with discontinuous buffers (Davis, 1964). Duplicate gels were run for each experiment. One gel was fixed and stained with Coomassie brilliant blue R 250. Proteins from the unstained duplicate gel were electrophoretically transferred onto a nitrocellulose membrane (Kirkegaard and Perry Laboratories,

Gaithersburg, Md.) in a Model TE 50 Transphor (Hoefer Scientific Instruments, San Francisco, Ca.) as previously described (Towbin et al., 1979; Lessley et al., 1985). Antigens were demonstrated using both adsorbed and non-adsorbed sera and autoradiography with ^{125}I-labelled protein A (Amersham, Arlington Heights, Ill.).

Isoelectrofocusing

The isoelectric point of cytotoxin was determined by isoelectrofocusing in polyacrylamide gels containing ampholytes (LKB Ampholine PAGplates, pH 3.5-9.5; LKB-Produkter AB, Bromma, Sweden). Samples were electrophoresed on a flat bed electrophoresis apparatus for 1.5 hours at 30 watts and then stained with Coomassie Brilliant Blue R250. Isoelectric point determinations were made by comparisons with known standards (Pharmacia Fine Chemicals, Uppsala, Sweden) which were run with each gel. Included in the standards were amyloglucosidase, soybean trypsin inhibitor, B-lactoglobulin A, bovine and human carbonic anhydrase B, horse myoglobin, lentil lectin, and trypsinogen.

Protein Assay

Protein concentrations of pooled and concentrated cytotoxic fractions were determined by the method of Bradford (1976) using the Bio-Rad procedure and reagents (Bio-Rad Chemical Division, Richmond, Ca.).

Results

Fractionation of Crude Cytotoxin

The procedure used for separation of cytotoxin is outlined in Figure 1. Complete results of the separation procedure are listed in the appendix. The initial step in fractionation was anion exchange on DEAE-Sephacel (Figure 2). Typically, anion exchange separations produced two large absorbance peaks, the first peak eluting sharply with 0.05 M NaCl followed by a broader peak eluting from 0.1M to 0.2M NaCl. Four regions of cytotoxicity were detected (Figure 3). Two major regions of cytotoxicity were associated with fractions 95-120 (Region 1) and fractions 135-180 (Region 2). The regions of minor cytotoxic activity at fractions 185-205 and 215-235 were not toxic upon subsequent gel filtration analysis and were not pursued further. By ELISA a broad, region of increased absorbance was shared by both Region 1 and Region 2.

Gel filtration of the concentrated fractions from Region 1 resulted in a large absorbance peak eluting between fractions 40 - 60. A single, broad peak of cytotoxicity occurred in association with this large absorbance peak. Maximum cytotoxic activity eluted at a position corresponding to a molecular weight of approximately 160 kDa. There was increased absorbance associated with these fractions on ELISA. Cytotoxic fractions obtained from gel filtration of Region 1 material were pooled and concentrated. In subsequent chromatographic separations of this pooled material, however, cytotoxicity could not be detected.

Gel filtration of the concentrated fractions from Region 2 yielded

Figure 1 Cytotoxin Purification Scheme.

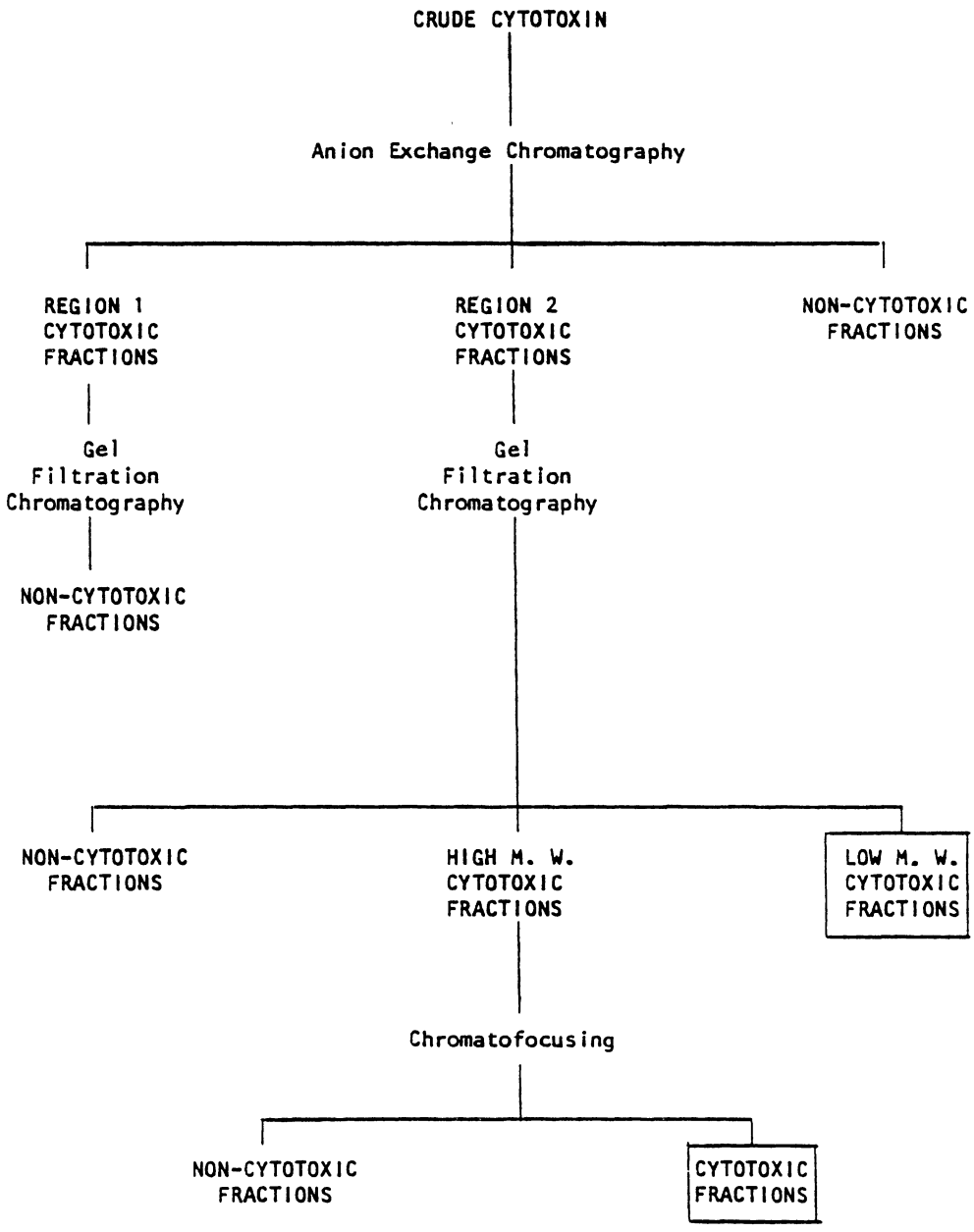
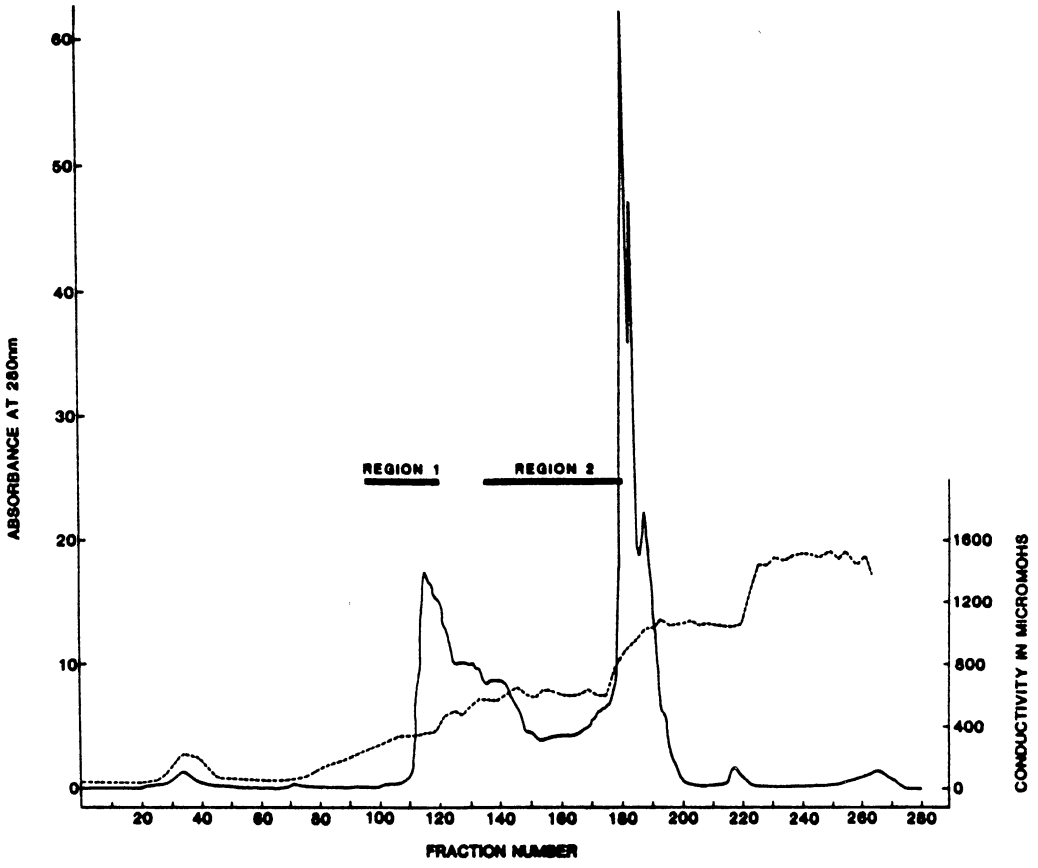
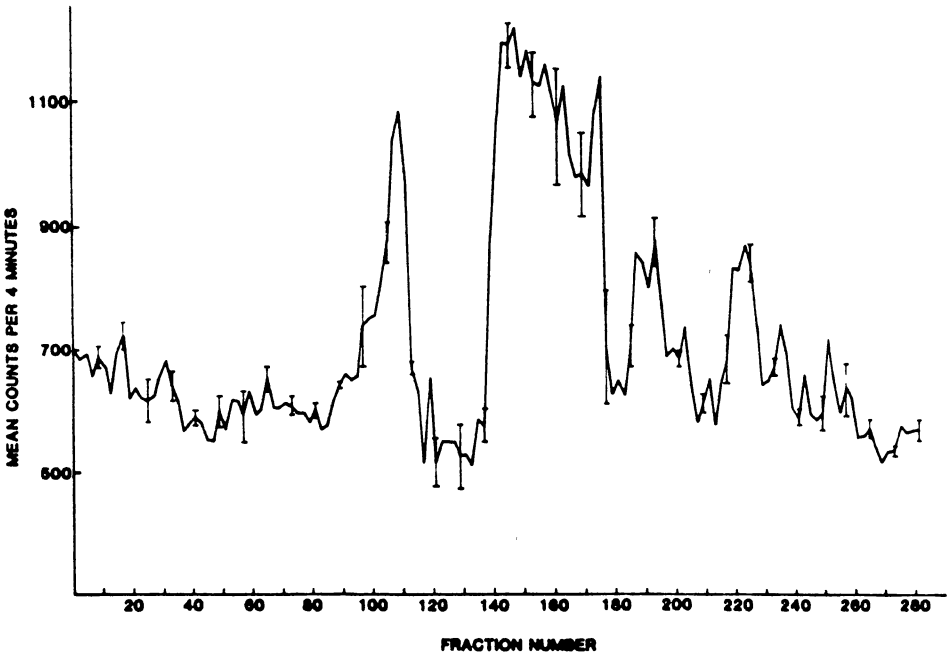

CRUDE CYTOTOXIN
Anion Exchange Chromatography
REGION 1 CYTOTOXIC FRACTIONS
REGION 2 CYTOTOXIC FRACTIONS
NON-CYTOTOXIC FRACTIONS
Gel Filtration Chromatography
Gel Filtration Chromatography
NON-CYTOTOXIC FRACTIONS
NON-CYTOTOXIC FRACTIONS
HIGH M. W. CYTOTOXIC FRACTIONS
LOW M. W. CYTOTOXIC FRACTIONS
Chromatofocusing
NON-CYTOTOXIC FRACTIONS
CYTOTOXIC FRACTIONS

Figure 2 Crude Cytotoxin - Anion Exchange Chromatography. Absorbance (——) and conductivity (·····) of chromatographic fractions. Region 1 and Region 2 indicate fractions demonstrating cytotoxicity.

Figure 3 Crude Cytotoxin - Cytotoxicity. ^{51}Cr release assay of fractions obtained from anion exchange chromatography.


ABSORBANCE AT 280nm
CONDUCTIVITY IN MICROMOHS
REGION 1
REGION 2
FRACTION NUMBER


MEAN COUNTS PER 4 MINUTES
FRACTION NUMBER

two major absorbance peaks at fractions 40 - 55 and 70 - 95 (Figure 4). A large plateaued peak of cytotoxic activity eluted between these two major areas of absorbance corresponding to a molecular weight of 160 kDa (Figure 5). Upon gel filtration of material from both Region 1 and Region 2, variable amounts of cytotoxicity were detected in the vicinity of fractions corresponding to a molecular weight of 30 kDa.

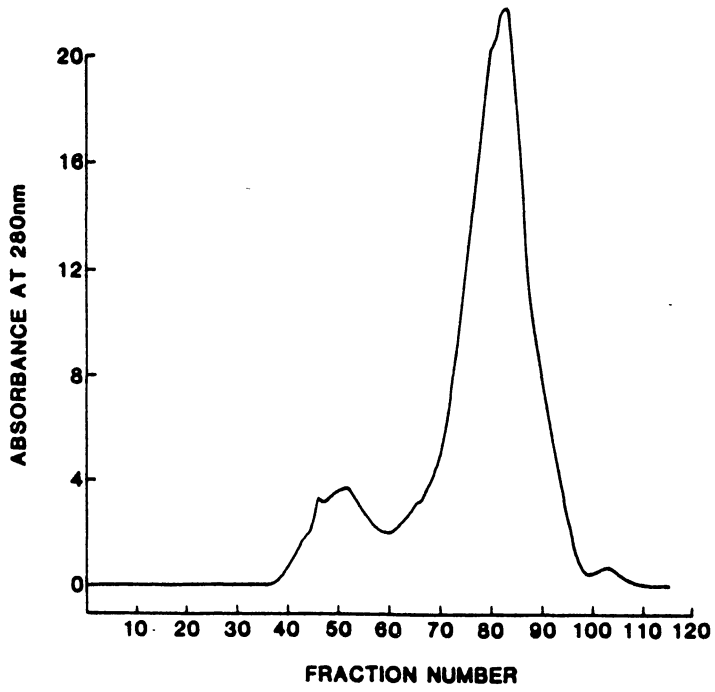
Pooled and concentrated cytotoxic fractions obtained from gel filtration separations of Region 2 material failed to bind to chromatofocusing columns equilibrated at pH 8.3 and pH 7.4. A substantial amount of non-cytotoxic material did bind at pH 8.3, however, resulting in additional purification of the cytotoxic component (Figures 6 and 7).

Additional manipulation of cytotoxin demonstrated retention of sufficient cytotoxicity at pH 4.5 for detection in dilute chromatographic fractions. At pH values over 9.5 detection of cytotoxicity was inconsistent. Dialysis of cytotoxin into 0.3M KH_2PO_4 resulted in decreased cytotoxic activity. Dialysis of this material back into 0.01M KH_2PO_4, however, resulted in the return of substantial amounts of cytotoxicity.

A quantitative summary of the purification procedure is presented in Table II. Increases in cytotoxic activity expressed as a function of protein concentration were not observed even as associated non-cytotoxic protein components of the sample were removed. The entire separation procedure resulted in a 97% reduction in protein content of the original sample with a 1.3% recovery of the total original cytotoxic activity.

Figure 4 Region 2 Material - Gel Filtration Chromatography. Absorbance of fractions obtained from gel filtration of anion exchange fractions 135-180 (Region 2).

Figure 5 Region 2 Material - Cytotoxicity. ^{51}Cr release assay of fractions obtained from gel filtration of anion exchange fractions 135-180 (Region 2).


ABSORBANCE AT 280nm
20
16
12
8
4
0
10 20 30 40 50 60 70 80 90 100 110 120
FRACTION NUMBER

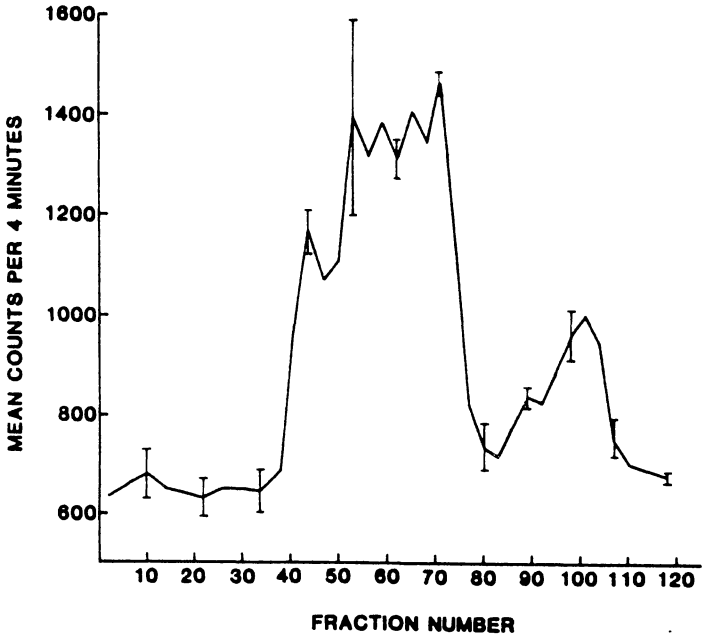
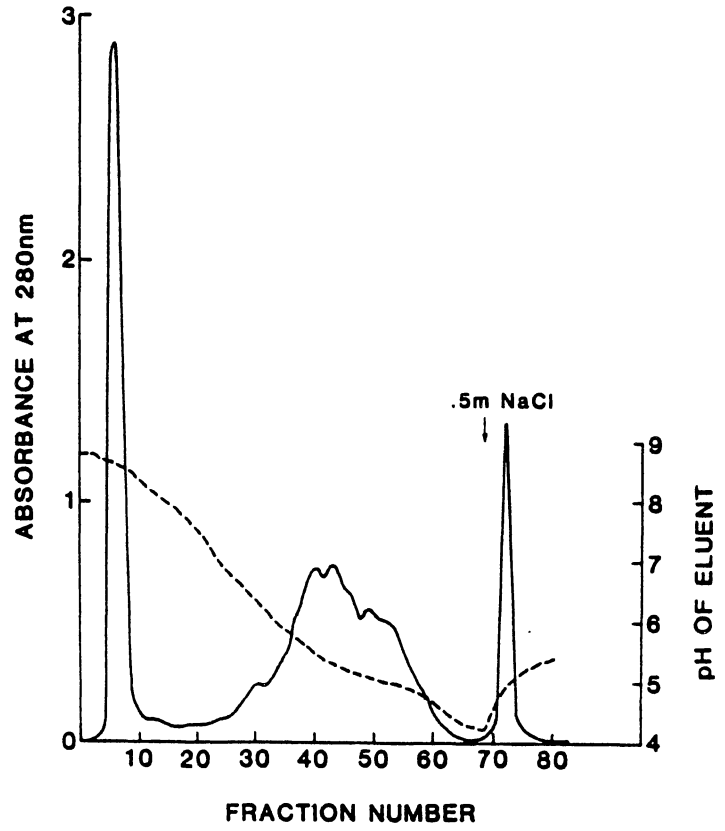

MEAN COUNTS PER 4 MINUTES
1600
1400
1200
1000
800
600
10 20 30 40 50 60 70 80 90 100 110 120
FRACTION NUMBER

Figure 6 Gel Filtration Cytotoxin - Chromatofocusing.
Absorbance of fractions obtained by chromatofocusing of Region 2 gel filtration fractions 35-70.

Figure 7 Gel Filtration Cytotoxin - Cytotoxicity.
^{51}Cr release assay of fractions obtained by chromatofocusing of Region 2 gel filtration fractions 35-70.


ABSORBANCE AT 280nm
3
2
1
0
.5m NaCl
pH OF ELUENT
9
8
7
6
5
4
10 20 30 40 50 60 70 80
FRACTION NUMBER

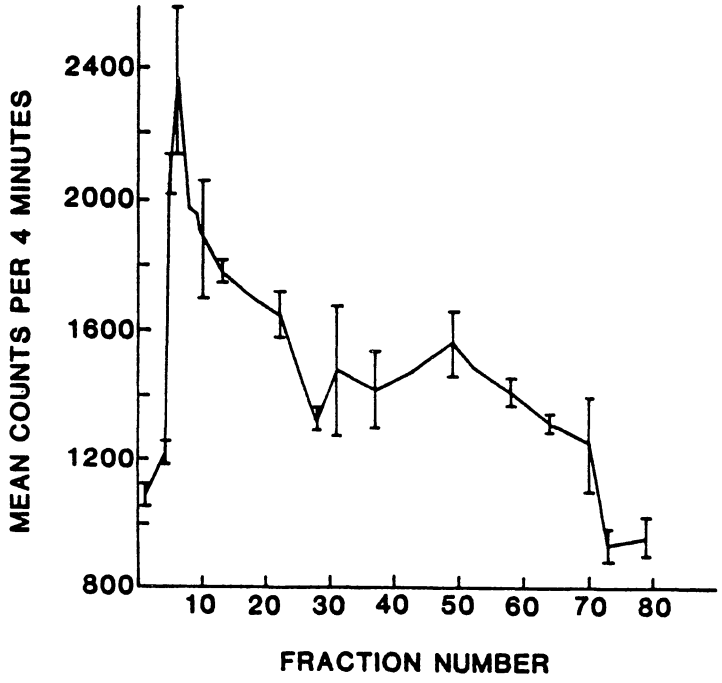

MEAN COUNTS PER 4 MINUTES
2400
2000
1600
1200
800
10 20 30 40 50 60 70 80
FRACTION NUMBER

TABLE II

CYTOTOXIN PURIFICATION PROCEDURE: SUMMARY

	Total ml	Protein mg/ml	Total Protein mg	Cytotoxin units /ml	Cytotoxin units /mg	Total Cytotoxin units	Cytotoxin Purification	Percent Cytotoxin Recovery
Crude CF	346	120	41,520	2,515	20.96	870,190	100	100
Ion Exchange								
Region 1	47	160	7,520	455	2.84	21,360	0.135	
Region 2	50	266	13,300	10,265	38.59	513,250	1.84	
								61.4
Gel Filtration								
Region 1	127	55	6,985	310	5.64	39,370	0.269	
Region 2	276	11.9	3,284	395	33.19	109,020	0.583	
								17.1
Chromatofocusing								
Region 2	94	14.8	1,391	120	8.11	11,280	0.387	
								1.3

Low Molecular Weight Cytotoxin

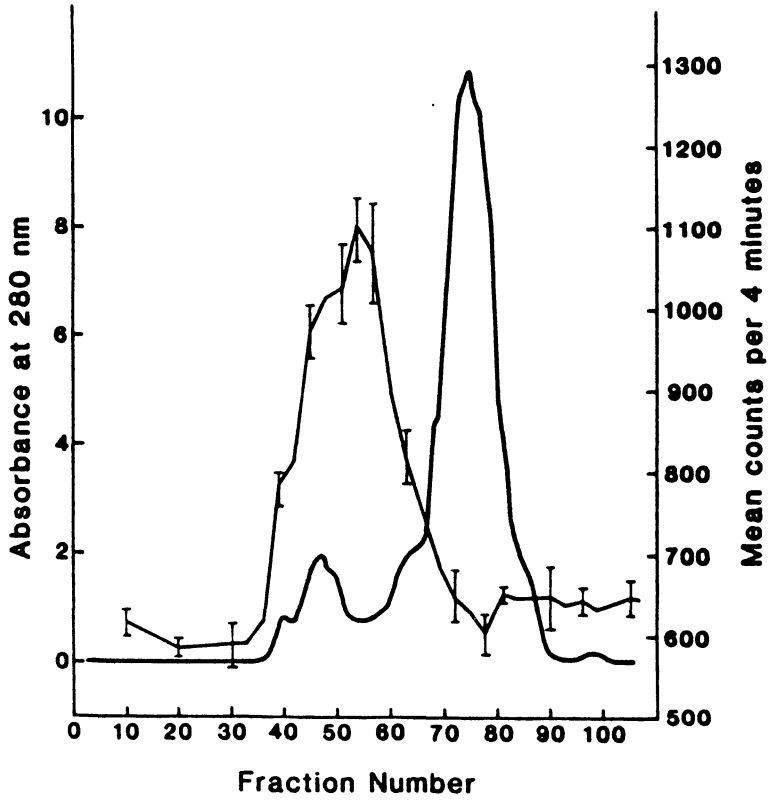
To further investigate the occasional occurrence of low molecular weight cytotoxic fractions in gel filtration experiments, a pooled and concentrated sample of anion exchange fractions from Region 2 was split into two portions. One sample was analyzed by gel filtration immediately and yielded absorbance and cytotoxicity profiles similar to those described previously (Figure 8). The second sample was allowed to age for 3 weeks at 4 C prior to application to the column. The aged sample yielded an absorbance and cytoxicity profile similar to the fresh sample, except that an additional moderately sized cytotoxic peak eluted at a position corresponding to a molecular weight of approximately 30 kDa (Figure 9). By ELISA, increased absorbance was not associated with this small molecular weight peak.

Cytotoxic Activity

Two types of titration curves were obtained during the determination of the cytotoxic activity present in each of the major separated, pooled cytotoxic regions (Figure 10). Material from early in the separation process usually showed an abrupt decline in cytotoxicity at low (1:2 and 1:4) dilutions, a plateau region, and a linear decline in cytotoxicity at high dilutions (>1:64). Titration curves of more purified material obtained later in the process generally exhibited linear declines in cytotoxicity which reached baseline values at high dilutions. The intensity of Region 2 cytotoxicity seemed to decline with time, but was easily detected throughout the separation process.

Figure 8 Region 2 Material (Fresh) - Gel Filtration Chromatography and Cytotoxicity. Absorbance (——) and ^{51}Cr release assay (-I-) profiles of fractions obtained from gel filtration of Region 2 Material. Region 2 Material was subjected to gel filtration immediately after it was obtained from anion exchange experiments.

Figure 9 Region 2 Material (Aged) - Gel Filtration Chromatography and Cytotoxicity. Absorbance (——) and ^{51}Cr release assay (-I-) profiles of fractions obtained from gel filtration of Region 2 Material. Region 2 Material was subjected to gel filtration 3 weeks after it was obtained from anion exchange experiments.


Absorbance at 280 nm
Mean counts per 4 minutes
Fraction Number
0
2
4
6
8
10
500
600
700
800
900
1000
1100
1200
1300
0 10 20 30 40 50 60 70 80 90 100

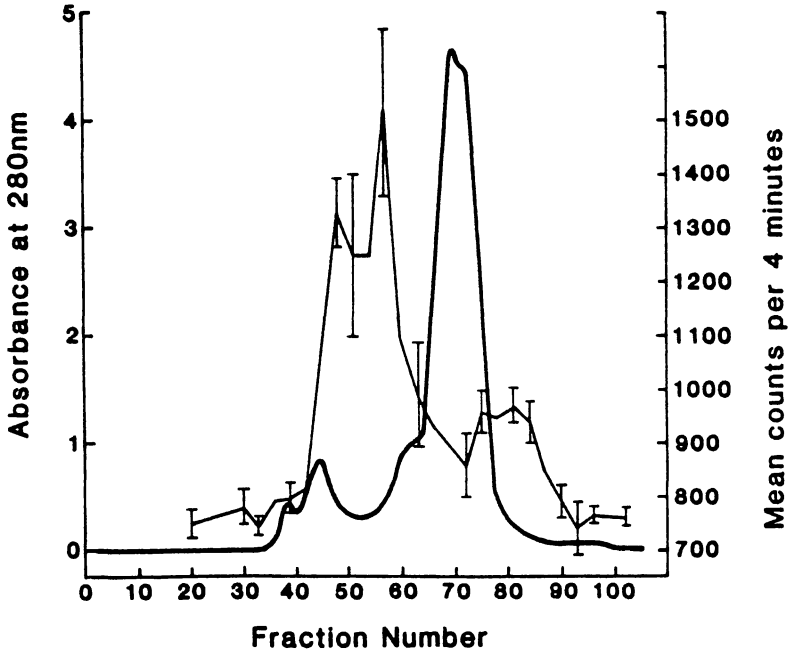
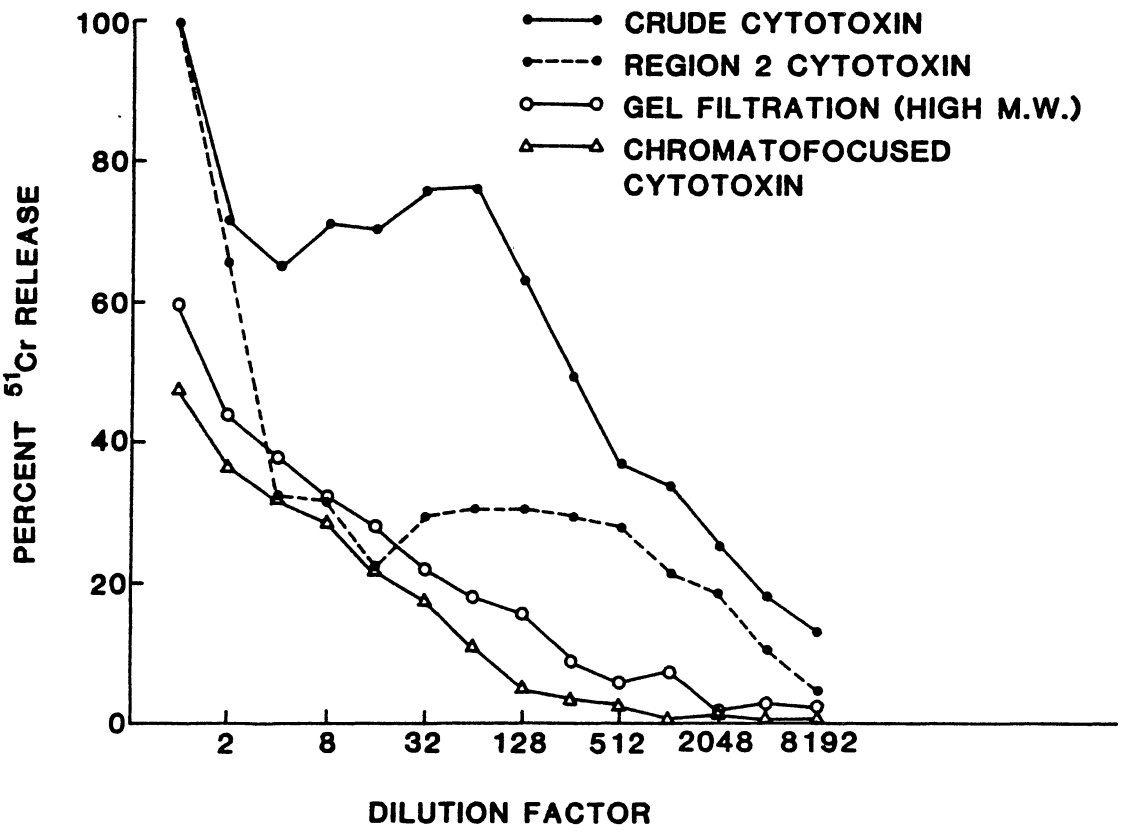

Absorbance at 280nm
Mean counts per 4 minutes
Fraction Number
0
1
2
3
4
5
700
800
900
1000
1100
1200
1300
1400
1500
0 10 20 30 40 50 60 70 80 90 100

Figure 10 Titration of Major Cytotoxic Regions.
^{51}Cr release assays of serial dilutions of cytotoxin preparations obtained at major steps in the purification procedure. Gel filtration (High M.W.) represents fractions 35-70 obtained from gel filtration of Region 2 Material. Chromatofocused cytotoxin is the final, purified cytotoxin.


CRUDE CYTOTOXIN
REGION 2 CYTOTOXIN
GEL FILTRATION (HIGH M.W.)
CHROMATOFOCUSED CYTOTOXIN
PERCENT 51Cr RELEASE
100
80
60
40
20
0
2
8
32
128
512
2048
8192
DILUTION FACTOR

Immunologic Assays

The ELISA used for detection of antigen associated with live *P. haemolytica* consistently demonstrated increased absorbance associated with the 160 kDa cytotoxic fractions obtained from gel filtration experiments.

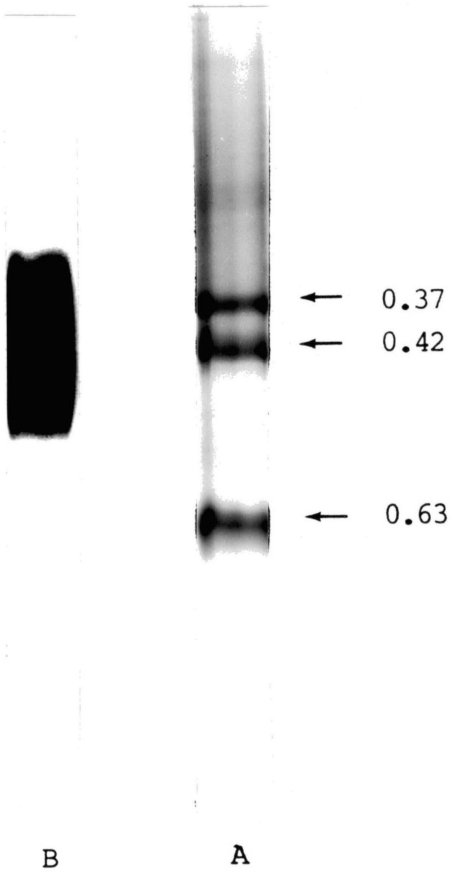
The ELISA used for detection of antibodies to purified cytotoxin in rabbit and mouse sera showed higher A_{405} values for sera obtained after inoculation compared to pre-inoculation sera. The mean pre-inoculation A_{405} in rabbits was 0.172 ± 0.010. Sera obtained following three inoculations had a mean A_{405} value of 1.668 ± 0.071. In mice, the mean pre-inoculation A_{405} was 0.162 ± 0.015. Sera obtained following three inoculations had a mean A_{405} value of 0.870 ± 0.098.

Immunodiffusion tests using rabbit serum demonstrated single lines of precipitation between the serum and all cytotoxin dilutions. In mice, two lines of precipitation were obtained between the serum and each cytotoxin dilution.

Electrophoresis and Immunoblotting

Non-denaturing PAGE of the purified material from Region 2 resulted in 3 protein bands (Figure 11). A major band occurred with a relative mobility (R_f) of 0.63. Two other bands migrated closely together with relative mobilities of 0.37 and 0.42. When BSA was electrophoresed in the same system, a mobility of 0.63 was obtained for the monomer. Immunoblotting with adsorbed sera resulted in a strong binding of antibody in association with the R_f 0.37 and 0.42 bands. No reaction occurred with the R_f=0.63 band.

Figure 11 Purified Cytotoxin - PAGE and Immunoblotting. PAGE (A) and immunoblot (B). Arrows represent molecular weights.


0.37
0.42
0.63
B
A

SDS-PAGE of the same material after complete denaturation and reduction resulted in the identification of four primary protein bands after staining with Coomasie (Figure 12). The first was a broad, faint band with an apparent molecular weight corresponding to 160 kDa. Two intensely stained bands were present having molecular weights of 66 kDa and 57 kDa, respectively. A faint, but very sharp band was also present at approximately 23 kDa. Several faint, indistinct bands were scattered among these major bands. Immunoblotting using unadsorbed serum resulted in a smeared zone of antibody binding from 160 to 80 kDa, with dark, distinct bands visible at 66 kDa and 57 kDa. A very faint reaction occurred at 23 kDa. In contrast, immunoblotting using the more specific adsorbed sera yielded two strong bands at 66 and 57 kDa (Figure 12). Only faint, indistinct background reactions were observed from 50 to 100 kDa. No reaction occurred with the 160 kDa band with either adsorbed or non-adsorbed sera.

SDS-PAGE of pooled fractions from the high molecular weight and low molecular weight regions of cytotoxicity obtained from gel filtration of fresh and aged Region 2 material is shown in Figure 13. Pooled samples of the material eluting between fractions 45 - 60 contained the four major protein bands previously described. There was a shift of staining intensity, however, from the 160 kDa band to the 57 kDa band in the aged sample relative to the fresh one. In the material eluting between fractions 70 - 85, bands common to both samples were present at 75, 66, 57, and 14 kDa. Bands at 45 kDa and 54 kDa were present in the aged sample, but not clearly distinguishable in the fresh one. The fresh sample, however, contained a substantially larger, more intense band at 57 kDa than that for the aged sample.

Figure 12 Purified Cytotoxin - SDS-PAGE and Immunoblotting. SDS-PAGE (A), and immunoblot (B). Arrows represent molecular weights.

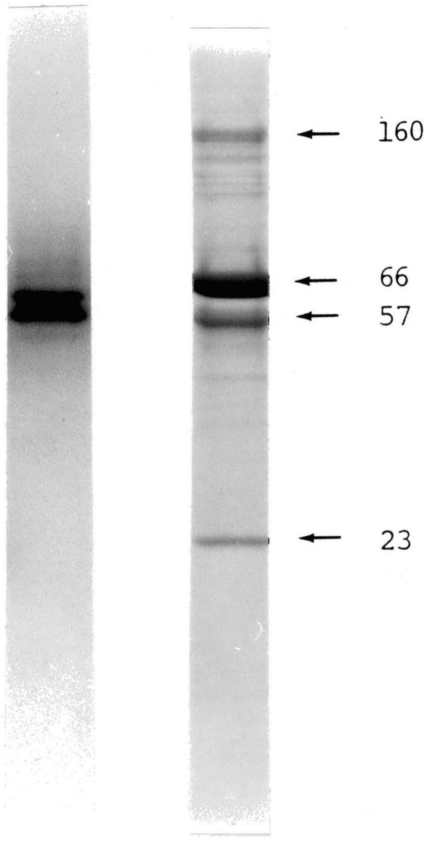
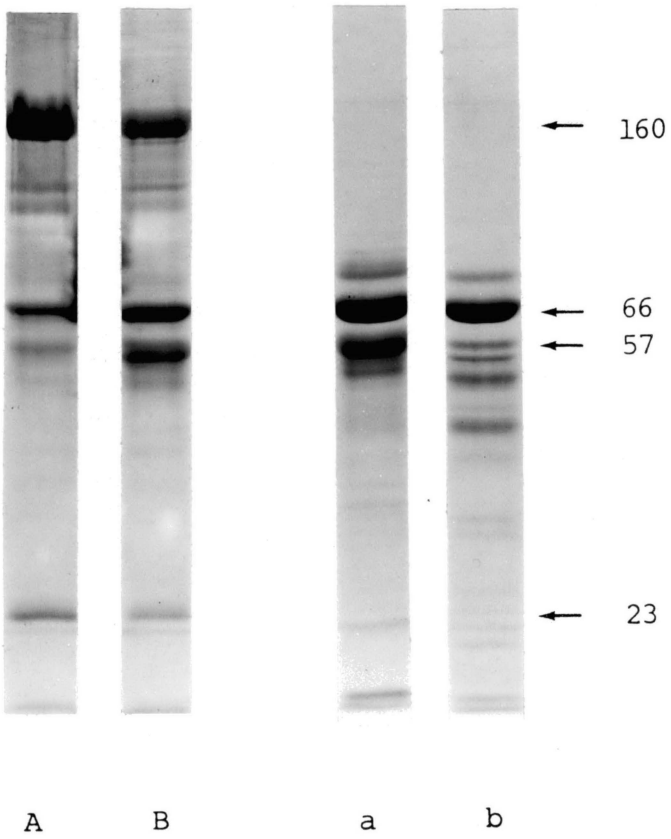

160
66
57
23
Б
А

Figure 13 Region 2 Material (Fresh and Aged) - SDS-PAGE and Immunoblotting. Fresh (A) and Aged (B) high molecular weight proteins (gel filtration fractions 35-70)。 Fresh (a) and aged (b) low molecular weight proteins (gel filtration fractions 90-110。


160
66
57
23
A
B
a
b

Immunoblotting of the high molecular weight material from both the fresh and aged samples with unadsorbed serum gave distinct bands at 66, 57, and 23 kDa. Lightly stained background reactions were present from 160 to 80 kDa. No antigenic bands were present with the low molecular weight material from either sample.

Isoelectrofocusing

Isoelectrofocusing resulted in the separation of purified cytotoxin into several prominent protein bands and some faint, less prominent protein bands. These bands migrated closely together and had isoelectric points ranging from 4.6-5.2.

Discussion

The results of these experiments have demonstrated a straightforward procedure for obtaining relatively purified P. haemolytica cytotoxin. The procedure requires three chromatographic steps and successfully removes a large portion of the BSA and other non-bacterial serum proteins which are necessary for efficient cytotoxin production. The final product, though not biochemically pure as determined by electrophoresis, retains its cytotoxic and immunologic properties, thus allowing further investigation of these characteristics.

On the basis of these studies native P. haemolytica cytotoxin appears to be a 160 kDa protein. In non-denaturing PAGE, cytotoxin separated into 2 protein bands which were associated with a broad region that bound antibodies present in adsorbed serum. A third band appeared to be BSA. Following denaturation, four distinct protein

bands were present in SDS-PAGE (160, 66, 57, and 23 kDa). Two of these (66 and 57 kDa), reacted with antibodies in adsorbed sera. These results suggest that cytotoxin is composed of subunits or is a single polypeptide which can undergo intrachain cleavage.

The results of ^{51}Cr assays of Region 2 gel filtration fractions demonstrated the consistent presence of cytotoxic activity associated with the native 160 kDa protein. In addition an approximately 30 kDa cytotoxic, but immunologically non-reactive substance, was found in occasional preparations. The incidence of this low molecular weight cytotoxic material increased in aged samples and appeared to occur as a result of degradation of the 160 kDa cytotoxin. The breakdown of the 160 kDa cytotoxin into smaller cytotoxic components under the mild conditions of this experiment suggest either an inherent instability of the molecule or the possible presence of proteolytic substances in Region 2 material. A neutral protease produced by P. haemolytica has been reported and could be at least partially responsible for this degradative process (Otulakowski et al., 1983). From the available evidence, it is not clear whether the low molecular weight cytotoxic material and the immunologically non-reactive 23 kDa band detected on SDS-PAGE are related.

Himmel et al. (1982) reported the isolation of an immunogenic and toxic 150 kDa protein from P. haemolytica in a dialysis culture system with brain-heart infusion and calf serum growth medium. This protein migrated as a single band on PAGE and was reduced to a 50 kDa and a 20 kDa band with SDS-PAGE. In some experiments where aged cells were used to inoculate the growth medium the 150 kDa protein was replaced by a highly immunogenic 50 kDa protein. Double immunodiffusion studies of

the 150 kDa and 50 kDa antigens demonstrated common antigenic determinants. These authors suggested that the 50 kDa antigen was a subunit of the 150 kDa protein.

The results of the current investigation are similar to those described above, except that an additional 66 kDa protein was detected. On the basis of molecular weight, this protein may have represented BSA. However, immunoblots of native cytotoxin separated by SDS-PAGE which were developed with adsorbed sera produced a strong reaction with this 66 kDa protein. In contrast, immunoblots of BSA separated by PAGE and developed with the same sera failed to result in any antibody binding. These findings suggest that the 66 kDa protein represents either a distinct component of cytotoxin, or may represent a complex of BSA with a small molecular weight fragment of cytotoxin. The 66 kDa antigen could also represent an intermediate form of one or more of the final cytotoxin breakdown components.

The 66, 57, and 23 kDa proteins obtained in this study appear to be distinct from the 42 and 30 kDa outer membrane proteins which have been extracted from P. haemolytica (Squire et al., 1984). Immunoblotting with hyperimmune anti-P. haemolytica serum of SDS-PAGE preparations of proteins separated from a saline extract of P. haemolytica resulted in the demonstration of 10 distinct antigens (Lessley et al., 1985). Included among these antigens were common bands at 62 and 24 kDa as well as a unique 56 kDa band which was present in one of the proteins. Whether any of these are related to the protein components isolated from cytotoxin is unknown. The saline extracts from which these antigens were obtained were not toxic to bovine neutrophils (Gentry, 1984).

Certain properties of various cytotoxin preparations have been described (Baluyut et al., 1981; Himmel et al., 1982), but the precise range of biochemical parameters necessary for cytotoxicity, immunogenicity, and many general characteristics of cytotoxin are still unclear. During the course of chromatographic experiments in this study several features of cytotoxin were observed. Cytotoxin retained detectable activity within an approximate pH range of 4 to 9. Previous reports demonstrated considerable losses of activity at pH 3 to 6, with moderate losses at pH 9 and 10 (Baluyut et al., 1981).

In the current study, high phosphate concentrations seemed to have a detrimental, but reversible effect on the cytotoxic activity of partially purified cytotoxin. Subsequent preliminary experiments evaluating the effect of various divalent cations on cytotoxicity failed to show any obvious effects (unpublished data). The presence of certain metal chelators, however, did seem to enhance cytotoxicity in some cases. The significance of these findings is unclear, but might indicate a certain sensitivity of cytotoxin to increases in ionic strength or reduction of divalent cation concentrations.

While native cytotoxin tends to form fairly distinct protein bands on PAGE, immunoblots using adsorbed sera demonstrated antibody binding over a broad area associated with these bands. This suggests that native cytotoxin may be heterogeneous in charge or molecular weight, possibly due to a high carbohydrate content. A heterogeneous charge is also suggested by the relatively wide range of ionic strengths over which cytotoxin eluted on anion exchange chromatography. The isoelectric point of purified cytotoxin was found to range between 4.6-

5.2. This is in contrast to the previously estimated value of 9.7 (Himmel et al., 1982).

Purified cytotoxin was found to be immunogenic in both rabbits and mice. Rabbits recognized a single antigen, whereas mice reacted to two distinct antigens.

A major problem encountered throughout these experiments was the progressive loss of cytotoxic activity as the purification process progressed. Some of this loss may have occurred due to physical absorption of cytotoxin to membranes used during concentration. It seems likely, however, that at least part of this loss resulted from degradation or alteration of cytotoxin. Similar declines in cytotoxicity with increased purification have been previously seen and attributed to the variability in cells used for the cytotoxicity assay and the presence of additional growth factors in less purified cytotoxic samples (Himmel et al., 1982). The cause of the rapid loss of cytotoxic activity in Region 1 material is unknown. Gel filtration showed it to be 160 kDa in size, but further analysis is necessary to determine if it is the same as the Region 2 cytotoxin or represents a different substance.

The shape of titration curves of pooled cytotoxic fractions obtained at each step of the separation procedure seemed to be dependent upon the protein concentration of the sample. Early samples containing large amounts of protein demonstrated prominent plateau regions whereby continued dilution had no effect on cytotoxicity. At higher dilutions, linear declines in cytotoxicity occurred. More purified samples, having lower protein concentrations, had rapid linear decreases in cytotoxicity with each increasing dilution. These results

suggest a possible buffering or stabilizing role for protein (mainly BSA) on the *in vitro* biological activity of cytotoxin. Possible mechanisms for this effect might be through protection of cytotoxin against proteolytic activity or stabilization of the basic structure of cytotoxin. Another possibility is that certain substances essential for the expression of cytotoxicity are removed during purification resulting in more rapid loss of activity with dilution.

In conclusion, the result of this study was the acquisition of mg quantities of a partially purified *P. haemolytica* cytotoxin which retained its immunogenic and toxic properties. Biological characteristics of the cytotoxin were demonstrated by analysis and titration of various chromatographed fractions derived from crude cytotoxin. The immunogenicity of cytotoxin and its components was demonstrated by immunoblotting, animal inoculation studies, ELISA, and immunodiffusion. Additional procedures are necessary, however, to obtain biochemically purified cytotoxin which still retains its biological and immunological properties. The precise role cytotoxin plays in pneumonic pasteurellosis will remain unclear until adequate amounts of relatively pure cytotoxin become available for continued *in vitro* use as well as for *in vivo* experimentation and vaccination studies.

Chapter III

SERUM ANTIBODY RESPONSE TO CYTOTOXIN

Introduction

Bovine pneumonic pasteurellosis (shipping fever) is a severe fibrinous pneumonia of feedlot cattle associated with infection by Pasteurella haemolytica biotype A serotype 1 (Collier, 1968; Lillie, 1974; Jensen et al., 1976). Various approaches used to try to prevent the disease have proven largely unsuccessful such that pneumonic pasteurellosis continues to be a major problem to the feedlot industry (Jensen et al., 1976; Martin et al., 1980).

Recent studies have concentrated on defining the role played by humoral immunity in resistance to shipping fever. Clinical use of P. haemolytica bacterins has not proven effective (Amstutz et al., 1981; Martin, 1983; Morter et al., 1982). Cattle given bacterins developed an antibody response to somatic antigens, but this response did not consistently result in protection against challenge by P. haemolytica (Confer et al., 1985a; Gentry et al., 1985). Live P. haemolytica vaccines given by aerosol or parenteral routes resulted in enhanced resistance to experimental challenge (Panciera et al., 1984). Similarly, enhanced resistance to challenge was noted in calves having had prior natural exposure to the organism (Confer et al., 1984b). Further studies using live P. haemolytica vaccines suggested a possible protective role for capsular antigens (Confer et al., 1984a). Several

studies have indicated that neutralizing antibody to P. haemolytica cytotoxin may play a role in increased protection against pneumonic pasteurellosis (Cho et al., 1984; Shewen and Wilkie, 1983; Gentry et al., 1985).

Cytotoxin is an approximately 160 kDa protein which has been incriminated in the pathogenesis of pneumonic pasteurellosis through its toxicity to ruminant alveolar macrophages and peripheral blood leukocytes (Baluyut et al., 1981; Shewen and Wilkie, 1982). Antibody to cytotoxin has previously been measured only by labor intensive cytotoxin neutralization assays. The purpose of this study was to develop an enzyme-linked immunosorbent assay (ELISA) to detect serum antibody to purified cytotoxin, to compare antibody responses detected by ELISA to those detected by neutralization assays, and to determine the ELISA's potential as a predictor of resistance to experimental pneumonic pasteurellosis.

Materials and Methods

Serum Samples

Sera were obtained from 5-8 month old calves which had been used in previous experiments evaluating the effect of various forms of vaccination on resistance to pneumonic pasteurellosis. (Confer et al., 1985a; Confer et al., 1984b; Panciera et al., 1984). Each calf was vaccinated subcutaneously with two doses of either PBS, P. haemolytica bacterin (aluminum hydroxide adjuvant), or 5×10^9 colony forming units (CFU) of live P. haemolytica (Corstvet et al., 1978; Panciera et al., 1984; Confer et al.; 1985a). Vaccinations were given on days 0

and 7. Serum samples were collected on days 0,7,14, and 21 and stored at -70° C. On day 21, calves were experimentally exposed by transthoracic injection with 5 ml of P. haemolytica containing approximately 1 x 10^9 CFU/ml (Panciera and Corstvet, 1984). On day 25, calves were sacrificed and lung lesions were evaluated to determine the extent of lung resistance to challenge exposure (Panciera et al, 1984). Numerical scores were awarded on the basis of morphologic criteria which included the size of the lesion and the degree of extension of inflammation from the original lesion site. A maximum score of 20 represented a severe lesion and lack of resistance whereas lower scores corresponded to increased resistance. A total of 12 calves from each vaccination group were used in the study.

Serologic Evaluation

Antibodies to somatic antigens of P. haemolytica were determined by a quantitative fluorometric immunoassay (FIAX, International Diagnostic Technology, Santa Clara, Calif.) using formalin-killed 22 hour cultures of P. haemolytica serotype 1 as the antigen. (Confer et al., 1983).

Serum cytotoxin neutralization titers were determined as previously described (Cho et al., 1984; Shewen and Wilkie, 1983; Gentry et al., 1985). In general, these determinations were made by preincubation of serial two-fold dilutions of test sera with crude cytotoxin followed by cytotoxicity assays using ^{51} Cr release from bovine leukocytes to measure cell viability.

Anticytotoxin ELISA

The antigen used in the ELISA was a partially purified cytotoxic protein obtained from supernatants of logarithmic- phase cultures of P. haemolytica. The protein was separated from crude P. haemolytica supernatants by anion exchange chromatography, gel filtration, and chromatofocusing as previously described (Chapter II), and was designated as purified cytotoxin. SDS-PAGE and immunoblotting of purified cytotoxin demonstrated two antigenic bands when reacted with antisera to live P. haemolytica which had been adsorbed with formalin-killed bacteria to remove antibody to capsular and somatic antigens.

Optimal conditions for the ELISA were determined from preliminary experiments using antigen coating concentrations of 0.01 ug, 0.1 ug, and 1 ug per 100 ul, serum dilutions from 1:50 to 1:1600, and conjugate dilutions of 1:200 and 1:400.

For routine assays, 100 ul of antigen diluted to 10 ug/ml in carbonate buffer, pH 9.6, was placed in each well of 96-well polystyrene plates (Nunc, Denmark) and incubated overnight at room temperature on a rocker platform. Plates were washed 3 times with PBS containing 0.05% Tween 20 (PBS-Tween). For each serum sample tested, 100 ul of a 1:400 dilution in PBS-Tween containing 1% bovine serum albumin (BSA) was added to duplicate wells and incubated for 1 hour. Following 3 washes with PBS-Tween, 100 ul of a 1:200 dilution of horseradish peroxidase conjugated, affinity purified anti-bovine IgG in PBS-Tween-1% BSA was placed in each well. After a 1 hour incubation, the plates were washed 6 times with PBS-Tween. For color development, 100 ul of substrate, consisting of of 0-phenylenediamine

and hydrogen peroxide in phosphate-citric acid buffer was added to each well and the plates were incubated for 45 minutes in the dark. The reaction was stopped by addition of 50 ul of 2.5M H_2SO_4, to each well and the absorbance at 490 nm (A_{490}) was determined on a manual ELISA reader (Bio-Tek, Burlington, Vermont). The reported A_{490} for each sample was the average of duplicate wells. To compare samples from different plates, A_{490} readings were standardized on the basis of a positive control (sera from a steer hyperimmunized with live P. haemolytica) and a negative control (PBS) which were determined on each plate.

Adsorption Experiments

Selected sera were used to study the specificity of the ELISA by preadsorption of the sera with several P. haemolytica antigens. For each sample, 100 ul of serum was diluted 1:200 in PBS-Tween-1% BSA and then incubated with no equal volume of either purified cytotoxin, a saline extract of P. haemolytica (Gentry et al., 1982), formalin-killed P. haemolytica, or PBS. Cytotoxin and capsular extract were used in concentrations of 1000 ug, 100 ug, 10 ug, and 1 ug per ml, whereas formalin-killed bacteria were used at 10^{10}, 10^9, 10^8, and 10^7 CFU equivalents as determined spectrophotometrically (Confer et al., 1983). Mixtures were incubated overnight at 4° C on a rocker platform in 96 well round bottom tissue culture plates (Corning). Adsorbed sera were then assayed using the ELISA procedure as previously described.

Statistical Evaluation

The antibody responses to cytotoxin determined using ELISA were

compared to FIAX titers, cytotoxin neutralization titers, and lesion scores by multiple linear regression analysis (Statistical Analysis System, Cary, N. C.). Group means for the different vaccinates were evaluated by analysis of variance and least significant differences.

Results

Serial two-fold dilutions of sera from 1:50 to 1:1600 gave absorbance readings typical of an antibody response curve (Figure 14). Preadsorbtion of sera with PBS or various concentrations of a saline extract of P. haemolytica and formalin-killed P. haemolytica had no effect on cytotoxin antibody response (Figure 15). Preadsorption of the same sera with cytotoxin, however, resulted in dramatic decreases in cytotoxin antibody response at the two higher concentrations of cytotoxin.

In calves vaccinated with live P. haemolytica, there was a marked increase in ELISA -detected antibody to cytotoxin (ELISA cytotoxin antibody) during the period from day 0 to 21 (Figure 16). During the same period, the bacterin group had an insignificant increase in cytotoxin antibody response, whereas the ELISA cytotoxin antibody response for the PBS group remained relatively constant. From day 7 to 21, a significant difference was seen between the responses of the live-vaccinates compared to both the bacterin and PBS groups. No significant difference was present between bacterin and PBS groups on any day.

The means and standard deviations for ELISA cytotoxin antibody responses, cytotoxin neutralizing titers, FIAX titers, and lesion scores for each vaccine group are given in Table III. Analysis of

Figure 14 Serum Cytotoxin Antibody ELISA - Serum Dilution Response. Each line represents doubling dilutions of different bovine sera tested in the ELISA.

ELISA - Serum Dilution Response

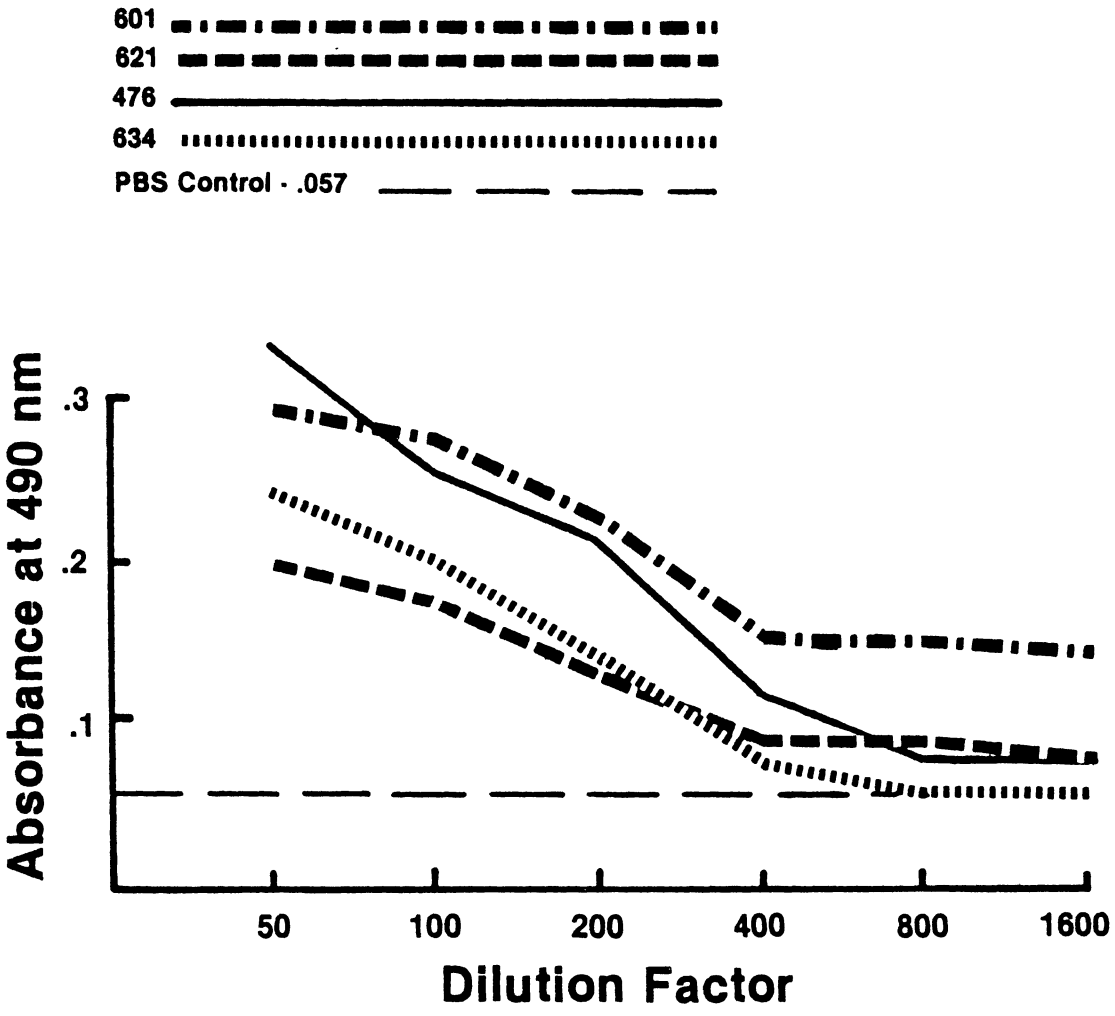


Figure 15 Serum Cytotoxin Antibody ELISA - Antigen Specificity. Increasing concentrations of cytotoxin and capsular extract and increasing numbers of formalized bacteria were absorbed with sera containing anticytotoxin antibody. These sera and unabsorbed sera were run in the ELISA.

ELISA - Antigen Specificity

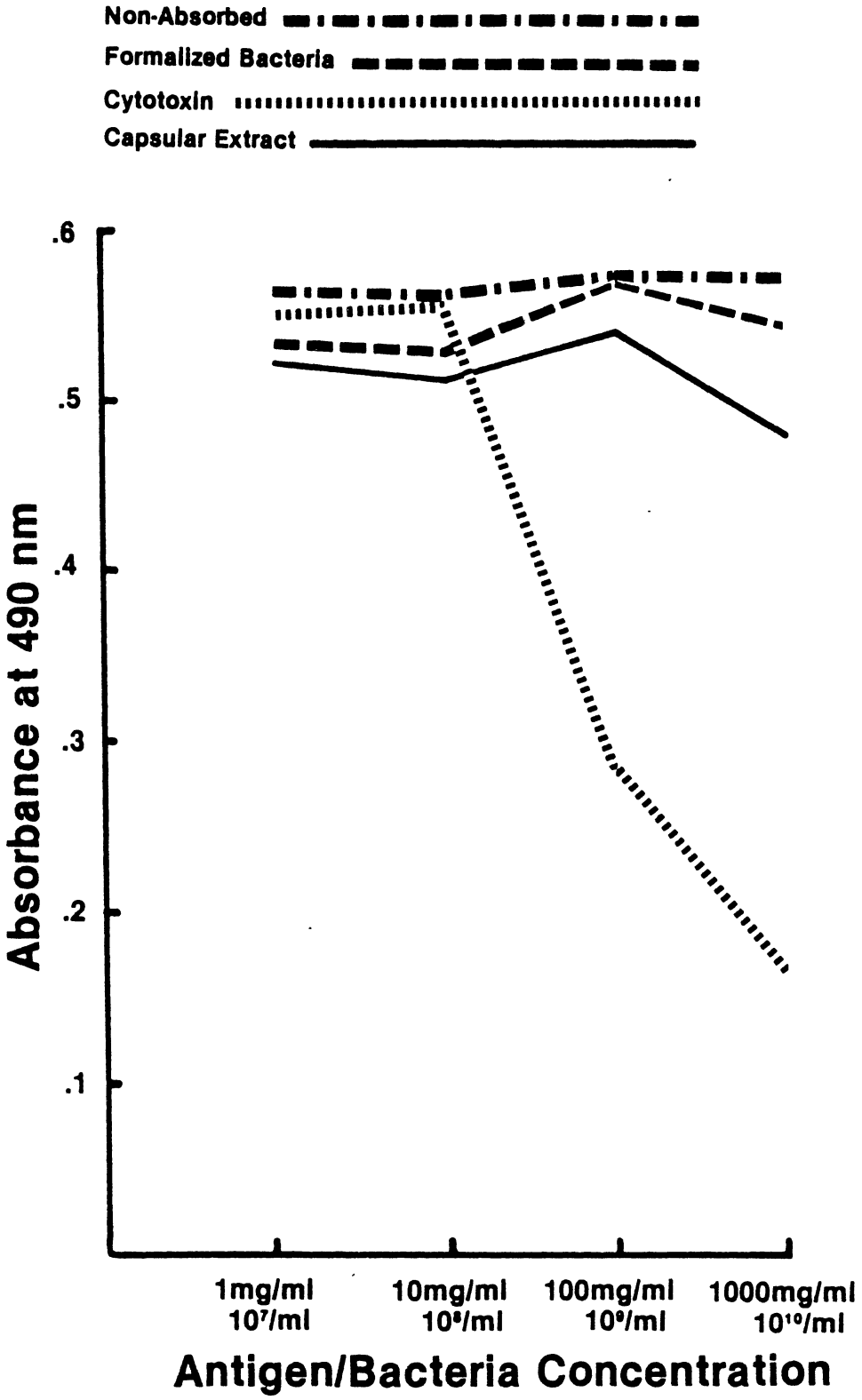


Figure 16 Serum Cytotoxin Antibody ELISA - Vaccination Response. Live received live *P. haemolytica* subcutaneously, bacterin received formalin-killed *P. haemolytica* subcutaneously, and PBS received PBS subcutaneously (controls).

ELISA - Vaccination Response

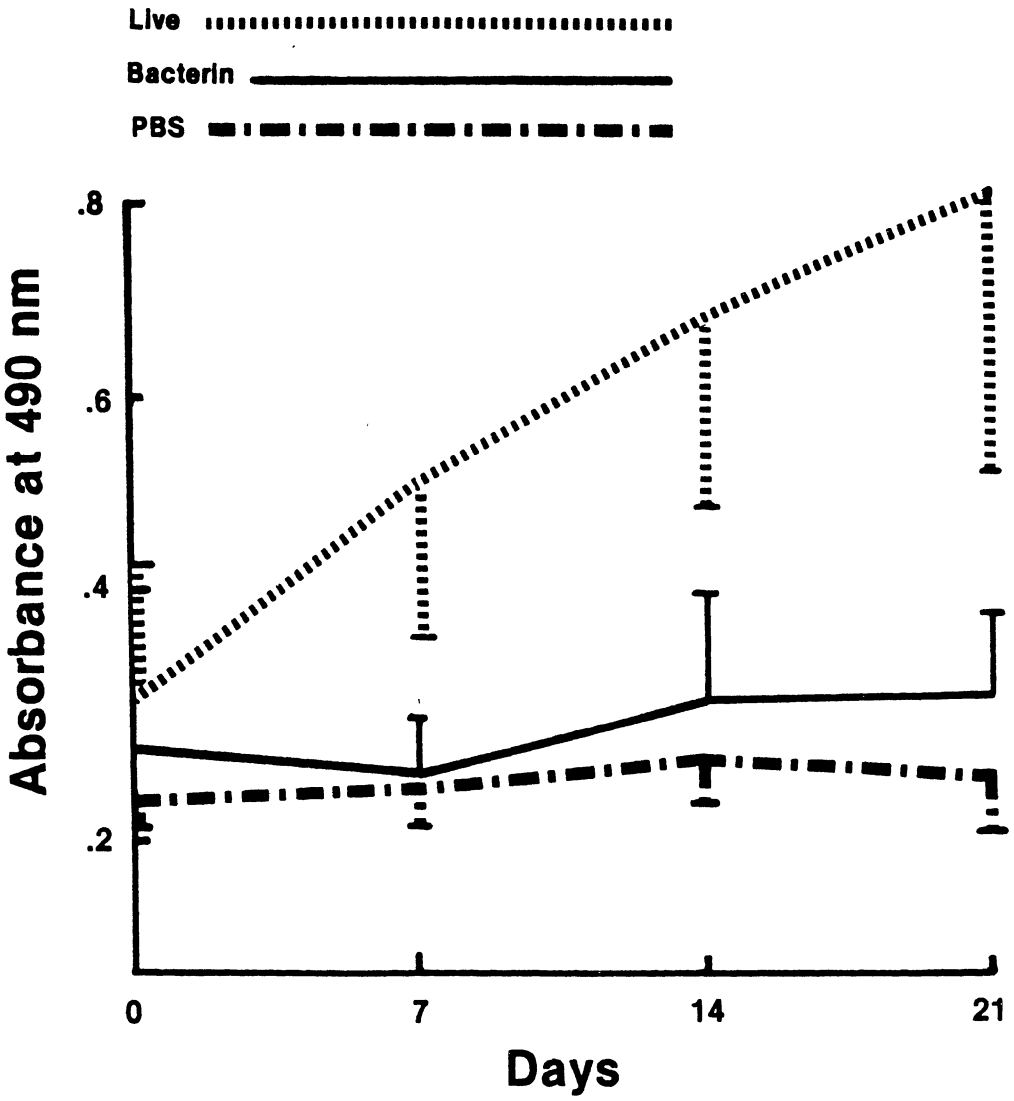


TABLE III

EFFECT OF VACCINATION ON SEROLOGIC PARAMETERS AND LESION SCORES

	PBS Vaccinates		Bacterin Vaccinates		Live Vaccinates	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
ELISA (A_{490})	0.39	0.18	0.30	0.10	0.70	0.22
Cytotoxin neutralization titer	6.4	5.1	8.5	5.2	88.0	36.4
FIAX titer	28.5	15.1	102.7	88.1	173.2	46.7
Lesion score	16.9	4.5	10.3	5.4	3.7	2.5

variance between these three groups showed a significant difference ($p < 0.0001$) between the means regardless of which of the four parameters was used as the dependent variable. The results of least significant difference analysis between groups for each parameter indicated that ELISA cytotoxin antibody responses and cytotoxin neutralizing titers for live vaccinates were signficantly different ($p < 0.05$) from both the bacterin and PBS groups. Significant differences were not detected between bacterin and PBS vaccinated mean responses. For FIAX titers and lesion scores, a significant difference ($p < 0.05$) was present between the means of all three groups.

Correlation coefficients (r) and probabilities (p) for all samples organized by parameter are given in Table IV. Directly measured values were used for comparisons of all parameters except the cytotoxin neutralization titer, which was transformed to the logarithm of the actual value. Correlation of ELISA cytotoxin antibody responses with cytotoxin neutralization, FIAX, and lesion score values were all significant at $p<0.01$. The strongest correlation was between ELISA cytotoxin antibody response and cytotoxin neutralization titers, whereas the lowest was between ELISA cytotoxin antibody response and FIAX titers. As an indicator of resistance to pneumonic pasteurellosis the ELISA had a better correlation with lesion score than did the FIAX. Significant correlations were not obtained between any parameters when samples were evaluated within individual vaccine groups.

Discussion

The result of this study was the development of a primary binding immunoassay (ELISA) which is suitable for screening large numbers of

TABLE IV

CORRELATION OF SEROLOGIC PARAMETERS AND LESION SCORES

	Cytotoxin neutralization titer		FIAX titer		Lesion score	
	r	p	r	p	r	p
ELISA (A_{490})	0.675	0.0001	0.425	0.0097	-0.509	0.0015
Cytotoxin neutralization titer	-	-	0.662	0.0001	-0.605	0.0001
FIAX titer	-	-	-	-	-0.473	0.0036

bovine sera for antibody to P. haemolytica cytotoxin. Previously reported ELISA's to P. haemolytica have used either sodium salicylate extracts (Burrells et al., 1979; Donachie et al., 1983), or saline extracts (Confer et al., 1985) of P. haemolytica as antigens. The current report is the first direct binding assay utilizing a purified cytotoxic P. haemolytica antigen. The ELISA results provide similar information to that obtained by cytotoxin neutralization and eliminates the need for serial dilutions of sera, preparation of living cells, and use of radioisotopes.

Preadsorption studies using various P. haemolytica antigens demonstrated the specificity of the ELISA reaction for cytotoxin. When preincubated with high concentrations of cytotoxin, immunoglobulin antigen binding sites specific for cytotoxin apparently became saturated, resulting in large decreases in ELISA absorbance. Similar high concentrations of saline extracts of P. haemolytica and formalin-killed P. haemolytica had only minor effects on absorbance. These results suggest that cytotoxin is not associated with structural components of the bacteria and further supports its classification as a true exotoxin (Shewen and Wilkie, 1985).

Evaluation of the sera of feedlot cattle for the ability to neutralize P. haemolytica cytotoxin demonstrated a signifiantly lower cytotoxin neutralizing activity in the serum of cattle which died of fibrinous pneumonia than in the sera of cattle which died for other reasons (Shewen and Wilkie, 1983). Similar associations between pneumonia and cytotoxin neutralization were found in studies of experimentally induced disease (Cho et al., 1984; Gentry et al., 1985). A direct positive correlation was demonstrated between

resistance to experimental challenge with P. haemolytica and serum cytotoxin neutralizing titers (Gentry et al., 1985). Antibodies to somatic antigens of P. haemolytica as determined by a modified indirect complement fixation test, however, appeared to be unrelated to the development of pneumonia (Cho et al., 1984).

The results of the current study supports these findings and suggest that exposure to live organisms is necessary to produce an immune response to cytotoxin and that this response is a better predictor of resistance to pneumonia than immune response to somatic antigens. ELISA cytotoxin antibody responses and cytotoxin neutralization titers for live-vaccinates were both significantly higher than those values for the bacterin group. In association with this, mean lesion scores for the live vaccinates were significantly smaller than scores obtained with controls or the group receiving a bacterin.

The relationship between the antigens of P. haemolytica and resistance to disease is becoming more clearly defined. Bacterins stimulate a somatic antibody response which does not consistently provide protection from disease, and in some cases has been incriminated as enhancing disease (Friend et al., 1977; Wilkie et al, 1980). In the current study, there was a significant reduction in the lesion scores of bacterin vaccinated animals compared to controls, however, indicating that some degree of protection was afforded by antibodies to somatic antigens. Animals which received live vaccine and possessed high levels of ELISA and cytotoxin neutralizing antibodies had lesion scores which were significantly lower than the bacterin group. Although additional protection was associated with

increased levels of cytotoxin antibodies, occasional animals with good neutralizing titers or ELISA antibody responses still developed severe pneumonic lesions. The reasons or factors involved in these cases are unknown.

The etiology and pathogenesis of pneumonic pasteurellosis are complex and multifactorial. A successful product for immunologic prevention of the disease will most likely have to take into account multiple antigenic features of infectious agents associated with the disease. The ELISA described in this report provides a helpful tool for more clearly defining the role played by cytotoxin in pneumonic pasteurellosis.

CHAPTER IV

SUMMARY AND CONCLUSIONS

The purpose of this study was to develop a procedure for the purification of Pasteurella haemolytica cytotoxin. Once obtained, purified cytotoxin was partially characterized biochemically and biologically. It was then used in immunologic studies to examine the role cytotoxin plays in the humoral response of the bovine to experimental pneumonic pasteurellosis.

The purification procedure which was developed consisted of a series of three common chromatographic techniques (anion exchange, gel filtration, and chromatofocusing). The procedure was straightforward, reproducible, and yeilded large quantities of relatively pure cytotoxin. Native cytotoxin was found to be a 160 dKa protein. Upon denaturation, this protein was reduced into four predominate protein bands. Bands at 160 kDa and 23 kDa were non-antigenic in the bovine, whereas bands at 57 kDa and 66 kDa proved to be antigenic as determined with immunoblots utilizing bovine anticytotoxin immunoglobulin. Under certain conditions, an approximately 30 kDa protein which was cytotoxic but non-antigenic, was also encountered.

Native cytotoxin was found to be biologically active through a pH range of 4.5-9.0. It was reversibly inactivated by high ionic strength and would undergo degradation with time. As well as being antigenic to the bovine, rabbits and mice also mounted antibody

responses against cytotoxin.

An enzyme-linked immunosorbent assay (ELISA) was developed to measure the bovine serum antibody reponse to cytotoxin. This assay was shown to be highly specific for cytotoxic antigens and yeilded information similar to that obtained by cytotoxin neutralization assays. Cattle vaccinated witth live Pasteurella haemolytica had significantly higher ELISA cytotoxin antibody titers and less severe pneumonic lesions upon experimental challenge than those cattle vaccinated with P. haemolytica bacterins. ELISA cytotoxin antibody titers were more strongly correlated with experimental pneumonic lesions than were titers to somatic antigens of P. haemolytica. These results demonstrated the ELISA to cytotoxin to be a more accurate predictor of resistance to pneumonic pasteurellosis than tests measuring somatic antigens.

The results of this study were three-fold. First, purification and characterization studies helped to more adequately define the nature of P. haemolytica cytotoxin. The purification procedure also allowed a second accomplishment, which was the accumulation of adequate amounts of purified cytotoxin for use in future experiments. Third, immunological studies demonstrated the antigenicity of cytotoxin and the importance of a strong anticytotoxin antibody response for protection against experimental pneumonic pasteurellosis.

Based upon the information obtained during this study, cytotoxin appears to be an important component involved in the pathogenesis of pneumonic pasteurellosis. Future studies should be directed towards additional purification of cytotoxin, development of techniques to yeild large amounts of both pruified cytotoxin and cytotoxin specific

antibodies for use in continued *in vitro* studies, and the use of cytotoxic antigens in *in vivo* vaccination trials.

BIBLIOGRAPHY

Abramson, J. S., G. S. Giebink, E. L. Mills, P. G. Quie. 1981. Polymorphonuclear leukocyte dysfunction during influenza virus infection in chinchillas. J. Infect. Dis. 143:836-845.

Aly, R., H. I. Shinefield, W. G. Strauss, H. I. Maibach. 1977. Bacterial adherence to nasal mucosal cells. Infect. and Immun. 17:546-549.

Amstutz, H. E., L. A. Horstmaan, R. L. Morter. 1981. Clinical evaluation of the efficacy of Haemophilus somnus and Pasteurella sp. bacterins. Bov. Pract. 16:106-108.

Andersen, B. R., J. L. Duncan. 1980. Activation of human neutrophil metabolism by streptolysin O. J. Infect. Dis. 141:680-685.

Andersen, B. R., D. E. van Epps. 1972. Supression of chemotactic activity of human neutrophils by Streptolysin O. J. Infect Dis. 125:353-359.

Andrews, P. 1965. The gel filtration behaviour of proteins related to their molecular weights over a wide range. Biochem. J. 96: 595-605.

Anonymous. 1968. Report of the panal for the symposium on immunity to the bovine respiratory disease complex. J. Am. Vet. Med. Ass. 152:713-719.

Babiuk, L. A., S. D. Acres. 1984. Models for bovine respiratory disease pp. 287-325 in "Bovine Respiratory Disease. A Symposium", ed. R. W. Loan. College Station: Texas A&M University Press.

Baggiolini, M. 1980. The Neutrophil. pp. 163-216 in "The Cell Biology of Inflammation", ed. G. Weismann. New York: Elsiever/North Holland Biomedical Press.

Baldwin, D. E., R. G. Marshall, G. E. Wessman. 1967. Experimental infection of calves with myxovirus parainfluenza-3 and Pasteurella haemolytica. Am. J. Vet. Res. 20:1773-1782.

Baluyut, C. S., R. R. Simonson, W. J. Bemrick, S. K. Maheswaran. 1981. Interaction of Pasteurella haemolytica with bovine neutrophils: Identification and partial characterization of a cytotoxin. Am. J. Vet. Res. 42:1920-1926.

Beachey, E. H. 1981. Bacterial adherence: Adhesin-receptor interactions mediating the attachment of bacteria to mucosal surfaces. J. Infect. Dis. 143:325-345.

Bendixen, P. H., P. E. Shewen, S. Rosendal, B. N. Wilkie. 1981. Toxicity of Haemophilus pleuropneumoniae for porcine lung macrophages, peripheral blood monocytes, and testicular cells. Infect. and Immun. 33:673-676.

Bennett, B. W. 1982. Efficacy of Pasteurella bacterins for yearling feedlot cattle. Bov. Pract. 3:26-30.

Benson, M. L., R. G. Thomson, V. E. O. Valli. 1978. The bovine alveolar macrophage II. In vitro studies with Pasteurella haemolytica. Can. J. Comp. Med. 42:368-369.

Berggren, K. A., C. S. Baluyut, R. R. Simonson, W. F. Bemrick, S. K. Maheswaran. 1981. Cytotoxic effects of Pasteurella haemolytica on bovine neutrophils. Am. J. Vet. Res. 42:1383-1388.

Bradford, M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Campos, M., C. R. Rossi, M. J. P. Lawman. 1982. Natural cell-mediated cytotoxicity of bovine mononuclear cells against virus-infected cells. Infect. and Immun. 36:1054-1059.

Carter, G. R. 1957. A bacterin for the prevention of shipping fever in Canada. Vet. Med. 52:254-255.

Carter, G. R. 1964. Pasteurella haemolytica: An importnt pathogen of sheep and cattle. Vet. Med. 59:722-724.

Cavalieri, S. J., I. S. Snyder. 1982. Effects of Eschericia coli alpha-hemolysin on human peripheral leukocyte function in vitro. Infect. and Immun. 37:966-974.

Cavalieri, S. J., I. S. Snyder. 1982. Effect of Eschericia coli alpha-hemolysin on human peripheral leukocyte viability in vitro. Infect. and Immun. 36:455-461.

Cho, H. J., J. G. Bohac, W. D. G. Yates, H. Bielefeldt-Ohmann. 1984. Anticytotoxin activity of bovine sera and body fluids against Pasteurella haemolytica A1 cytotoxin. Can. J. Comp. Med. 48: 151-155.

Cluff, L. E. 1971. Effects of lipopolysaccharides on susceptibility to infections. pp. 399-413 in "Microbial Toxins", Vol. V, ed. S. Kadis, G. Weinbaum, S. J. Ajl. New York: Academic Press.

Collier, J. R. 1968. Pasteurellae in bovine respiratory disease. J. Am. Vet. Med. Ass. 152:824-828.

Confer, A. W., J. C. Fox, P. R. Newman, G. W. Lawson, R. E. Corstvet. 1983. A quantitative fluorometric assay for the measurement of antibody to Pasteurella haemolytica in cattle. Can. J. Comp. Med. 47:37-42.

Confer, A. W., R. J. Panciera, R. E. Corstvet, F. A. Rummage, R. W. Fulton. 1984a. Bovine pneumonic pasteurellosis: Effect of culture age of Pasteurella haemolytica used as a live vaccine. Am. J. Vet. Res. 45:2543-2545.

Confer, A. W., R. J. Panciera, R. W. Fulton. 1984b. Effect of prior natural exposure to Pasteurella haemolytica on resistance to experimental bovine pneumonic pasteurellosis. Am. J. Vet. Res. 45:2622-2624.

Confer, A. W., R. J. Panciera, R. W. Fulton, M. J. Gentry, J. A. Rummage. 1985a. Effect of vaccination with live or killed Pasteurella haemolytica on resistance to experimental bovine pneumonic pasteurellosis. Am. J. Vet. Res. 46:342-347.

Confer, A. W., B. A. Lessley, R. J. Panciera, R. W. Fulton, J. A. Kreps. 1985b. Serum antibodies to antigens derived from a saline extract of Pasteurella haemolytica: Correlation with resistance to experimental bovine pneumonic pasteurellosis. Submitted for publication.

Coonrod, J. D., K. Yoneda. 1983. Detection and partial characterization of antibacterial factor(s) in alveolar lining material of rats. J. Clin. Invest. 71:129-141.

Corstvet, R. E., R. J. Panciera, H. B. Rinker. 1973. Survey of tracheas of feedlot cattle for Haemophilus somnus and other selected bacteria. J. Am. Vet. Med. Ass. 163:870-873.

Corstvet, R. E., R. J. Panciera, P. Newman. 1978. Vaccination of calves with Pasteurella multocida and Pasteurella haemolytica. Proc. Am. Ass. Vet. Lab. Diagn. 21:67-90.

Corstvet, R. E., M. J. Gentry, P. R. Newman, J. A. Rummage, A. W. Confer. 1982. Demonstration of age-dependent capsular material on Pasteurella haemolytica serotype 1. J. Clin. Micro. 16: 1123-1126.

Couch, R. B. 1981. The effects of influenza on host defenses. J. Infect. Dis. 144:284-291.

Coyle-Dennis, J. E., L. H. Lauerman. 1978. Biological and biochemical characteristics of Fusobacterium necrophorum leukocidin. Am. J. Vet. Res. 39:1790-1793.

Coyle-Dennis, J. E., L. H. Lauerman. 1979. Correlations between leukocidin production and virulence of two isolates of Fusobacterium necrophorum. Am. J. Vet. Res. 40:274-276.

Davies, D. H., L. E. Carmichael. 1973. Role of cell-mediated immunity in the recovery of cattle from primary and recurrent infections with infectious bovine rhinotracheitis virus. Infect. and Immun. 8:510-518.

Davies, P., R. J. Bonney. 1980. The secretion of hydrolytic enzymes by mononuclear phagocytes. pp. 497-542 in "The Cell Biology of Inflammation", ed. G. Weissmann. New York:Elsevier/North Holland Biomedical Press.

Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. 121:404-427.

Densen, P., G. L. Mandell. 1980. Phagocyte stategy vs. Microbial Tactics. Rev. Infect. Dis. 2:817-838.

Donachie, W., C. Burrells, A. McL. Dawson. 1979. Specificity of the enzyme-linked immunosorbent assay (ELISA) for antibodies in the sera of specific pathogen-free lambs vaccinated with Pasteurella haemolytica antigens. Vet. Micro. 8:199-205.

Duncan, J. R., R. G. Thomson. 1970. Influence of immunization procedures on upper respiratory tract immunity in cattle. Can. J. Comp. Med. 34:94-100.

Engelbrecht, H. 1968. Present status of immunization procedures for pasteurellosis. J. Am. Vet. Med. Ass. 152:881-884.

Fales, W. H., J. F. Warner, G. W. Teresa. 1971. Effects of Fusobacterium necrophorum leukotoxin on rabbit peritoneal macrophages in vitro. Am. J. Vet. Res. 38:491-499.

Fauve, R., J. Alouf, A. Delaunay, M. Raynaud. 1966. Cytotoxic effects in vitro of highly purified Streptolysin O on mouse macrophages. J. Bacteriol. 92:1150-1153.

Filion, L. G., P. S. Wilson, H. Bielefeldt-Ohmann, L. A. Babiuk, R. G. Thomson. 1984. The possible role of stress in the induction of pneumonic pasteurellosis. Can. J. Comp. Med. 48:268-274.

Frank, G. H., L. B. Tabatabai. 1981. Neuraminidase activity of Pasteurella haemolytica isolates. Infect. and Immun. 32: 1119-1122.

Frank, G. H., P. C. Smith. 1983. Prevalence of Pasteurella haemolytica in transported calves. Am J. Vet. Res. 44:981-985.

Fridovich, I. 1978. The biology of oxygen radicals. Science 201: 875-880.

Friend, S. C., R. G. Thomson, B. N. Wilkie. 1977. Pulmonary lesions induced by Pasteurella haemolytica in cattle. Can. J. Comp. Med. 41:219-223.

Friend, S. C., B. N. Wilkie, R. G. Thomson, D. A. Barnum. 1977. Bovine pneumonic pasteurellosis: Experimental induction in vaccinated and non-vaccinated calves. Can. J. Comp. Med. 41:77-83.

Gale, C., A. H. Hamdy, A. L. Trapp. 1963. Studies on an experimental vaccine for prophylaxis of shipping fever. J. Am. Vet. Med. Ass. 142:884-887.

Gemmell, C. G., P. K. Peterson, D. J. Schmeling, P. G. Quie. 1982. Effect of Staphylococcal alpha-toxin on phagocytosis of Staphylococci by human polymorphonuclear leukocytes. Infect. and Immun. 38:975-980.

Gentry, M. J., R. E. Corstvet, R. J. Panciera. 1982. Extraction of capsular material from Pasteurella haemolytica. Am. J. Vet. Res. 43:2070-2073.

Gentry, M. J. 1984. The role of capsular material of Pasteurella haemolytica Type 1 in bovine pneumonic pasteurellosis. PhD thesis, Oklahoma State University.

Gentry, M. J., A. W. Confer, R. J. Panciera. 1985. Serum neutralization of cytotoxin from Pasteurella haemolytica, serotype 1 and resistance to experimental bovine pneumonic pasteurellosis. Vet. Immuno. and Immunopath. in press.

Gerber, J. D., A. E. Marron, C. J. Kucera. 1978. Local and systemic cellular and antibody immune responses of cattle to infectious bovine rhinotracheitis virus vaccines administered intranasally or intramuscularly. Am. J. Vet. Res. 39:753-760.

Gilka, F., R. G. Thomson, M. Savan. 1974. The effect of edema, hydrocortisone acetate, concurrent viral infection, and immunization on the clearance of Pasteurella haemolytica from the bovine lung. Can. J. Comp. Med. 38:251-259.

Gilmour, N. J., W. B. Martin, J. M. Sharp, D. A. Thompson, P. W. Wells. 1979. The development of vaccines against pneumonic pasteurellosis in sheep. Vet. Rec. 104:15.

Gladstone, G. P., A. Yoshida. 1967. The cytopathic action of purified Staphylococcal S-haemolysin. Brit. J. Exp. Path. 48:11-19.

Glorioso, J. C., G. W. Jones, H. G. Rush, L. J. Pentler,, C. A. Darif, J. E. Coward. 1982. Adhesion of Type A Pasteurella multocida to rabbit pharyngeal cells and its possible role in rabbit respiratory tract infections. Infect. and Immun. 35:1103-1109.

Graham, W. R. 1953. The pathology of shipping fever in feedlot cattle. J. Am. Vet. Med. Ass. 123:198-203.

Green, G. M., E. H. Kass. 1965. The influence of bacterial species on pulmonary resistance to infection in mice subjected to hypoxia cold stress, and ethanolic intoxication. Brit. J. Exp. Path. 46: 360-366.

Grey, C. L., R. G. Thomson. 1971. Pasteurella haemolytica in the tracheal air of calves. Can. J. Comp. Med. 35:121-128.

Hamdy, A. H., A. L. Trapp. 1964. Immunization of cattle against shipping fever: Experimental exposure. Am. J. Vet. Res. 25: 1648-1652.

Hamdy, A. H., N. B. King, A. L. Trapp. 1965. Attempted immunization of cattle against shipping fever: A field trial. Am. J. Vet. Res. 26:897-902.

Hamdy, A. H., A. L. Trapp. 1967. Investigation of nasal microflora of feedlot calves before and after weaning. Am. J. Vet. Res. 28: 1019-1025.

Hammerschmidt, D. E. 1983. Activation of the complement system and of granulocytes in lung injury: The adult respiratory distress syndrome. pp. 147-172 in "Advances in inflammation research" vol. 5 ed. G. Weissmann. New York:Raven Press.

Henson, P. M., K. McCarthy, G. L. Larsen, R. O. Webster, R. B. Dreisin, T. E. King, J. O. Shaw. 1979. Complement fragments, alveolar macrophages, and alveolitis. Am. J. Path. 97:93-110.

Hesse, R. A., T. E. Toth. 1983. Effects of bovine parainfluenza-3 virus on phagocytosis and phagosome-lysosome fusion of cultured bovine alveolar macrophages. Am J. Vet. Res. 44:1901-1907.

Himmel, M. E., M. D. Yates, L. H. Lauerman, P. G. Squire. 1982. Purification and partial characterization of a macrophage cytotoxin from Pasteurella haemolytica. Am. J. Vet. Res. 43:764-767.

Hirayama, T., I. Kato. 1983. A rapid stimulation of phosphatidylinositol metabolism in rabbit leukocytes by Pseudomonal leukocidin. F. E. B. S. 157:46-50.

Hirayama, T., M. Noda, F. Matsuda, M. Nagamori, I. Kato. 1984. Binding of Pseudomonal leukocidin to rabbit polymorphonuclear leukocytes. Infect. and Immun. 46:631-634.

Hirsch, J. G., A. W. Bernheimer, G. Weissmann. 1963. Motion picture study of the toxic action of Streptolysins on leukocytes. J. Exp. Med. 118:223-228.

Hoerlein, A. B., S. P. Saxena, M. E. Mansfield. 1961. Studies on shipping fever of cattle II. Prevalence of Pasteurella species in nasal secretions from normal calves and calves with shipping fever. Am. J. Vet. Res. 22:470-472.

Hurd, J., R. B. Heath. 1975. Effect of cyclophosphamide on infections in mice caused by virulent and avirulent strains of influenza virus. Infect. and Immun. 11:886-889.

Jakab, G. J. 1974. Effect of sequential inoculations of Sendai virus and Pasteurella pneumotropica in mice. J. Am. Vet. Med. Ass. 164:723-728.

Jakab, G. J., G. M. Green. 1976. Defect in intracellular killing of Staphylococcus aureus within alveolar macrophags in Sendai virus infected murine lungs. J. Clin. Invest. 57:1533-1539.

Jakab, G. J., G. A. Warr, P. L. Sannes. 1980. Alveolar macrophage ingestion and phagosome-lysosome fusion defect associated with virus pneumonia. Infect. and Immun. 27:960-968.

Jakab, G. J., G. A. Warr. 1981. Immune-enhanced phagocytic dysfunction in pulmonary macrophages infected with parainfluenza 1 (Sendai) virus. Am. Rev. Resp. Dis. 124:575-581.

Jakab, G. J. 1982. Immune impairment of alveolar macrophage phagocytosis during influenza virus pneumonia. Am. Rev. Resp. Dis. 126:778-782.

Jakab, G. J. 1984. Viral-bacterial interactions in respiratory tract infections: A review of the mechanisms of virus-induced suppression of pulmonary antibacterial defenses. pp. 223-286 in "Bovine Respiratory Disease. A Symposium", ed. R. W. Loan. College Station:Texas A&M University Press.

Jensen, R., R. E. Pierson, P. M. Braddy, D. A. Saari, L. H. Lauerman, J. J. England, H. Keyvanfar, J. R. Collier, D. P. Horton, A. E. McChesney, A. Benitez, R. M. Christie. 1976. Shipping fever pneumonia in yearling feedlot cattle. J. Am. Vet. Med Ass. 169: 500-506.

Jericho, K. W. F., S. E. Magwood, P. H. G. Stockdale. 1976. Prevention of experimental bovine pneumonic pasteurellosis by exposure to IBR virus. Can. Vet. J. 17:194-195.

Jericho, K. W. F., E. V. Langford. 1978. Pneumonia in calves produced with aerosols of bovine herpesvirus 1 and Pasteurella haemolytica Can. J. Comp Med. 42:269-277.

Johanson, W. G., J. H. Higuchi, T. R. Chaudhuri, D. E. Woods. 1980. Bacterial adherence to epithelial cells in bacillary colonization of the respiratory tract. Am. Rev. Resp. Dis. 121:55-63.

Johnson, M. K., D. Boese-Marrazzo, W. A. Pierce, Jr. 1981. Effects of pneumolysin on human polymorphonuclear leukocytes and platelets. Infect. and Immum. 34:171-176.

Kaehler, K. L., R. J. F. Markham, C. C. Muscoplat, D. W. Johnson. 1980. Evidence of cytocidal effects of Pasteurella haemolytica on bovine peripheral blood mononuclear leukocytes. Am. J. Vet. Res. 41:1690-1693.

Keiss, R. E., D. H. Will, J. R. Collier. 1964. Skin toxicity and hemodynamic properties of endotoxin derived from Pasteurella haemolytica. Am. J. Vet. Res. 25:935-942.

Kelley, K. W. 1984. Immune reponses and plasma hormone concentrations in cold-exposed, xeranol-implanted calves. Am. J. Vet. Res. 45: 2617-2621.

King, N. B., C. Gale. 1963. Studies on myxovirus parainfluenza-3 vaccine for prevention of shipping fever in cattle. J. Am. Vet. Med. Ass. 142:881-883.

Kingdon, G. C., C. P. Sword. 1970. Effects of Listeria monocytogenes hemolysin on phagocytic cells and lysosomes. Infect. and Immun. 1:356-362.

Kleinerman, E. S., C. A. Daniels, R. P. Polisson, R. Snyderman. 1976. Effect of virus infection on the inflammatory reponse: Depression of macrophage accumulation in influenza infected mice. Am. J. Path. 85:373-382.

Kleinerman, E. S., R. Snyderman, C. A. Daniels. 1975. Depressed monocyte chemotaxis during influenza infection. Lancet 2:1063-1066.

Kleinerman, E. S., R. Snyderman, C. A. Daniels. 1974. Depression of human monocyte chemotaxis by herpes simplex and influenza viruses. J. Immunol. 113:1562-1567.

Kucera, C. J., J. C. S. Wong, T. J. Feldner. 1983. Challenge exposure of cattle vaccinated with a chemically altered strain of Pasteurella haemolytica. Am. J. Vet. Res. 44:1848-1852.

LaForce, F. M. 1976. Effect of alveolar lining material on phagocytic and bactericidal activity of lung macrophages against Staphylococcus aureus. J. Lab. Clin. Med. 88:691-699.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T 4. Nature (London) 227: 680-685.

Larsen, G. L., K. McCarthy, R. O. Webster, J. Henson, P. M. Henson. 1980. A differential effect of C5a and C5a des arg in the induction of pulmonary inflammation. Am. J. Path. 100:179-192.

Larson, H. E., R. Blades. 1976. Impairment of human polymorphonuclear leukocyte function by influenza virus. Lancet 2:285.

Lessley, B. A., A. W. Confer, D. A. Mosier, M. J. Gentry, J. A. Durham, J. A. Rummage. 1985. Saline extracted antigens of Pasteurella haemolytica: Separation by chromatofocusing, preliminary characterization, and evaluation of immunogenicity. Vet. Immuno. and Immunopath. in press.

Lillie, L. E., R. G. Thomson. 1972. The pulmonary clearance of bacteria by calves and mice. Can. J. Comp. Med. 36: 129-137.

Lillie, L. E. 1974. The bovine respiratory disease complex. Can. Vet. J. 15:233-242.

Lockard, V. G., J. B. Grogan, J. G. Brunson. 1973. Alterations in the bactericidal ability of rabbit alveolar macrophages as a result of tumbling stress. Am. J. Path. 70:57-68.

Lopez, A., R. G. Thomson, M. Savan. 1976. The pulmonary clearance of Pasteurella haemolytica in calves infected with bovine parainfluenza-3 virus. Can. J. Comp. Med. 40: 385-391.

Magwood, S. E., D. A. Barnum, R. G. Thomson. 1969. Nasal bacterial flora of calves in healthy and in pneumonia prone herds. Can. J. Comp. Med. 33:237-243.

Maheswaran, S. K., K. A. Berggren, R. R. Simonson, G. E. Ward, C. C. Muscoplat. Kinetics of interaction and fate of Pasteurella haemolytica in bovine alveolar macrophages. Infect. and Immun. 30:254-262.

Markham, R. J. F., B. N . Wilkie. 1980. Interaction between Pasteurella haemolytica and bovine alveolar macrophages: Cytotoxic effect on macrophages and impaired phagocytosis. Am. J. Vet. Res. 41:18-22.

Markham, R. J. F., B. N. Wilkie. 1980. Influence of broncho-alveolar washing supernatants and stimulated lymphocyte supernatants on the uptake of Pasteurella haemolytica by cultured bovine alveolar macrophages. Am. J. Vet. Res. 41:443-446.

Martin, S. W., A. H. Meek, D. G. Davis, R. G. Thomson, J. A. Johnson, A. Lopez, L. Stephens, R. A. Curtis, J. F. Prescott, S. Rosendal, M. Savan, A. J. Zubaidy, M. R. Bolton. 1980. Factors associated with mortality in feedlot cattle: The Bruce county beef cattle project. Can. J. Comp. Med. 44:1-10.

Martin, S. W. 1983. Vaccination: Is it effective in preventing respiratory disease or influencing weight gains in feedlot calves? Can. Vet. J. 24:10-19.

Matsumoto, M., J. A. Schmitz, B. Syuto, B. J. Watrous, D. E. Mattson. 1984. Immunogenicity of a soluble antigen against Pasteurella haemolytica-associated pneumonia in calves. Vet. Res. Comm. 8:117-130.

Matsuoka, T., C. Gale, E. E. Ose, R. N. Berkman. 1966. Calfhood immunization against an experimental parainfluenza and Pasteurella challenge. Can. J. Comp. Med. 30:228-232.

Matsuoka, T., T. M. Folkerts, C. Gale. 1972. Evaluation in calves of an inactivated bovine rhinotracheitis and parainfluenza-3 vaccine combined with Pasteurella bacterin. J. Am. Vet. Med. Ass. 160:333-337.

McGee, M. P., A. Kreger, E. S. Leake, S. Harshman. 1983. Toxicity of Staphylococcal alpha toxin for rabbit alveolar macrophages. Infect. and Immun. 39:439-444.

Milligan, T. W., C. J. Baker, D. C. Straus, S. J. Mattingly. 1978. Association of elevated levels of extracellular neuraminadase with clinical isolates of type III, Group B Streptococci. Infect. and Immun. 21:738-746.

Morter, R. L., H. E. Amstutz, R. A. Crandell. 1982. Clinical evaluation of prophylactic regimens for bovine respiratory disease. Bov. Pract. 17:56-58.

Mukkur, T. K. S. 1977. Demonstration of cross-protection between Pasteurella multocida type A and Pasteurella haemolytica serotype 1. Infect. and Immun. 18:583-585.

Noda, M.,T. Hirayama, I. Kato, F. Matsuda. 1980. Crystallization and properties of Staphylococcal leucocidin. Biochim. Biophys. Acta. 633:33-44.

Noda, M., I. Kato, T. Hirayama, F. Matsuda. 1980. Fixation and inactivation of Staphylococcal leukocidin by phosphatidyl choline and ganglioside Gm1 in rabbit polymorphonuclear leukocytes. Infect. and Immun. 29:678-684.

Noda, M., I. Kato, F. Matsuda, T. Hirayama. 1981. Mode of action of Staphylococcal leukocidin: Relationship between binding of 125 I-labelled S and F components of leukocidin to rabbit polymorphonuclear leukocytes and leukocidin activity. Infect. and Immun. 34:362-367.

Noda, M., I. Kato, T. Hirayama, F. Matsuda. 1982. Mode of action of Staphylococcal leukocidin: Effect of the F and S components on the activities of membrane associated enzymes of polymorphonuclear leukocytes. Infect. and Immun. 35:38-45.

Nagy, L. K., C. W. Penn. 1976. Protection of cattle against experimental haemorrhagic septicaemia by the capsular antigens of Pasteurella multocida, types B and E. Res. Vet. Sci. 20:249-253.

Nathan, C. F., H. W. Murray, Z. A. Cohn. 1980. The macrophage as an effector cell. New Engl. J. Med. 303:622-626.

Nonoyama, S., H. Kojo, Y. Mine, M. Nishida, S. Goto, S. Kuwahara. 1979. Inhibitory effect of Pseudomonas aeruginosa on the phagocytic and killing activities of rabbit polymorphonuclear leukocytes: Purification and characterization of an inhibitor of polymorphonuclear leukocyte function. Infect. and Immun. 24:394-398.

Ofek, I., S. Bergner-Rabinowitz, I. Ginsburg. 1970. Oxygen stable hemolysins of Group A Streptococci VII. The relation of the leukotoxic factor to Streptolysin S. J. Infec. Dis. 122:517-522.

Ofek, I, S. Bergner-Rabinowitz, I. Ginsburg. 1972. Oxygen stable hemolysins of Group A Streptococci VIII. Leukotoxic and antiphagocytic effects of Streptolysins S and O. Infect. and Immun. 6:459-464.

Otulakowski, G. L., P. E. Shewen, A. E. Udoh, A. Mellors, B. N. Wilkie. 1983. Proteolysis of sialoglycoprotein by Pasteurella haemolytica cytotoxic culture supernatent. Infect. and Immun. 42:64-70.

Palotay, J. L., S. Young, S. A. Lovelace, J. H. Newhall. 1963. Bovine respiratory infections II. Field trial using bacterial vaccine products as a means of prophylaxis. Am. J. Vet. Res. 24:1137-1142.

Panciera, R. J., R. E. Corstvet, A. W. Confer, C. N. Gresham. 1984. Bovine pneumonic pasteurellosis: Effect of vaccination with live Pasteurella species. Am. J. Vet. Res. 45:2538-2542.

Pass, D. A., R. G. Thomson, G. C. Ashton. 1971. Regional histological variations of the nasal mucosa in cattle. Can. J. Comp. Med. 35:212-217.

Paton, J. C., A. Ferrante. 1983. Inhibition of human polymorphonuclear leukocytes respiratory burst, bactericidal activity, and migration by pneumolysin. Infect. and Immun. 41:1212-1216.

Paton, J. C., R. A. Lock, D. J. Hansman. 1983. Effect of immunization with pneumolysin on survival time of mice challenged with Streptococcus pneumoniae. Infect. and Immun. 40:548-552.

Ramphal, R., P. M. Small, J. W. Shands, W. Fixchlschweiger, P. A. Small. 1980. Adherence of Pseudomonas aeruginosa to tracheal cells injured by influenza infection of by endotracheal intubation. Infect. and Immun. 27:614-619.

Ray, P. K. 1977. Bacterial neuraminidase and altered immunologic behaviour of treated mammalian cells. Adv. Appl. Microbiol. 21:227-267.

Rehmtulla, A. J., R. G. Thomson. 1981. A review of the lesions in shipping fever of cattle. Can. Vet. J. 22:1-8.

Rhoades, K. R., K. L. Heddlestone, P. A. Rebers. 1967. Experimental hemorrhagic septicemia, gross and microscopic lesions resulting from acute infection and from endotoxin administration. Can. J. Comp. Med. 31:226-233.

Roberts, D. S. 1967. The pathogenic synergy of Fusobacterium necrophorum and Corynebacterium pyogenes I. Influence of the leukocidal exotoxin of Fusobacterium necrophorum. Brit. J. Exp Path. 48:665-673.

Sanford, B. A., A. Shelokov, M. A. Ramsey. 1978. Bacterial adherence to virus-infected cells: A cell culture model of bacterial superinfection. J. Infect. Dis. 137:176-181.

Sawyer, W. D. 1969. Interaction of influenza virus with leukocytes and its effect on phagocytosis. J. Infect. Dis. 119:541-556.

Scharmann, W. 1976. Formation and isolation of leucocidin from Pseudomonas aeruginosa. J. Gen. Microbiol. 93:283-291.

Scharmann, W. 1976. Purification and characterization of leucocidin from Pseudomonas aeruginosa. J. Gen. Microbiol. 93:292-302.

Scharmann, W. 1976. Interaction of purified leukocicin from Pseudomonas aeruginosa with bovine polynorphonuclear leukocytes. Infect. and Immun. 13:1046-1053.

Scharmann, W. 1976. Cytotoxic effects of leukocidin from Pseudomonas aeruginosa on polymorphonuclear leukocytes from cattle. Infect. and Immun. 13:836-843.

Scharmann, W., R. Jacob, J. Porstendorfer. 1976. The cytotoxic action of leucocidin from Pseudomonas aeruginosa on human polymorphonuclear leukocytes. J. Gen Microbiol. 93:303-308.

Schiefer, B., G. E. Ward, R. E. Moffatt. 1978. Correlation of microbiological and histological findings in bovine fibrinous pneumonia. Vet. Path. 15:313-321.

Schipper, I. A., C. B. Buornson, G. E. Strum. 1962. Summary of four-year study on shipping fever prevention. Vet. Med. 57:225-228.

Shewen, P. E., B. N. Wilkie. 1982. Cytotoxin of Pasteurella haemolytica acting on bovine leukocytes. Infect. and Immun. 35:91-94.

Shewen, P. E., B. N. Wilkie. 1983. Pasteurella haemolytica cytotoxin neutralizing activity in sera from Ontario beef cattle. Can. J. Comp. Med. 47:497-498.

Shewen, P. E., B. N. Wilkie. 1985. Evidence for the Pasteurella haemolytica cytotoxin as a product of actively growing bacteria. Am. J. Vet. Res. 46:1212-1214.

Slauson, D. O. 1982. The mediation of pulmonary inflammatory injury. Adv. Vet. Sci. Comp. Med. 26:99-153.

Smith, J. E. 1977. Microbial surfaces in relation to pathogenicity. Bacteriol. Rev. 41:475-500.

Snell, J. D. 1966. Effects of inhaled endotoxin. J. Lab. Clin. Med. 67:624-632.

Squire, P. G., D. W. Smiley, R. B. Croskell. 1984. Identification and extraction of Pasteurella haemolytica membrane proteins. Infect. and Immun. 45:667-673.

Stankus, R. P., R. M. Cashner, J. E. Salvaggio. 1978. Broncho-pulmonary macrophage activation in the pathogenesis of hypersensitivity pneumonitis. J. Immunol. 120:685-688.

Stockdale, P. H. G., E. V. Langford, C. leQ Darcel. 1979. Experimental bovine pneumonic pasteurellosis I. Prevention of the disease. Can. J. Comp. Med. 43:262-271.

Stockdale, P. H. G., K. W. F. Jericho, W. D. G. Yates, C. leQ Darcel, E. V. Langford. 1979. Experimental bovine pneumonic pasteurellosis II. Genesis and prevention. Can. J. Comp. Med. 43:272-279.

Tadayon, R. A., L. H. Lauerman. 1981. The capacity of various fractions of Pasteurella haemolytica to stimulate protective immunity in mice and hamsters. Vet. Microbiol. 6:85-93.

Taichman, N. S., R. T. Dean, C. J. Sanderson. 1980. Biochemical and morphological characterization of the killing of human monocytes by a leukotoxin derived from Actinobacillus actinomycetemcomitans. Infect. and Immun. 28:258-268.

Thomson, R. G., M. L. Benson, M. Savan. 1969. Pneumonic pasteurellosis of cattle: Microbiology and immunology. Can. J. Comp. Med. 33:194-206.

Thomson, R. G. 1974. Pathology and pathogenesis of the common diseases of the respiratory tract of cattle. Can. Vet. J. 15:249-251.

Thomson, R. G., S. Chander, M. Savan, M. L. Fox. 1975. Investigation of factors of probable significance in the pathogenesis of pneumonic pasteurellosis in cattle. Can. J. Comp. Med. 39:194-207.

Thomson, R. G. 1980. A perspective on respiratory disease in feedlot cattle. Can. Vet. J. 21:181-185.

Thomson, R. G. 1980. The pathogenesis of shipping fever in cattle. Proc. Am. Ass. Bov. Pract. 13:103-112.

Towbin, H., T. Staehelin, J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Nat. Acad. Sci. U.S. 76:4350-4354.

Tsai, C. C., W. P. McArthur, P. C. Baehni, B. F. Hammond, N. S. Taichman. 1979. Extraction and partial characterization of a leukotoxin from a plaque-derived gram-negative micro-organism. Infect. and Immun. 25:427-439.

van Epps, D. E., B. R. Andersen. 1974. Streptolysin O inhibition of neutrophil chemotaxis and mobility: Nonimmune phenomenon with species specificity. Infect. and Immun. 9:27-33.

van Heyningen, W. E. 1970. Bacterial protein toxins- General characteristics in "Microbial Toxins" Part I. pp.1-26. ed. S. J. Ajl, S. Kadis, T. C. Montie, New York: Academic Press.

van Heyningen, W. E., C. C. J. Carpenter, N. F. Pierce, W. B. Greenough III. 1971. Deactivation of cholera toxin by ganglioside. J. Infect. Dis. 124:415-418.

Voller, A., D. E. Bidwell, A. Bartlett. 1979. The enzyme linked immunosorbent assay (ELISA): A guide with abstracts of microplate applications. pp. 18-19, 35-40. Dynatech Labs., Alexandria, Va.

Walker, R. D., T. W. Schultz, F. M. Hopkins, M. J. Bryant. 1984. Growth phase-dependent phagocytosis of Pasteurella haemolytica by bovine pulmonary macrophages. Am. J. Vet. Res. 45:1230-1234.

Ward, P. D., W. H. Turner. 1980. Identification of Staphylococcal Panton-Valentine leukocidin as a potent dermonecrotic toxin. Infect. and Immun. 27:393-397.

Warr, G. A., G. J. Jakab. 1979. Alteration in lung macrophage antimicrobial activity associated with viral pneumonia. Infect. and Immun. 26:492-497.

Warshauer, D., E. Goldstein, T. Akers, W. Lippert, M. Kim. 1977. Effect of influenza viral infection on the ingestion and killing of bacteria by alveolar macrophages. Am. Rev. Resp. Dis. 115:269-277.

Watson, B. B., J. C. Lavizzo. 1973. Extracellular antigens from Listeria monocytogenes II. Cytotoxicity of hemolytic and lipolytic antigens of Listeria for cultured mouse macrophages. Infect. and Immun. 7:753-758.

Wells, M. A., P. Albrecht, F. A. Ennis. 1981. Recovery from a viral respiratory infection I. Influenza pneumonia in normal and T-deficient mice. J. Immunol. 126:1036-1041.

Wilkie, B. N., R. J. F. Markham, P. E. Shewen. 1980. Response of calves to lung challenge exposure with Pasteurella haemolytica after parenteral or pulmonary immunization. Am. J. Vet. Res. 41:1773-1778.

Woodin, A. M. 1959. Fractionation of a leucocidin from Staphylococcus aureus. Biochem. J. 73:225-235.

Woodin, A. M. 1960. Purification of the two components of leucocidin from Staphylococcus aureus. Biochem. J. 75:158-165.

Woodin, A. M. 1970. Staphylococcal leucocidin in "Microbial Toxins" Part III. pp.327-354. ed. S. J. Ajl, S. Kadis, T. C. Montie. New York: Academic Press.

Woods, G. T., M. E. Mansfield, D. Segre, J. C. Holper, C. A. Brandly, C. Barthel. 1962. The role of viruses in respiratory diseases of cattle III. Respiratory disease in beef calves vaccinated before weaning with bovine myxovirus parainfluenza-3 (SF-4) vaccine. Am. J. Vet. Res. 23:832-835.

Woods, G. T., M. E. Mansfield, G. F. Cmarik, G. Marquis. 1974. A four-year clinical and serologic study of the use of inactivated parainfluenza-3 virus vaccines and Pasteurella sp. bacterins in beef calves. Vet. Med./Sm. An. Clin. 69:474-478.

Yates, W. D. G. 1982. A review of infectious bovine rhinotracheitis, shipping fever pneumonia, and viral-bacterial synergism in respiratory disease of cattle. Can. J. Comp. Med. 46:225-263.

Yates, W. D. G., P. H. G. Stockdale, L. A. Babiuk, R. J. Smith. 1983. Prevention of experimental bovine pneumonic pasteurellosis with an extract of Pasteurella haemolytica. Can. J. Comp. Med. 47:250-256.

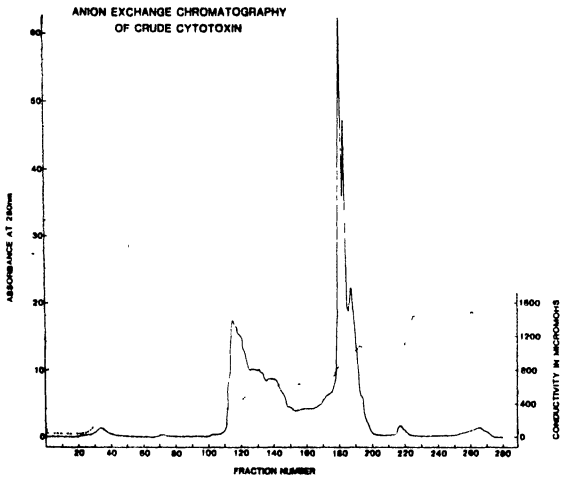
Zambon, J. J., C. DeLuca, J. Slots, R. J. Genco. 1983. Studies of leukotoxin from Actinobacillus actinomycetemcomitans using the promyelocytic HL-60 cell line. Infect. and Immun. 40:205-212.

APPENDIX

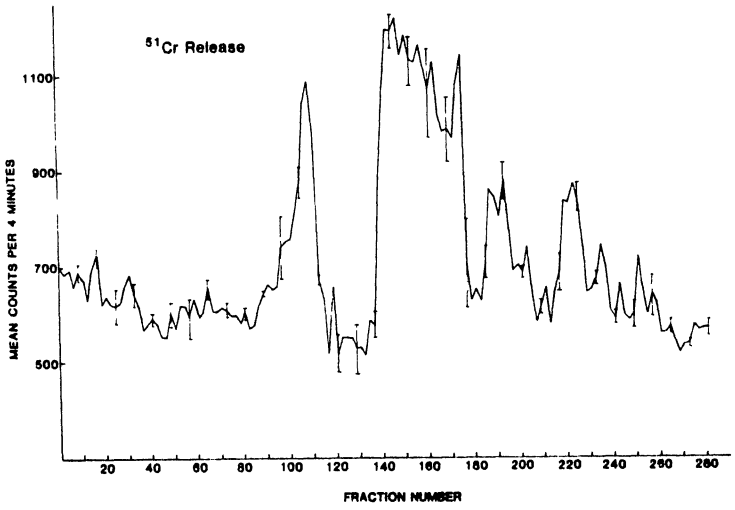
INITIAL CHARACTERIZATION OF CYTOTOXIN

Figure 17 Anion Exchange Chromatography of Crude Cytotoxin: ^{51}Cr release; Antigenicity. Absorbance profile (A), ^{51}Cr release profile (B), and antigenicity (ELISA) profile (C).

A


ANION EXCHANGE CHROMATOGRAPHY
OF CRUDE CYTOTOXIN
ABSORBANCE AT 280nm
CONDUCTIVITY IN MICROMHOS
FRACTION NUMBER

B


51Cr Release
MEAN COUNTS PER 4 MINUTES
FRACTION NUMBER

C

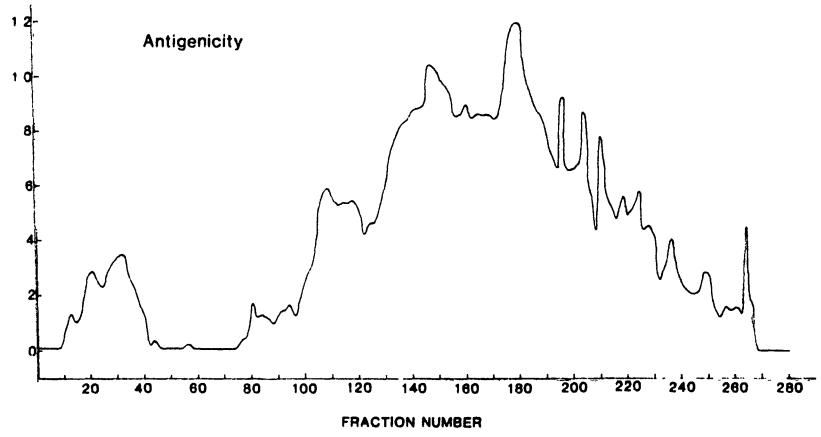
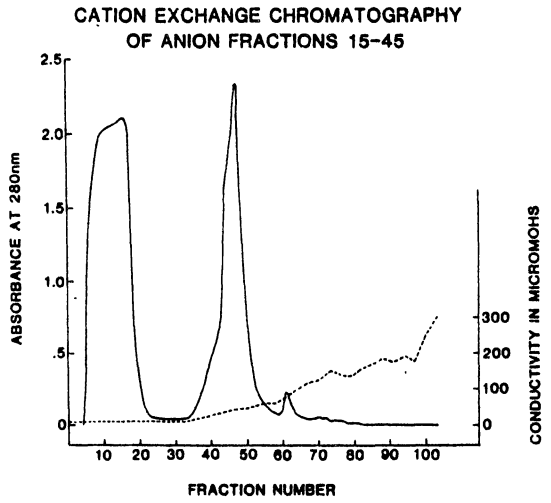
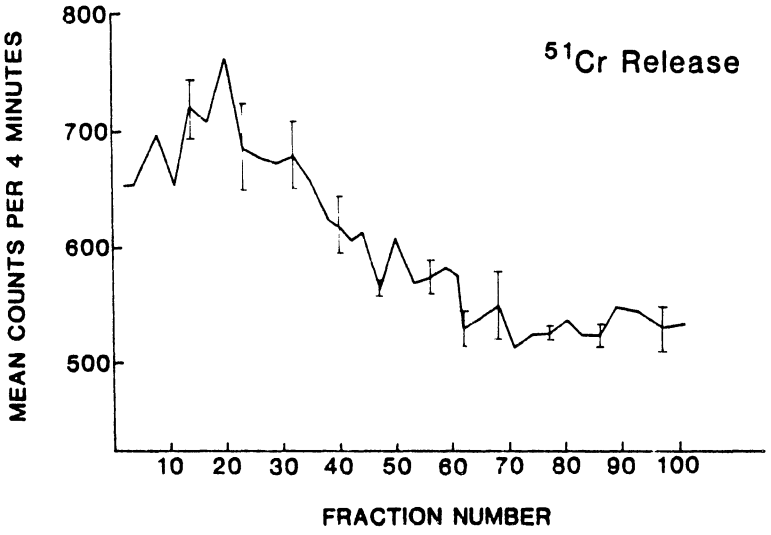

Antigenicity
FRACTION NUMBER

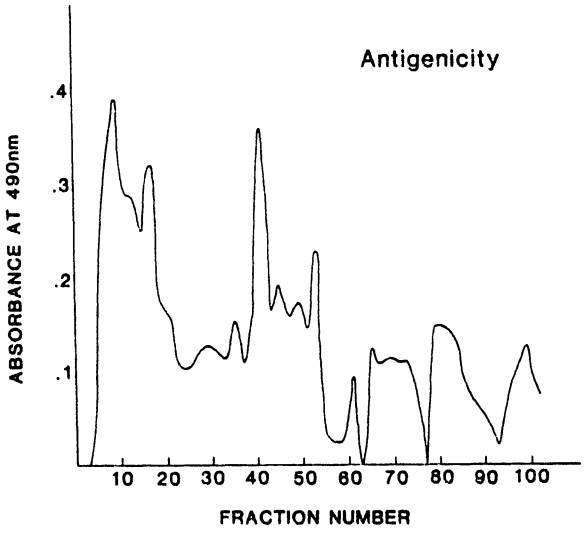
Figure 18 Cation Exchange Chromatography of Anion Fractions 15-45: ^{51}Cr release; Antigenicity. Absorbance profile (A), ^{51}Cr release profile (B), and antigenicity (ELISA) profile (C).


CATION EXCHANGE CHROMATOGRAPHY
OF ANION FRACTIONS 15-45
ABSORBANCE AT 280nm
CONDUCTIVITY IN MICROMOHS
FRACTION NUMBER

A

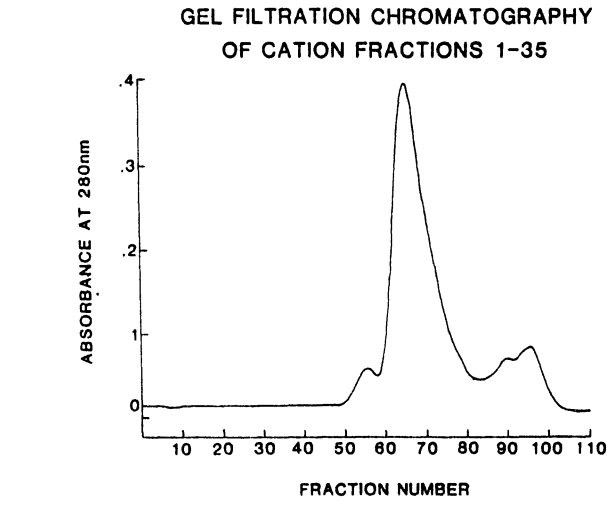

^{51}Cr Release
MEAN COUNTS PER 4 MINUTES
FRACTION NUMBER

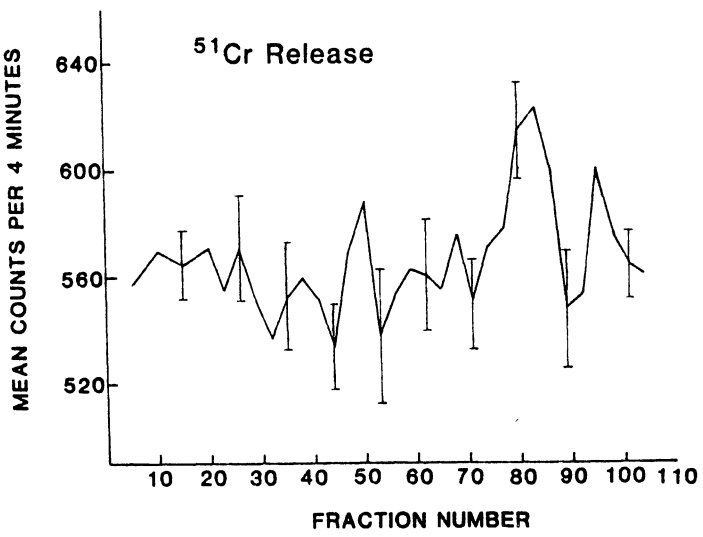
B


Antigenicity
ABSORBANCE AT 490nm
FRACTION NUMBER

C

Figure 19 Gel Filtration Chromatography of Cation Fractions 1-35: ^{51}Cr release; Antigenicity. Absorbance profile (A), ^{51}Cr release profile (B), and antigenicity (ELISA) profile (C).


GEL FILTRATION CHROMATOGRAPHY
OF CATION FRACTIONS 1-35
A
ABSORBANCE AT 280nm
.4
.3
.2
1
0
10 20 30 40 50 60 70 80 90 100 110
FRACTION NUMBER


B
^{51}Cr Release
MEAN COUNTS PER 4 MINUTES
640
600
560
520
10 20 30 40 50 60 70 80 90 100 110
FRACTION NUMBER

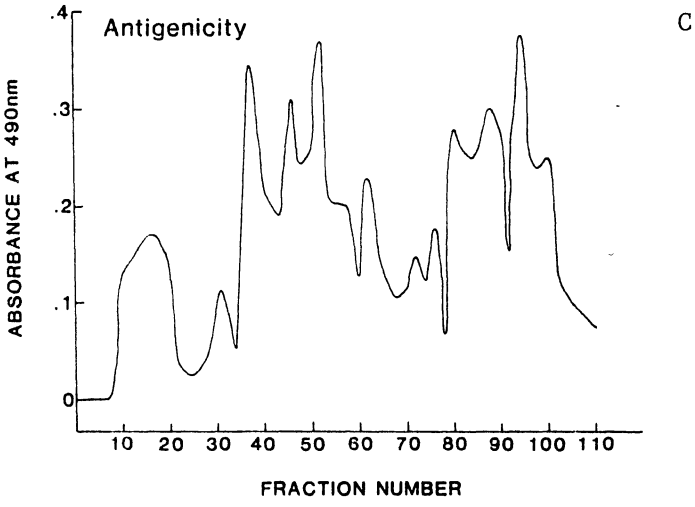
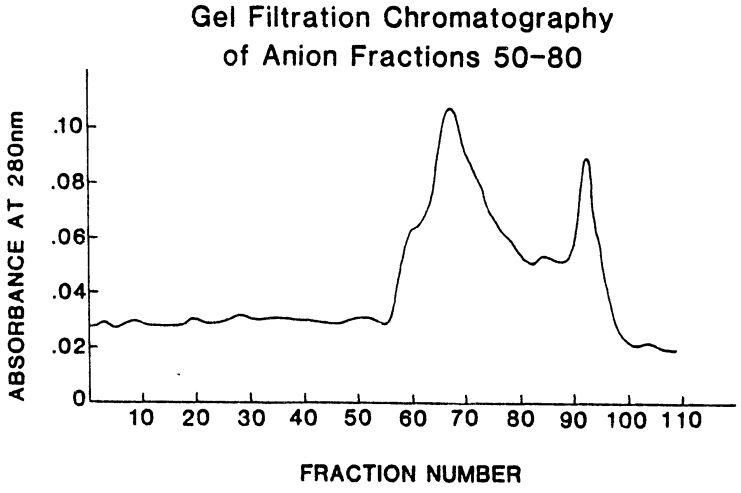
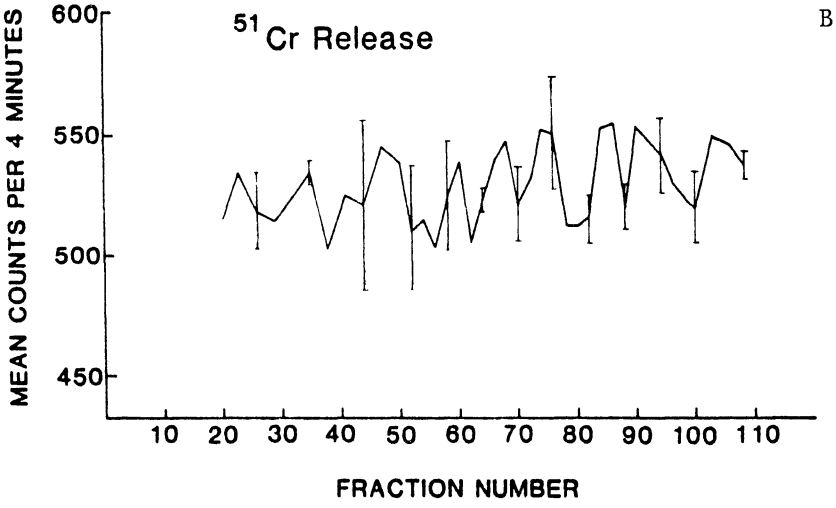

C
Antigenicity
ABSORBANCE AT 490nm
.4
.3
.2
.1
0
10 20 30 40 50 60 70 80 90 100 110
FRACTION NUMBER

Figure 20 Gel Filtration Chromatography of Anion Fractions 50-80: ^{51}Cr release; Antigenicity. Absorbance profile (A), ^{51}Cr release profile (B), and antigenicity (ELISA) profile (C).


Gel Filtration Chromatography
of Anion Fractions 50-80
A
ABSORBANCE AT 280nm
.10
.08
.06
.04
.02
0
10 20 30 40 50 60 70 80 90 100 110
FRACTION NUMBER


B
MEAN COUNTS PER 4 MINUTES
600
550
500
450
51Cr Release
10 20 30 40 50 60 70 80 90 100 110
FRACTION NUMBER

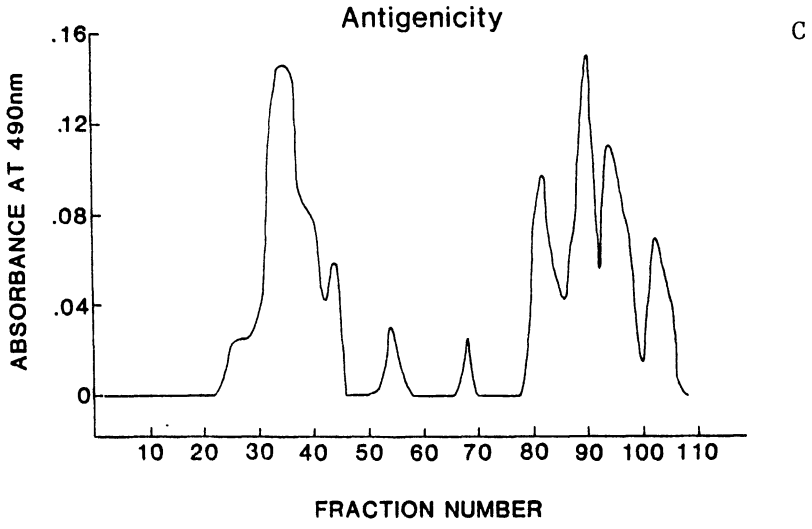
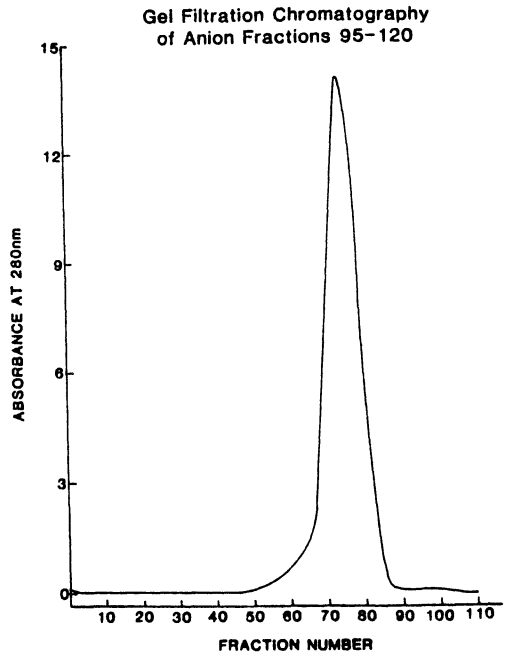
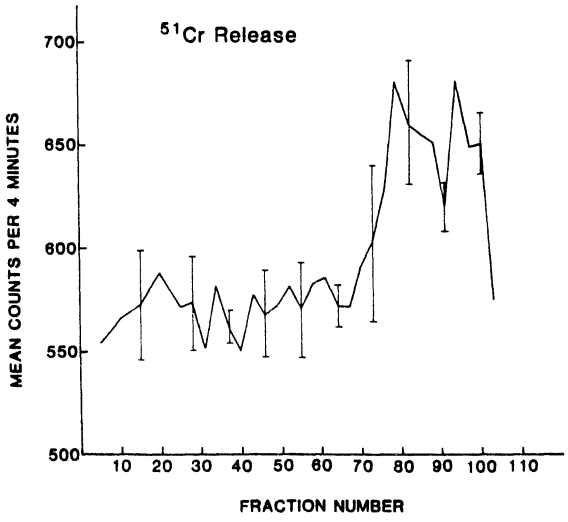

Antigenicity
C
ABSORBANCE AT 490nm
.16
.12
.08
.04
0
10 20 30 40 50 60 70 80 90 100 110
FRACTION NUMBER

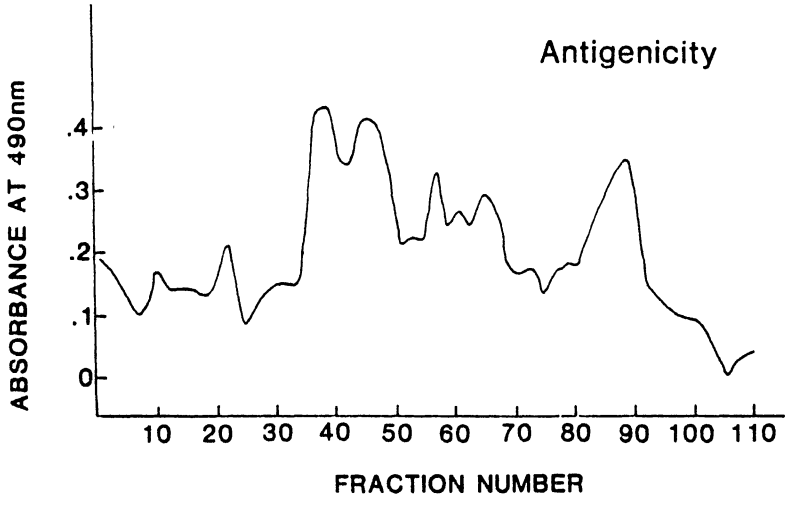
Figure 21 Gel Filtration Chromatography of Anion Fractions 95-120; ^{51}Cr release; Antigenicity. Absorbance profile (A), ^{51}Cr release profile (B), and antigenicity (ELISA) profile (C).


Gel Filtration Chromatography
of Anion Fractions 95-120
ABSORBANCE AT 280nm
15
12
9
6
3
0
10 20 30 40 50 60 70 80 90 100 110
FRACTION NUMBER

A

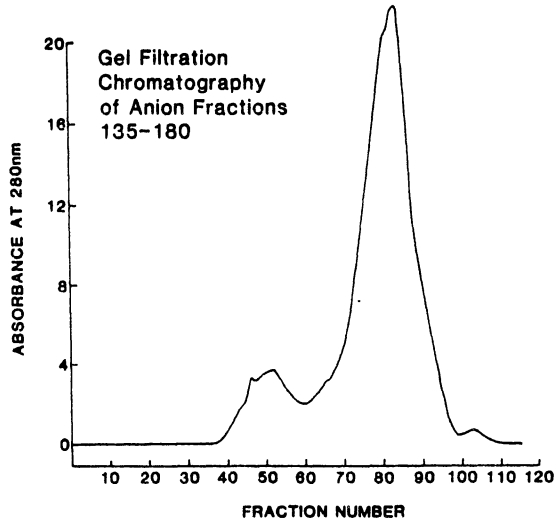

^{51}Cr Release
MEAN COUNTS PER 4 MINUTES
700
650
600
550
500
10 20 30 40 50 60 70 80 90 100 110
FRACTION NUMBER

B

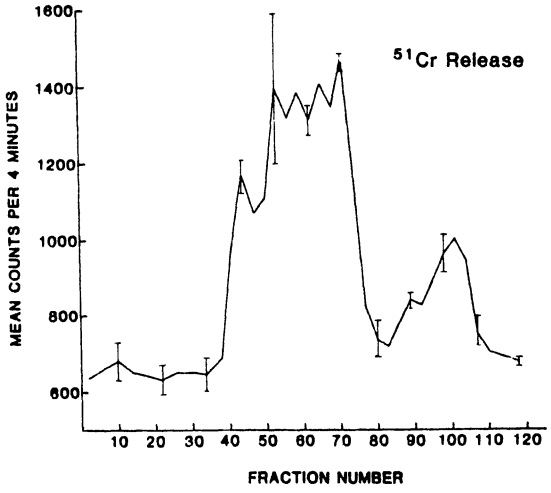

Antigenicity
ABSORBANCE AT 490nm
.4
.3
.2
.1
0
10 20 30 40 50 60 70 80 90 100 110
FRACTION NUMBER

C

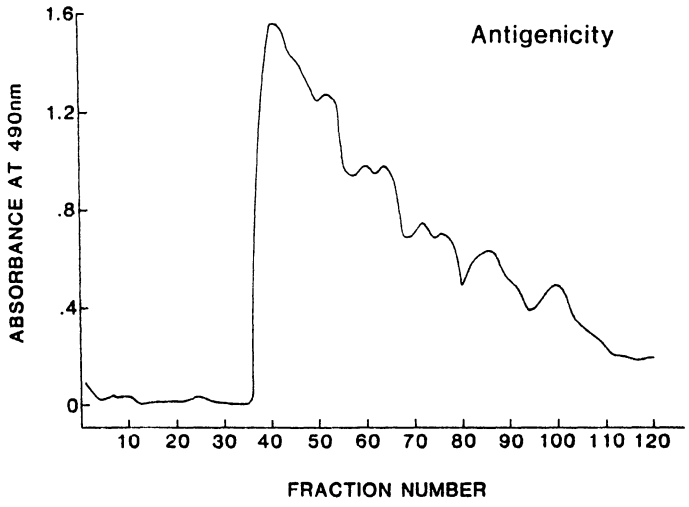
Figure 22 Gel Filtration Chromatography of Anion Fractions 135-180: ^{51}Cr release; Antigenicity. Absorbance profile (A), ^{51}Cr release profile (B), and antigenicity (ELISA) profile (C).


Gel Filtration
Chromatography
of Anion Fractions
135-180
ABSORBANCE AT 280nm
20
16
12
8
4
0
10 20 30 40 50 60 70 80 90 100 110 120
FRACTION NUMBER

A

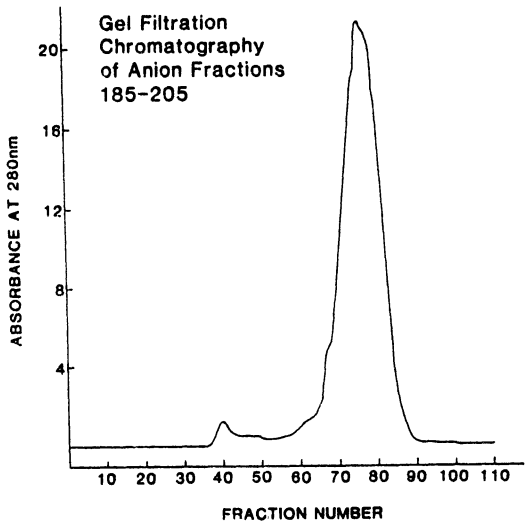

^{51}Cr Release
MEAN COUNTS PER 4 MINUTES
1600
1400
1200
1000
800
600
10 20 30 40 50 60 70 80 90 100 110 120
FRACTION NUMBER

B

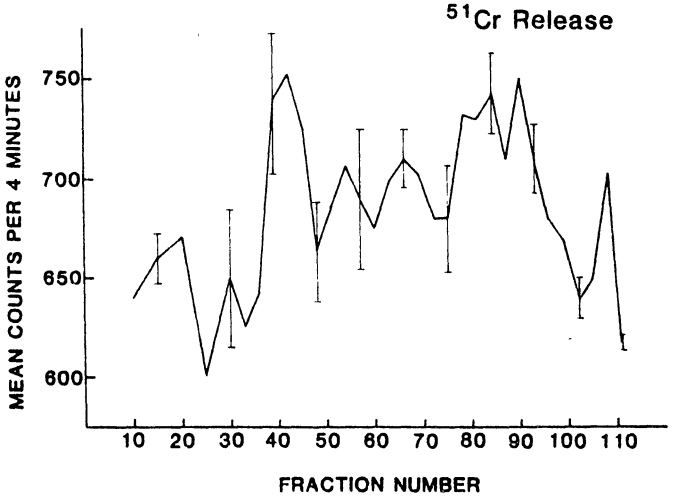

Antigenicity
ABSORBANCE AT 490nm
1.6
1.2
.8
.4
0
10 20 30 40 50 60 70 80 90 100 110 120
FRACTION NUMBER

C

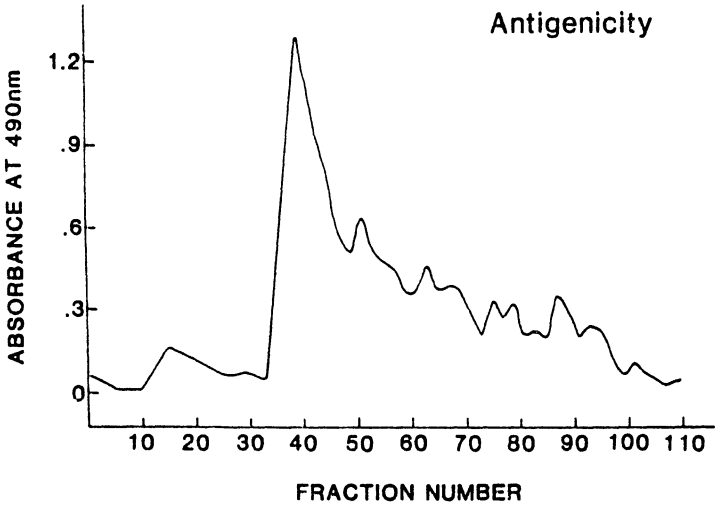
Figure 23 Gel Filtration Chromatography of Anion Fractions 185-205: ^{51}Cr release; Antigenicity. Absorbance profile (A), ^{51}Cr release profile (B), and antigenicity (ELISA) profile (C).


Gel Filtration
Chromatography
of Anion Fractions
185-205
ABSORBANCE AT 280nm
20
16
12
8
4
10 20 30 40 50 60 70 80 90 100 110
FRACTION NUMBER

A

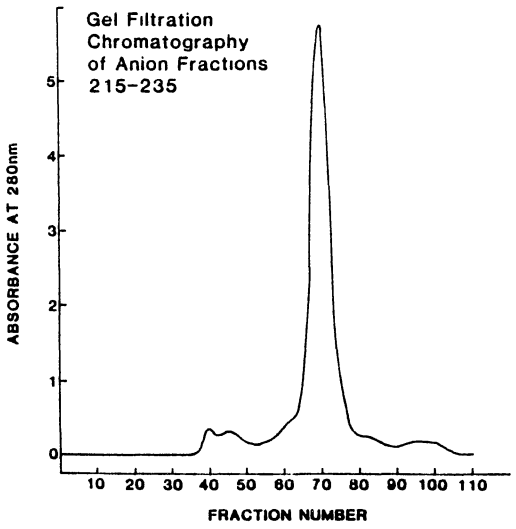

^{51}Cr Release
MEAN COUNTS PER 4 MINUTES
750
700
650
600
10 20 30 40 50 60 70 80 90 100 110
FRACTION NUMBER

B

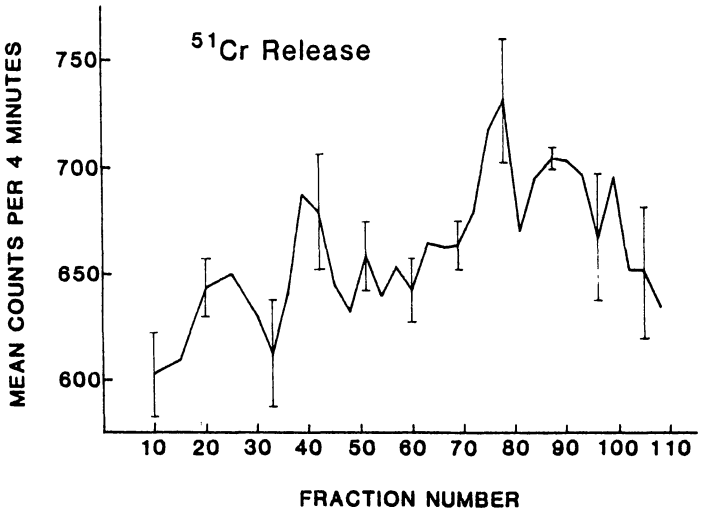

Antigenicity
ABSORBANCE AT 490nm
1.2
.9
.6
.3
0
10 20 30 40 50 60 70 80 90 100 110
FRACTION NUMBER

C

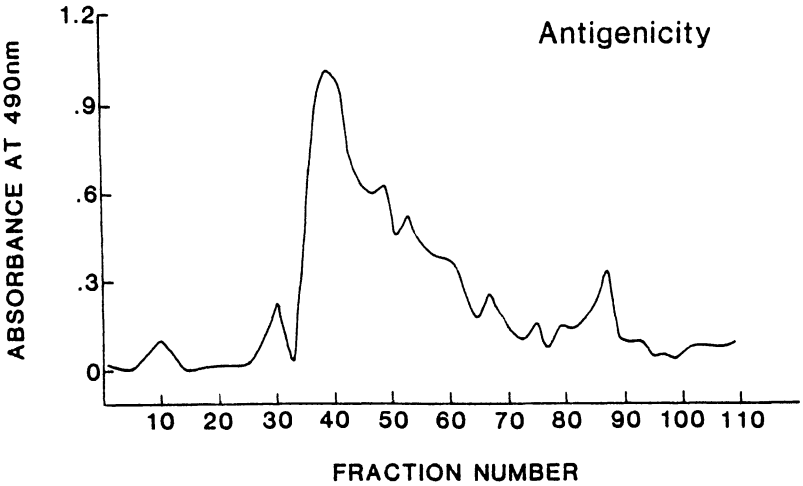
Figure 24 Gel Filtration Chromatography of Anion Fractions 215-235: 51Cr release; Antigenicity. Absorbance profile (A), ^{51}Cr release profile (B), and antigenicity (ELISA) profile (C).


Gel Filtration
Chromatography
of Anion Fractions
215-235
ABSORBANCE AT 280nm
0
1
2
3
4
5
10 20 30 40 50 60 70 80 90 100 110
FRACTION NUMBER

A

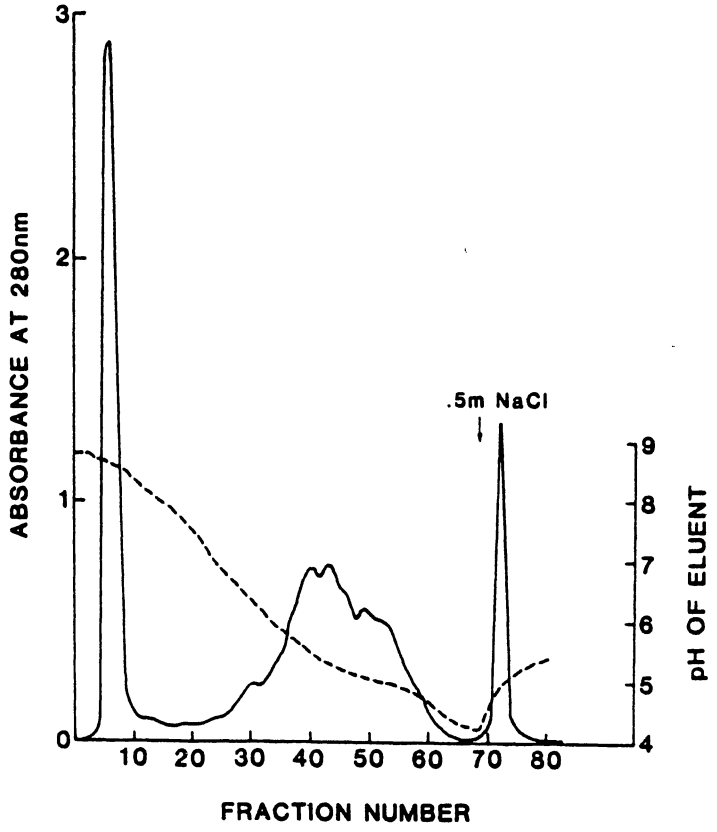

^{51}Cr Release
MEAN COUNTS PER 4 MINUTES
600
650
700
750
10 20 30 40 50 60 70 80 90 100 110
FRACTION NUMBER

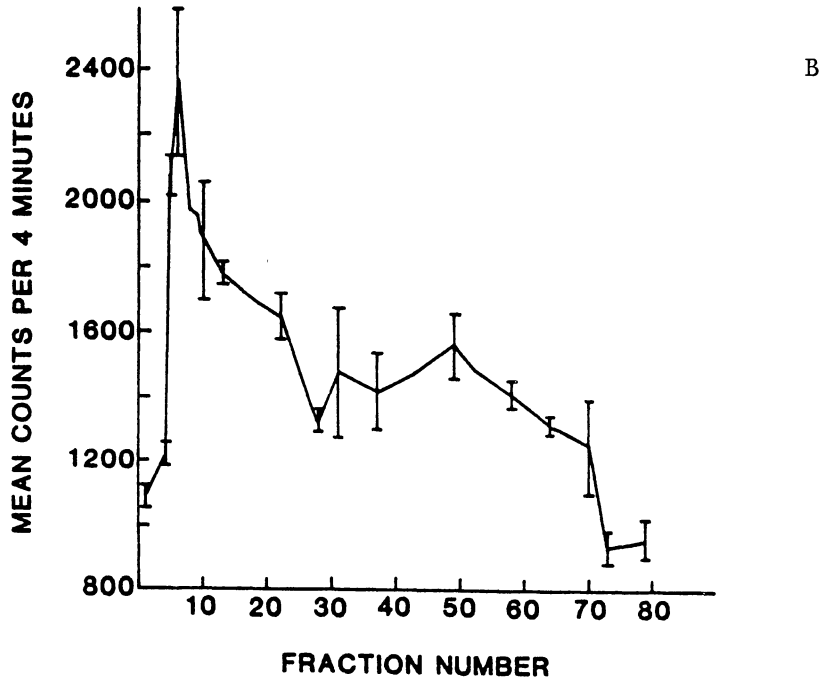
B


Antigenicity
ABSORBANCE AT 490nm
0
.3
.6
.9
1.2
10 20 30 40 50 60 70 80 90 100 110
FRACTION NUMBER

C

Figure 25 Chromatofocusing of Fractions 35-70 obtained from Gel Filtration of Anion Fractions 135-180: ^{51}Cr release. Absorbance profile (A), and ^{51}Cr release profile (B).


A
ABSORBANCE AT 280nm
3
2
1
0
.5m NaCl
pH OF ELUENT
9
8
7
6
5
4
10 20 30 40 50 60 70 80
FRACTION NUMBER


B
MEAN COUNTS PER 4 MINUTES
2400
2000
1600
1200
800
10 20 30 40 50 60 70 80
FRACTION NUMBER

VITA 2

Derek Alan Mosier

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