Viral-bacterial synergism in the bovine respiratory disease complex

by

Robert E. Briggs

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INTRODUCTION

The bovine respiratory disease (BRD) complex is a major cause of mortality and morbidity in feedlot cattle despite prophylactic immunization with bacterial and viral products.[1,2] Bovine respiratory disease, often referred to as shipping fever, is prevalent among calves after weaning, shipping, mixing with other calves, and placement in the feedlot. Factors thought to play a role in the development of BRD include stress (crowding, temperature extremes, transportation, airborne pollutants, etc.) and infectious agents, including several bacteria and viruses.[2] The economically important effects of BRD are usually associated with bacterial pneumonia, primarily by *Pasteurella haemolytica*.[3,4]

The pathogenesis and etiology of BRD are only partially understood. *P. haemolytica* is a normal inhabitant of the upper respiratory tract of cattle.[4,5,6] During shipment or experimental viral infection, the numbers of *P. haemolytica* in the upper respiratory tract often dramatically increase.[4,6] Although acute BRD is generally associated with pulmonary infection with *P. haemolytica*, efforts to reproduce lesions of BRD by challenge with *P. haemolytica* alone have met with little success.[5-11] One study showed that 90 percent of *P. haemolytica* experimentally instilled into the lung are cleared within 4 hours.[12] During viral infection, however, pneumonia often results from experimental *P. haemolytica* challenge.[6,13-18] These observations have led to the hypothesis that multiple factors, involving the interaction of stressors with virus, lead to a state of enhanced susceptibility to

bacterial infection. If host immune functions are in fact compromised by virus infection or stress, and since virus infection and/or conditions of stress often lead to large populations of *P. haemolytica* in the upper respiratory tract, it should not be surprising that calves which contract BRD tend to undergo bacterial pneumonia caused by *P. haemolytica*. The purpose of this report is to review the evidence that certain viruses associated with BRD alter host pulmonary immune defense mechanisms and to characterize the nature of those alterations. In addition, a genetic procedure which might identify subtypes of *P. haemolytica* in outbreaks of BRD is investigated.

Explanation of thesis format

This thesis is composed of three sections relating to viral-bacterial synergism in BRD. The first section is a critical review of bovine pulmonary immunologic defense mechanisms, the alterations of those mechanisms caused by viral infections, and the relationship between viral infection and bacterial pneumonia in the BRD complex. The second section is a manuscript describing research, designed and performed by this author (with technical assistance), concerning the functional alteration of polymorphonuclear leukocytes during infection of cattle with two respiratory viral agents. The third section is a manuscript describing a retrospective study, designed and performed by this author (with technical assistance), concerning epidemiologic aspects of the bacterium most frequently incriminated in severe bovine respiratory disease, *Pasteurella haemolytica*. Since stress- or virus-induced

alterations of cattle directly affect the survival and proliferation of *P. haemolytica* in both the lungs and upper respiratory passages, the epidemiologic aspects of *P. haemolytica* in bovine respiratory disease are intimately related to the topic of viral-bacterial synergism. A comprehensive literature review of all aspects of viral-bacterial synergism in bovine respiratory disease, however, would be exceedingly complex. The literature review in section 1, therefore, is limited to the topic of viral effects on pulmonary immunologic function.

SECTION I. VIRAL IMPAIRMENT OF IMMUNOLOGIC FUNCTIONS INVOLVED IN BOVINE RESPIRATORY DISEASE: A REVIEW

Viral impairment of immunologic functions involved in bovine respiratory disease: A review

Robert E. Briggs, D.V.M.

From the National Animal Disease Center, Agricultural Research Service, United States Department of Agriculture, Ames, IA 50010

INTRODUCTION

Respiratory disease is particularly prevalent during the first 45 days after calves have been weaned, transported, and placed in the feedlot.[1] During this period calves encounter stressful conditions, nutritional changes, and exposure to a number of infectious agents from other co-mingled calves. There is ample evidence that stress and nutrition play a role in immunologic function.[2-4] Under such conditions, calves would be increasingly susceptible to viral infection, whether by new virus in a naive calf, re-infection of a previously exposed animal, or recrudescence of latent viral infections. Viral infections are frequently incriminated in cases of severe or fatal bacterial infection in man and animals.[5-9] The evidence suggesting viral infection plays an important role in the etiology of severe bacterial pneumonia in cattle is substantial.[5,10-12]

Four viruses which have received the most attention concerning their involvement in the bovine respiratory disease (BRD) complex are infectious bovine rhinotracheitis (IBR) virus, parainfluenza-3 (PI-3) virus, bovine viral diarrhea (BVD) virus, and bovine respiratory syncytial virus (RSV). Each has been demonstrated to have effects on the bovine immune system, *in vivo* and *in vitro*. This report will briefly discuss the pulmonary immune defense mechanisms of probable relevance to bovine respiratory disease, review the evidence that certain viruses, associated with BRD, can alter pulmonary defense mechanisms, and characterize the nature of the alterations.

HOST PULMONARY DEFENSE MECHANISMS

Bacteria which are deposited in the lungs may either be eliminated or they may multiply to result in pulmonary disease. The capacity of the lung to eliminate bacteria is determined by a complex and interdependent set of extracellular and cellular factors. Extracellular factors include the cough reflex, the bronchoconstriction reflex, the mucociliary blanket, and the alveolar milieu. Cellular factors include lymphocytes, pulmonary alveolar macrophages (PAM), and polymorphonuclear leukocytes (PMN). The mucociliary blanket physically protects underlying epithelium and filters many incoming particles from the airways. The ciliary action distal to the alveoli elevates mucus and trapped particles to be swallowed or expectorated. The alveolar milieu, and to some extent airway secretions, contain such soluble components as surfactant, antibody of IgA, IgG, and IgM isotypes, bacteriostatic iron-containing compounds, complement components, and fibronectin.[13-16] An important role of several components is related to agglutination of particles and to augmentation of phagocytic function by opsonizing particles. Bactericidal and bacteriostatic activity may occur by action of certain extracellular components without additional cellular activity.

Bacteria which descend into the alveolus are rapidly engulfed by normal phagocytic cells of the lungs.[17] It is felt that bactericidal mechanisms within the lung are more important than mucociliary transport in removal of pathogenic bacteria. This conclusion is based on the observations that the rate of bacterial killing within the healthy lung greatly exceeds the rate of mucociliary transport out of the lungs, and

that the period of increased susceptibility to bacterial pneumonia does not coincide with the time of maximal inhibition of mucociliary clearance during viral infection.[18] The consensus of opinion seems to be that suppression of cellular defenses in the lungs, and suppression of systems which augment those defenses, is a primary factor in predisposing to bacterial infection.

The first line of cellular defense in the lung is the pulmonary alveolar macrophage. This cell, a specialized member of the mononuclear phagocyte system, is normally capable of a number of important immune functions.[19] The PAM performs non-specific defense by the same processes as are performed by the PMN. These processes include adherence, chemotaxis, phagocytosis, metabolic burst, killing, and enzymatic degradation of ingested material. The PAM, as well as PMN and certain lymphocyte types, also can perform antibody-dependent cellular-cytotoxicity (ADCC) in which large, nonphagocytosable, targets are destroyed.

In addition to its direct immunologic functions, there is evidence that the PAM has a central role in the overall pulmonary immune response. The PAM secretes soluble immunoregulatory molecules, processes antigens, and presents antigens to lymphocytes.[20-23] Certain lymphocytes, in response to PAM and antigen stimulation, secrete lymphokines capable of enhancing PAM activity.[24,25] The enhancement is non-specific and boosts PAM metabolic activity, phagocytosis, killing, and ADCC. Although the PAM has the capacity to perform many important immune functions, only non-specific mechanisms play important roles early in the course of

initial infectious exposure. Several days may be required for the induction of antigen specific defense, and lymphokine-mediated enhancement of macrophage function.

Often in concert with the alveolar macrophage, lymphocytes play an important role in pulmonary defense by producing specific antibodies and by augmenting the cellular immune response.[19] Lymphocytes are found in both pulmonary interstitial structures and in the alveolar lumen.[16,26,27] Lymphocyte traffic to and from the bovine lung has not been demonstrated, but movement to alveolar structures from blood, bronchus associated lymphoid tissue, and regional lymph nodes is likely.[28,29] The subtypes of lymphocytes obtained from human alveolar structures are similar to those found in blood. For T lymphocytes, populations consistent with helper, suppressor, and cytotoxic subtypes have been identified.[16,30-32] For B lymphocytes, surface immunoglobulins of IgM, IgD, IgG, and IgA have been identified.[16] There is evidence that lung lymphocytes function in a manner similar to blood lymphocytes. Lung lymphocytes respond to mitogens and antigens and in the mixed lymphocyte reaction.[16,20,33] Human lung lymphocytes also produce lymphokines when activated, which include macrophage migration inhibition factor, leukocyte inhibition factor, interleukin-2, interferon, and monocyte chemotactic factor.[16,34-36]

Soon after pulmonary exposure to *P. haemolytica*, there are impressive influxes of PMN into the lung, which may bolster the lungs' innate defense mechanisms.[37,38] Polymorphonuclear leukocytes originate in the bone marrow, and are released into peripheral blood circulation.

Under appropriate circumstances, chemotactic substances are generated or released from host cells and/or infectious agents. The substances diffuse to form a gradient along which PMN orient and move.[39] It is by this process that peripheral blood PMN quickly migrate into pulmonary tissue early in the course of pulmonic infection. Upon making contact with an appropriate target surface, the PMN may initiate phagocytosis or ADCC, depending on the size of the target. Efficiently recognized target surfaces are coated with opsonins, substances which promote phagocytosis. Important opsonins include certain subclasses of antibody, certain complement fragments, and perhaps fibronectin fragments and pulmonary surfactant.[40-42] Contact with the opsonin-target complex triggers a chain of events in the PMN (or PAM) which leads to internalization of the target particle, or to ADCC. Once internalized, the particles are located within phagosomes. Potent mixtures of degradative and bactericidal enzymes and substances are released into the phagosomes from 3 types of granules in which the substances are stored.[43] This process occurs by movement of the granules into the vicinity of the phagosome and subsequent fusion of their respective membranes, and is known as phagosome-lysosome fusion. The process of migration, phagocytosis, and phagosome-lysosome fusion require normal cytoskeletal function, which implies the necessity of normal microfilament and microtubule function.[44,45]

Greatly enhancing the bactericidal activity of the PMN (and PAM) is the oxidative burst which is stimulated following phagocytosis (in fact stimulated by many nonphagocytosable substances as well). During the

respiratory burst, oxygen consumption is sharply increased with associated production of hydrogen peroxide and related oxygen compounds. The respiratory processes occur close to the membranes enclosing the phagolysosome.[46] Many of the products produced are short lived, highly reactive, and very toxic both to the host and to infectious agents.[45,47]

From the foregoing discussion it is evident that a great number of mechanisms play a role in defending the lung against invading microorganisms. Initially, only non-specific mechanisms are available (which handle most of the organisms likely to invade the lung). After a period of days, specific mechanisms provided by involvement of lymphocytes supplant the non-specific mechanisms and greatly enhance potential antimicrobial defense. There are a number of possible ways, however, to impair at least some of the host defense mechanisms. A great deal of evidence suggests that stress or viral factors play a role in suppression of host defense and enhanced susceptibility to bacterial pneumonia.

HISTORY OF THE CONCEPT OF VIRAL-BACTERIAL SYNERGISM

The concept of viral-bacterial synergism dates back to the 19th century when influenza pandemics led to great numbers of human fatalities.[7] It was noted that mortality during influenza epidemics is associated with complicating bacterial pneumonia. In the 1960s, as many as 50% of the pneumonia cases in humans were found to be associated with evidence of viral infection.[8] Bacterial colonization of nasal passages was also correlated with viral upper respiratory tract infection in human infants.[48] Non-respiratory related cases of viral-bacterial synergism are abundant in the literature. The most prominent example of this currently is the acquired immunodeficiency syndrome (AIDS) in humans. Human immunodeficiency virus (HIV), formerly called human T cell-lymphotropic virus type III, directly affects a subset of T lymphocytes and results in marked susceptibility to many opportunistic infectious agents.[6] Parvovirus of cats or dogs often results in lymphopenia and suppressed immunity.[49,50] In mink, parvovirus can result in Aleutian disease, which profoundly affects humoral immunity.[49] Many other viruses, including measles, canine distemper, cytomegalovirus, Epstein-Barr, and infectious bursal agent can affect the immune function of humans or animals.[51-63] With evidence that many viruses have potential of disrupting normal immune functions, it is natural that some interest be directed at cattle and BRD.

EXPERIMENTAL EVIDENCE OF VIRAL-INDUCED IMMUNOLOGIC EFFECTS

Viral infection may result in enhanced susceptibility to bacterial disease by a number of possible mechanisms. The direct physical effect of complete or abortive viral replication can damage physical barriers such as the mucociliary blanket. Surface structures of airways may be altered to permit enhanced bacterial attachment and possible colonization. Constituents of mucus or alveolar fluid may be altered to allow more rapid bacterial replication or impeded migration of host phagocytic cells, as may occur in pulmonary edema. Macrophages or lymphocytes may become infected with the virus and undergo functional impairment or destruction. Only selected subsets of immune cells may be affected, as is the case with HIV which affects T lymphocytes responsible for delayed-type hypersensitivity and provision of helper functions for other T cell responses.[6]

In addition to direct viral effects, alterations of immune function may secondarily result from the activity of soluble or particulate factors of viral or host origin. Many viruses result in fever, malaise, tachypnea, elevated plasma cortisol, and interferon production. Plasma cortisol has effects on both humoral and cell-mediated immunity.[11,64] Interferon has been shown to affect chemotaxis, superoxide anion production, and cytotoxicity of neutrophils, as well as lectin-induced lymphocyte proliferation in the bovine system.[65-67] Immune complexes have been shown to inhibit a number of phagocytic cell functions, perhaps by exhausting those cells' functional capabilities.[68] These secondary effects often are observed slightly after peak titers of virus occur in

the host. Further research of virus-host interaction may uncover other common themes of immunologic alteration.

EVIDENCE OF VIRAL-BACTERIAL SYNERGISM IN BOVINE RESPIRATORY DISEASE

Following infection with endemic viruses such as PI-3 virus and IBR virus, some cattle experience inapparent infection, others develop mild symptoms and recover, and still others develop severe disease and do not survive.[69-77] The outcome of viral infection apparently involves many factors, the definition of which are largely speculative. There is ample evidence that severe forms of respiratory disease observed in the field result only when bacterial pneumonia is present.[12,71,78-80] Experimental infection with PI-3 virus, IBR virus, bovine RSV, or BVD virus do not generally lead to severe respiratory disease unless a bacterial agent is also involved.[69] The lesions of shipping fever pneumonia are described in terms of bacterial involvement.[10,79] Yet, numerous attempts to reproduce shipping fever with bacterial agents alone have met with very limited success.[78,81-86] These observations give support to the shipping fever equation, proposed by Hoerlein in 1964, which suggests that pneumonic pasteurellosis results from a combination of stress, viral infection and bacteria.[87]

The equation makes particular sense in consideration of the anatomy of the bovine respiratory tract and observations of bacterial nasal flora. Cattle suffer respiratory viral infections much more commonly during or shortly after shipment into the feedlot. During this same period, *Pasteurella haemolytica* can often be isolated in high concentration from upper respiratory tract secretions.[12] Since *P. haemolytica* can be demonstrated in inspired tracheal air of animals harboring these organisms in their upper respiratory tract,[88,89] the

lungs of shipped calves are doubtlessly exposed to higher numbers of potentially pathogenic organisms during and after shipment. The missing factor in the equation is how the *Pasteurellae* colonize the lung once they get there. It is assumed that a combination of stress and viral infection leads to reduced immunologic functions of the lung, and the bacteria which shower down from the upper respiratory tract can more easily colonize. There is a great deal of research currently being done to elucidate the mechanisms of the putative viral-induced immunologic deficits involved in bovine respiratory disease.

EFFECTS OF SPECIFIC VIRAL INFECTIONS IN CATTLE

Infectious bovine rhinotracheitis virus

Infectious bovine rhinotracheitis, caused by bovine herpesvirus 1, was first described by Miller in 1955 as a respiratory disease of feedlot cattle.[69] It was soon found that IBR virus could cause a wide variety of clinical manifestations other than those of the respiratory system, including reproductive, ocular, alimentary, and encephalitic symptoms.[69] This virus can result in rather impressive respiratory symptoms and pathologic lesions without synergistic infection with bacterial agents. Of the known viral agents involved in BRD, IBR virus shows the most promise of reproducing lesions and symptoms of shipping fever under laboratory conditions. Aerosol exposure to IBR virus will, in many cases, facilitate infection of the lung by an otherwise ineffective challenge with *P. haemolytica*, resulting in lesions and symptoms similar to field cases of shipping fever.[5,90-94] Evidence is accumulating that this enhanced susceptibility might be due, at least in part, to host immunosuppression by IBR virus.

Infection with IBR virus results in extensive loss of tracheal epithelium resulting in a reduction in the efficacy of the mucociliary defense system.[95,96] There are variable amounts of cellular debris and exudate which accumulate in the upper airways, and which may be inhaled and facilitate development of pneumonia.[69] Infectious bovine rhinotracheitis virus elicits a rapid rise in nasal interferon levels and a marginal rise in serum interferon levels.[65,97] There often is a reduction in peripheral blood leukocyte counts accompanying infection,

though secondary bacterial infection may quickly reverse that situation.[5,98]

Infectious bovine rhinotracheitis virus can replicate in the bovine PAM *in vitro*.[99,100] This infection results in impairment of immune receptor functions as well as ADCC activity of the PAM.[101] Efforts to reproduce these results *in vivo* with PAMs lavaged from infected animals, however, show that only a small proportion of PAMs become infected.[102] Their functional activity was not measurably altered in those studies. Later studies provided evidence that PAMs, lavaged from IBR virus-infected calves, may become activated subsequent to viral challenge.[103] These activated macrophages exhibited higher levels of Fc-mediated phagocytosis, higher levels of certain enzyme activities, and greater levels of prostaglandin E_2 production. Cellular cytotoxicity, interleukin-1 production, and chemotactic factor release were each reduced.

Peripheral blood lymphocytes from IBR virus-infected calves exhibit transiently depressed natural cytotoxicity against a xenogeneic cell line (YAC-1), but not against IBR virus-infected bovine fibroblasts.[65] The level of natural cytotoxicity against YAC-1 parallels the peripheral blood monocyte counts, which is evidence that population shifts may account for alterations of activity. Lymphocyte responses to the mitogens phytohemagglutinin and concanavalin A are also depressed during IBR virus infection.[65,104] The mechanism of this depression is not clear, but apparently other factors along with plasma interferon levels or viral induction of suppressor cell activity are involved.

Peripheral blood PMN from IBR virus-infected calves show reduced random migration and reduced directed migration toward a chemotactic stimulus.[65,98,105] There is evidence of an increase in phagocytic activity directed toward opsonized particles, and there may be a transient enhancement of oxidative metabolism as well.[65,98] Each of the alterations of PMN function observed during IBR virus infection has also occurred following *in vivo* exposure of cattle to bovine gamma interferon, which has been shown to activate bovine PMN (Roth JA, Iowa State University, Ames, Iowa: Personal communication, 1988). It is possible, then, that although PMN might be slow to emigrate into the infected alveolar lumen, they are activated and possess enhanced bactericidal activity.

Parainfluenza-3 virus

Bovine PI-3 virus was originally isolated from cattle with shipping fever, and reported by Reisinger et al. in 1959.[106] This virus is ubiquitous in much of the world, and most uncomplicated natural infections are mild or asymptomatic.[69] Colostrum deprived or otherwise seronegative calves often develop mild or moderate symptoms of respiratory disease following experimental exposure to PI-3 virus.[69,77,107] Isolation of PI-3 virus from cattle with respiratory disease is common.[69,77,108] Infection with PI-3 virus has been shown to predispose calves to more severe respiratory symptoms upon subsequent exposure to *P. haemolytica*[73]; a later study did not confirm that finding.[109] Impaired clearance of *P. haemolytica* has been reported

following PI-3 virus infection[110], but other investigators have failed to duplicate that observation.[111,112] While it's presence in BRD cannot be denied, many investigators currently are not convinced that PI-3 virus plays a large role.

Parainfluenza-3 virus is capable of replicating in bovine PAMs *in vitro*.[99] This infection has been shown to inhibit glass adherence, phagosome-lysosome fusion, Fc mediated phagocytosis, and cytotoxic activity.[113,114] Depression of *in vitro* lymphocyte responses to the mitogen phytohemagglutinin has been demonstrated following PI-3 virus infection of calves.[115] It is also reported that oxidative and myeloperoxidase activity of peripheral blood PMN is reduced in PI-3 virus infected-calves.[98]

Bovine viral diarrhea virus

Bovine viral diarrhea was first described by Olafson et al. in 1946 as an acute and often fatal gastro-intestinal disease similar to rinderpest.[116] Other cases were concurrently described which were called mucosal disease.[117] Some time later it was concluded that both syndromes were attributable to the same virus. A number of strains of BVD virus have been identified which vary antigenically and differ in cell culture cytopathogenicity, the latter characteristic giving rise to the categorization of strains as cytopathogenic and noncytopathogenic.[118,119] Cross neutralization occurs to only a limited extent between viral strains.[120] Depending on the strain of virus and time of exposure, a broad spectrum of clinical syndromes are possible in cattle, ranging from

mild or asymptomatic to severe. The gastro-intestinal, respiratory, lymphatic, reproductive (developing fetus), and integumentary systems are affected by BVD virus.[69,121] Classically, mucosal disease manifests itself as an acute, rapidly fatal disease with fever, profuse liquid diarrhea and buccal ulcers. Mucosal disease may sometimes be diagnosed as respiratory disease due to prominent signs of fever, tachypnea, and nasal discharge with minimal signs of diarrhea and mucosal lesions.[69] The virus has an affinity for lymphocytes and rapidly dividing cells.[69] This often results in leukopenia, and necrotizing effects on lymph nodes and Peyer's patches.[69]

Probably due to limited cross-neutralization between strains of BVD virus[119,120], and the phenomenon of persistent BVD virus infection[122], re-infection or superinfection with multiple strains of BVD virus is possible.[123-126] Experimental evidence suggests that the variability of clinical syndromes attributable to BVD virus may be due to immunopathologic interactions by the host to the two or more strains of BVD virus present in the superinfected animal.[126] Study of this virus thus involves not only primary infections in naive animals with various strains of BVD virus, but also infections with various strains in persistently infected animals, or in animals previously exposed to other strains.

Experimental exposure (to cytopathogenic, noncytopathogenic, or modified live vaccinal strains of BVD virus) of animals naive to BVD virus results in acute infection with few clinical signs, and a brief febrile response. If no complications by secondary infection occur,

recovery is rapid with accompanying rise in neutralizing antibody titer.[69,123,127-129] Exposure *in utero* results in a number of well documented developmental abnormalities, including repeat breeding, abortion, stillbirth, and weak calves.[69,130,131] Fetuses exposed to noncytopathogenic BVD virus prior to approximately day 125 of gestation may become persistently infected, becoming immuno-tolerant to that strain of BVD virus and persistently viremic (often without obvious untoward effects).[129,131,132] Persistent BVD virus infection is a considerable liability even in otherwise clinically normal calves. Whereas naive calves are relatively refractory to cytopathogenic BVD virus exposure, calves which are persistently infected can develop mucosal disease upon exposure to an appropriate cytopathogenic strain of BVD virus.[123,124,131] Superinfection with a given combination of cytopathic and noncytopathic BVD virus does not necessarily produce overt disease in persistently infected animals, however, and may result in neutralizing antibodies specific for the cytopathic strain.[126,131,133] Only appropriate combinations of cytopathic and noncytopathic BVD virus are capable of reproducing symptoms of mucosal disease in the laboratory (Bolin SR, National Animal Disease Center, Ames, Iowa: Personal communication, 1987).

In contrast to the acute BVD-mucosal disease syndrome, chronic BVD is characterized by an insidious onset and lesser severity.[69,130,131,134] Noncytopathic BVD virus is isolated from cattle which die from chronic BVD, as it is from cattle which succumb to mucosal disease.[123-125,134-136] It is possible, therefore, that a similar

pathogenetic mechanism exists between mucosal disease and chronic BVD, and that severity of the disease may depend on particular immunologic relationships between the host and the combination of BVD virus strains.[126]

It has been reported that dexamethasone can result in high susceptibility to severe disease by BVD virus, presumably by immunosuppression.[137] Attempts to reproduce this effect indicate that BVD virus acts in concert with dexamethasone, and that another disease agent, such as coccidia, may be involved in production of symptoms (Bolin SR, National Animal Disease Center, Ames, Iowa: Personal communication, 1987). Cattle given ACTH (which increases serum cortisol levels) along with modified-live BVD virus are more severely immunosuppressed than are cattle given modified-live BVD virus alone.[138]

In the field, infection with BVD virus commonly results in mild or inapparent symptoms.[129] It has been reported that BVD virus is the most common viral agent isolated from pneumonic lungs of cattle suffering shipping fever in the Texas Panhandle.[139] In that study most seroconversions to BVD virus were associated with seroconversion to *P. haemolytica* serotype 1. The predominant pathologic lesion was severe fibrinous pneumonia, in which cases only BVD virus and *P. haemolytica* were isolated. There were few lesions characteristic of BVD-mucosal disease.

Infection of otherwise normal cattle with cytopathogenic BVD virus results in a reduction in peripheral blood neutrophils and T lymphocytes.[127,128] During this period a bacteremia can be detected in

most animals.[140] There is a suppression of plasma cell development and immunoglobulin synthesis by splenic lymphoid cells infected with BVD virus *in vitro*.[141] Infection of cattle with cytopathogenic BVD virus, or a modified live vaccinal strain, reduces lymphocyte blastogenic responses to pokeweed mitogen[141], a mixed B and T cell mitogen, and to phytohemagglutinin, a T cell mitogen.[138,142] Cattle persistently infected with BVD virus also exhibit reduced lymphocyte activity.[143] There is evidence that a low molecular weight soluble mediator may be responsible for some of the effects of BVD virus on lymphocyte function. Bovine fetal lung cells infected with BVD virus *in vitro* elaborate a substance which inhibits bovine lymphocyte mitogenesis by concanavalin A.[144] Indomethacin, a cyclooxygenase pathway inhibitor, inhibits the elaboration of the suppressive factor(s). This provides some evidence that prostaglandins may be involved. *In vitro* infection of bovine monocytes with cytopathogenic or noncytopathogenic strains of BVD virus have also been shown to inhibit monocyte random and directed migration.[145]

Peripheral blood PMN from cattle experimentally infected with cytopathogenic or noncytopathogenic BVD virus exhibit reduced myeloperoxidase-hydrogen peroxide-halide activity.[127] This effect occurs without significant impairment of overall oxidative activity (measured by nitroblue tetrazolium reduction or native chemiluminescence) or phagocytic activity (measured by paraffin oil uptake). Serum levels of myeloperoxidase, however, are greater in BVD virus-infected cattle, indicating that degranulation may be enhanced during infection, and that

reduced levels of PMN myeloperoxidase-hydrogen peroxide-halide activity may result from enzyme depletion.[146] Vaccination of cattle with a modified live vaccine strain of BVD virus has also been shown to inhibit both myeloperoxidase-hydrogen peroxide-halide activity and ADCC, but has no significant effect on phagocytic activity (measured by *S. aureus* uptake).[138] Neutrophils of cattle persistently infected with BVD virus, on the other hand, demonstrate reduced phagocytic activity (measured by *S. aureus* uptake) without evidence of impaired myeloperoxidase-hydrogen peroxide-halide or ADCC activity.[143] Depressed lymphocyte blastogenic response to phytohemagglutinin, depressed PMN iodination, and enhanced *S. aureus* ingestion by PMNs has been demonstrated in a bull with symptoms of chronic BVD.[134] Although the reduction in lymphocyte responsiveness is consistent with persistent BVD virus infection, the PMN functional capacities differ from those noted in both acute and persistently infected cattle.

Bovine respiratory syncytial virus

Isolation of bovine RSV was reported in 1970 from cattle undergoing respiratory disease in Switzerland.[147] Soon thereafter, the virus was isolated from BRD outbreaks in Japan and the United States.[148-150] The virus is distinct from, but antigenically related to, human respiratory syncytial virus, a major cause of bronchiolitis and pneumonia in infants and young children.[151] Some cases of pneumonia caused by bovine RSV may result from a hypersensitivity reaction.[152] In these cases, re-infection or persistent infection may result in symptoms of atypical interstitial

pneumonia. Infection of cattle with bovine RSV is common, as indicated by the high percentage of cattle with serum antibody titers.[151] Under natural conditions, the severity of an infection may range from asymptomatic to fatal. Secondary bacterial infections are common in cattle and experimental bovine RSV infection has been shown to act synergistically with *P. haemolytica* infection in lambs.[153,154]

Scanning electron microscopy has demonstrated that ciliated respiratory epithelium is severely damaged after experimental infection with bovine RSV.[151] Experimental infection of calves results in severe damage to ciliated tracheal epithelium.[151] A small number of PAMs become infected by bovine RSV *in vitro*.[99] It has been shown that such *in vitro* infections with bovine RSV may not affect phagocytosis of latex beads, but do result in impaired Fc-receptor-mediated phagocytosis of antibody-coated sheep red blood cells (in spite of normal Fc-receptor function indicated by rosetting of SRBC), and increased levels of PAM lysosomal enzyme activity (measured by intracellular acid phosphatase.)[155] Pulmonary alveolar macrophages lavaged from calves experimentally infected with bovine RSV, however, exhibit enhanced ingestion of latex beads.[156] Human RSV infection *in vitro* has been shown to be associated with impaired lymphocyte function.[157]

Other viruses

Adenoviruses, enteroviruses, rhinoviruses, and others have been isolated from cases of BRD. There are apparently no reports concerning their effects on immunologic function in cattle.

SUMMARY

There is ample evidence that impairment of host pulmonary defense mechanisms by stressors and/or viruses are prerequisite for establishment of bacterial pneumonia in the BRD complex. Even under conditions favorable to development of bacterial pneumonia, however, only selected organisms successfully colonize the bovine lung, notably *Pasteurella haemolytica* serotype 1. The predominance of this single bacterial species and serotype suggests that despite immunologic impairment, there remain sufficient pulmonary defenses to prevent infection by the majority of bacteria contained in the bovine upper respiratory passages. It is evident, therefore, that specific bacterial virulence factors allow certain bacteria to take particular advantage of host pulmonary immunologic impairment. Whereas the bacterial virulence factors are insufficient by themselves to allow pulmonary colonization, when in concert with stressors and/or viruses they may often prove sufficient.

Due to the complexity of the pulmonary immune mechanisms and to the complexity of the infectious agents which can alter those mechanisms, it is difficult to assess the precise biological events and interactions necessary for the development of pneumonia. The importance of understanding these events, however, is underscored by the marginal efficacy of the many immunizing and chemotherapeutic agents presently used in efforts to prevent or to treat BRD. While managment procedures to reduce stress and minimize exposure to infectious agents would reduce the incidence of BRD, current marketing procedures of cattle do not allow many such measures to be implemented. Basic research into the cellular

and molecular mechanisms of virulence and immunosuppression involved in the BRD complex may eventually allow effective prophylaxis against the more serious forms of this disease.

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SECTION II. EFFECTS OF INFECTION WITH PARAINFLUENZA-3 VIRUS AND INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS ON NEUTROPHIL FUNCTIONS IN CALVES

Effects of infection with parainfluenza-3 virus and infectious bovine rhinotracheitis virus on neutrophil functions in calves

Robert E. Briggs, D.V.M

Marcus Kehrli, D.V.M.

Glynn H. Frank, D.V.M., Ph.D.

From the National Animal Disease Center, Agricultural Research Service, United States Department of Agriculture, Ames, IA, 50010

ABSTRACT

Calves were challenge-exposed in separate experiments with parainfluenza-3 (PI-3) virus or infectious bovine rhinotracheitis (IBR) virus. Blood neutrophils were assayed for functional activity every other day for at least three weeks by random migration, *Staphylococcus aureus* ingestion, antibody-dependent cell-mediated cytotoxicity, cytochrome-c reduction, iodination, and native chemiluminescence.

Exposure to PI-3 virus resulted in a brief febrile response and no other clinical signs. No alterations in total or differential WBC were detected. Chemiluminescence and iodination activities were reduced from activities before exposure.

Exposure to IBR virus resulted in mild clinical signs and a febrile response of several days' duration. Total WBC and mononuclear cell counts were reduced. Random migration was reduced, whereas *S aureus* ingestion was enhanced.

We concluded that infection of calves with IBR virus and PI-3 virus might directly or indirectly result in alterations of neutrophil function. The functional alterations are apparently different for each virus. These virus-induced alterations in neutrophil function might predispose calves to secondary bacterial pneumonia.

INTRODUCTION

In the bovine respiratory disease (BRD) complex, stress or viral infection are thought to precede bacterial pneumonia,[1] usually caused by *Pasteurella* spp.[2] Although various viruses, chlamydia, mycoplasmas, and bacteria have been implicated in the generation of BRD, there has been little success experimentally inducing the disease with any single agent.[3] Experimentally induced infection with infectious bovine rhinotracheitis (IBR) virus or parainfluenza-3 (PI-3) virus, then inoculation with *Pasteurella haemolytica* a few days later, has produced clinical BRD in several cases.[3-6] Results of studies in animals other than cattle with cytomegalovirus, influenza virus, and parainfluenza virus indicate that viral infection can predispose to bacterial infection.[3,7,8] The suspected mechanisms of this viral effect vary among viruses and host species. McGuire and Babiuk[9] found that experimental infection of calves with IBR virus reduced lymphocyte blastogenic response to phytohemagglutinin, and reduced random and directed migration of neutrophils *in vitro*. They concluded that a delay in recruitment of neutrophils into infected lungs might allow bacteria to colonize. The goal of the study reported here was to further define the alteration of neutrophil function after infection of cattle with IBR virus, and to determine whether infection with PI-3 virus might influence neutrophil function in cattle.

MATERIALS AND METHODS

Animals

Twelve colostrum-deprived Angus-type steer calves, approximately 1 year old, were used. The calves were allotted to treatment and nonexposed groups of six each. The same groups of calves were used for both experiments, one with IBR virus and one with PI-3 virus. Hemagglutination inhibition (HI) titers to PI-3 virus before experimental exposure ranged from 1:20 to 1:40. All calves were seronegative to IBR virus by the serum neutralization (SN) test. Three weeks after exposure, viral antibody titers were measured again.

Virus

Each virus was grown in secondary embryonic bovine kidney cells, then stored at -70 C. The titer of the PI-3 virus preparation was 10^8 $TCID_{50}$/ml. Titers of the IBR virus preparations were $10^{5.8}$ and 10^8 $TCID_{50}$/ml for first and second exposures, respectively.

Exposure

The PI-3 virus was aerosolized by use of 2 nebulizers (3 um droplets, each aerosolizing 0.1 ml/min, DeVilbiss) into a 1 m^3 chamber, from which each calf breathed for 15 minutes via a face mask. Three months later, the experiment was repeated with IBR virus. Two ml of IBR virus suspension was sprayed from an air-powered atomizer (352 g/cm^2) into each nostril of each calf in the experimental group. Because of only mild febrile responses and clinical signs, the calves were

re-exposed to IBR virus 4 days after the initial exposure. To evaluate clinical effect of infection, rectal temperatures were taken daily and clinical signs were subjectively assessed every other day. Blood was collected every other day into an EDTA-containing tube for total and differential WBC counts.

Neutrophil isolation

In each experiment, blood was collected every other day for one week prior to exposure and for at least two weeks after exposure. From each calf, 250 ml of blood was drawn into centrifuge bottles containing 30 ml of double strength acid-citrate-dextrose solution. Neutrophils were isolated by hypotonic lysis, as previously described.[10] Briefly, serum, buffy coat, and a liberal portion of the packed cell layer were removed by aspiration. Erythrocytes in the remaining packed cell layer (about 50 ml) were treated twice with lysis by addition of 2 volumes of phosphate-buffered water (0.0132M), then 45 seconds later by addition of 1 volume of triple strength phosphate buffered saline solution (2.7% saline, 0.0132M phosphate). After a final wash in phosphate buffered saline (0.0132M, PBS), the cells were resuspended at 5×10^7/ml in PBS. Purity was assessed by differential counting of the neutrophil preparation.

Neutrophil function assays

Evaluation of random migration under agarose, *Staphylococcus aureus* ingestion, antibody-dependent cell-mediated cytotoxicity (ADCC),

cytochrome-c reduction, iodination, and native chemiluminescence were done essentially as described by others.[10-12] All test samples were incubated at 39 C. Cytochrome-c reduction was done in triplicate and included control wells without neutrophils. The ADCC assay was done in quadruplicate and included control wells without neutrophils. Native chemiluminescence was not replicated, but included a nonstimulated control sample (without opsonized zymosan) for each neutrophil sample. All other assays were run in duplicate. *Staphylococcus aureus* ingestion and iodination included control tubes without neutrophils for total and background radioactivity determinations.

Statistical analysis

The data were split into two periods, a control period from 1 week before exposure through 2 days after exposure, and a period of infection from 4 through 11 days after exposure. Postexposure day 2 was included in the control period because there was little evidence of clinical disease at that time, and no effect on neutrophil function was evident. Exposed calves were considered to be in the viral incubation stage. No differences between exposed and nonexposed groups were evident 2 weeks or more after exposure. An analysis of variance procedure was used to account for overall differences between control and treatment groups, calves within each group, and date of sampling. Treatment effect was judged from the treatment group by time interaction. The error term was residual variation of calf within group within day of sampling. Conservative degrees of freedom were used (1 for each calf rather than 1

for each blood sample evaluated) in an F-test to determine significant treatment effect. Sufficient divergence of the virus exposed group from the control group, after virus exposure, thus would indicate a significant treatment effect. This statistical model would account for the effects of possible preexisting differences in neutrophil activity between groups and between calves within groups.

RESULTS

Clinical response to infection

Five of 6 calves exposed to PI-3 virus were febrile (rectal temperature > 39.4 C), 3 of which were febrile for 3 or more days (Fig 1). All 6 calves were febrile by 5 days after exposure to IBR virus (Fig 1). Five of these calves remained febrile for 8 or more days. Calves had other mild clinical signs of disease including tachypnea, swelling and reddening of nasal mucosa, and moderate mucoid nasal discharge. Postexposure HI titers to PI-3 virus ranged from 1:160 to $\geq$ 1:640 in the exposed group. Postexposure SN titers to IBR virus were $\geq$ 1:256 in the exposed group (except in one calf that had a titer of 1:128). Calves in the nonexposed group remained healthy and had no increases in viral antibody titer to IBR virus or to PI-3 virus.

Blood leukocyte counts

Mean blood leukocyte counts for control calves during each experiment were within an accepted normal range[13] (Table 1). Total and differential counts of PI-3 virus-exposed calves did not change significantly after exposure and remained within an accepted normal range[13] (Fig 2). Total WBC count and mononuclear cell count decreased significantly ($P<0.02$) in the IBR virus-exposed calves after exposure, although leukocyte counts remained within an acceptable normal range[13] (Fig 3).

Neutrophil preparation purity

Neutrophil preparations had a mean > 90% of polymorphonuclear neutrophils (range, 75 to 99%). The samples with less purity were associated with less cell yield, and greater numbers of contaminating mononuclear cells. A few samples contained up to 18% eosinophils. An analysis of variance procedure was used to determine whether the variation of eosinophil, mononuclear cell, or neutrophil percentages were correlated with measurable functional differences. No significant association was found at these degrees of contamination.

Neutrophil function factors

Mean values of neutrophil function factors for control calves during each experiment were used to establish baseline values for each factor (Table 1). Exposure to PI-3 virus caused no significant change in random migration, *S aureus* ingestion, cytochrome-c reduction, or ADCC. However, both chemiluminescence and iodination were reduced ($P<0.02$) from amounts before infection (Fig 4). Infection with IBR virus caused no significant change in cytochrome-c reduction, ADCC, chemiluminescence, or iodination. However, random migration was reduced ($P<0.02$) and *S aureus* ingestion was enhanced ($P<0.01$) from amounts before infection (Fig 5).

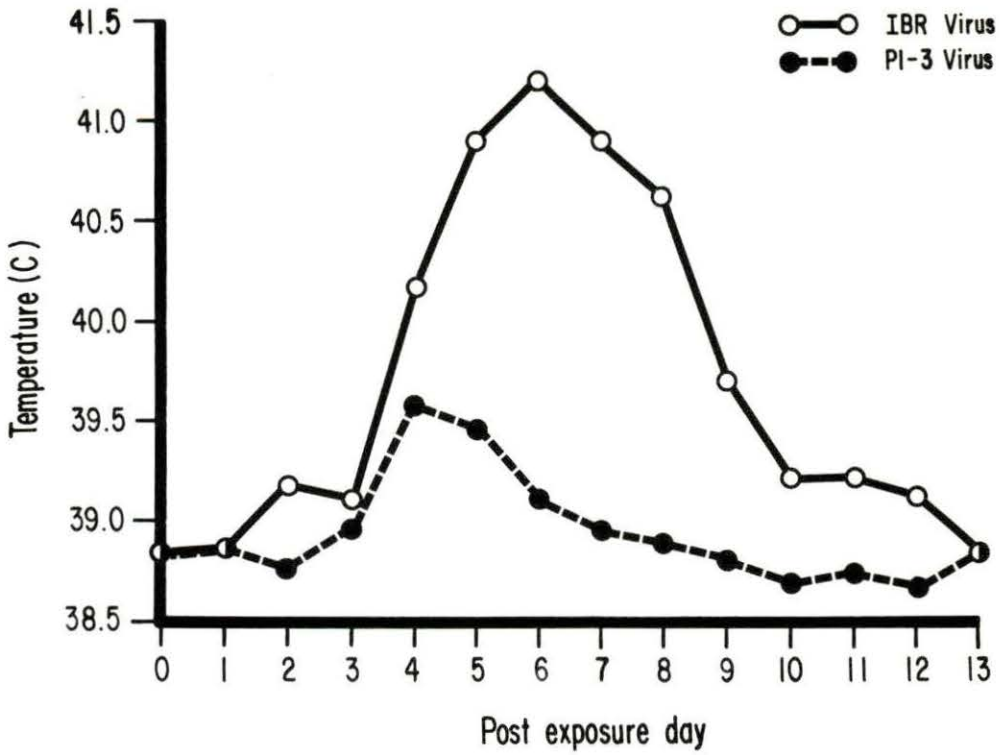

FIGURE 1. Mean rectal temperature for exposed calves after exposure to infectious bovine rhinotracheitis virus or parainfluenza-3 virus

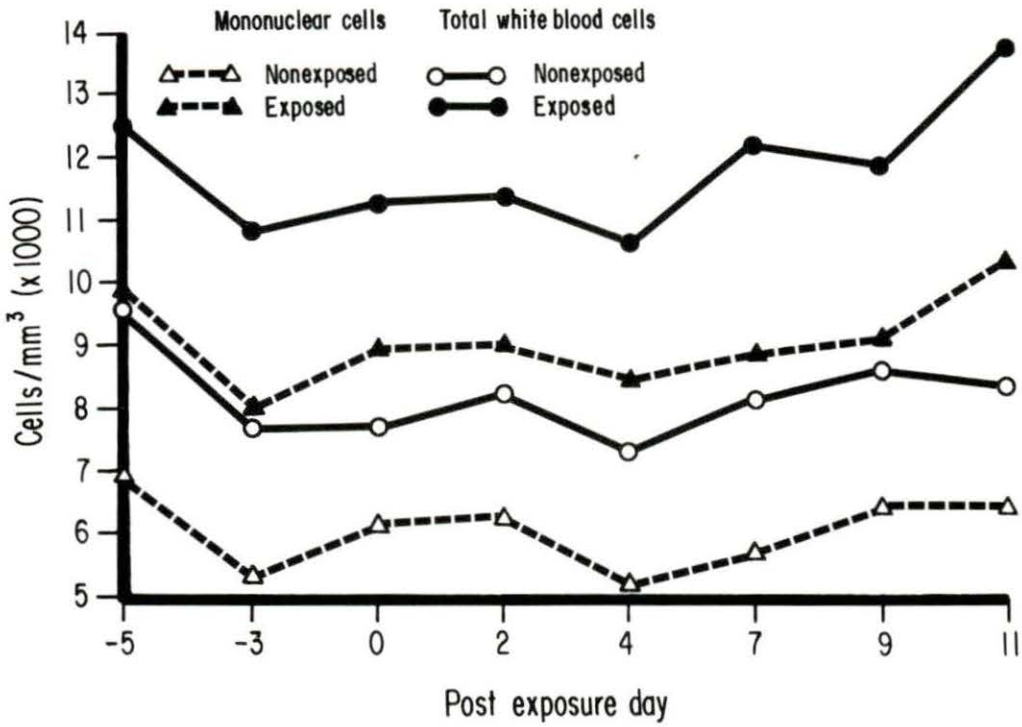

FIGURE 2. Total WBC and mononuclear cell counts before and after exposure to parainfluenza-3 virus

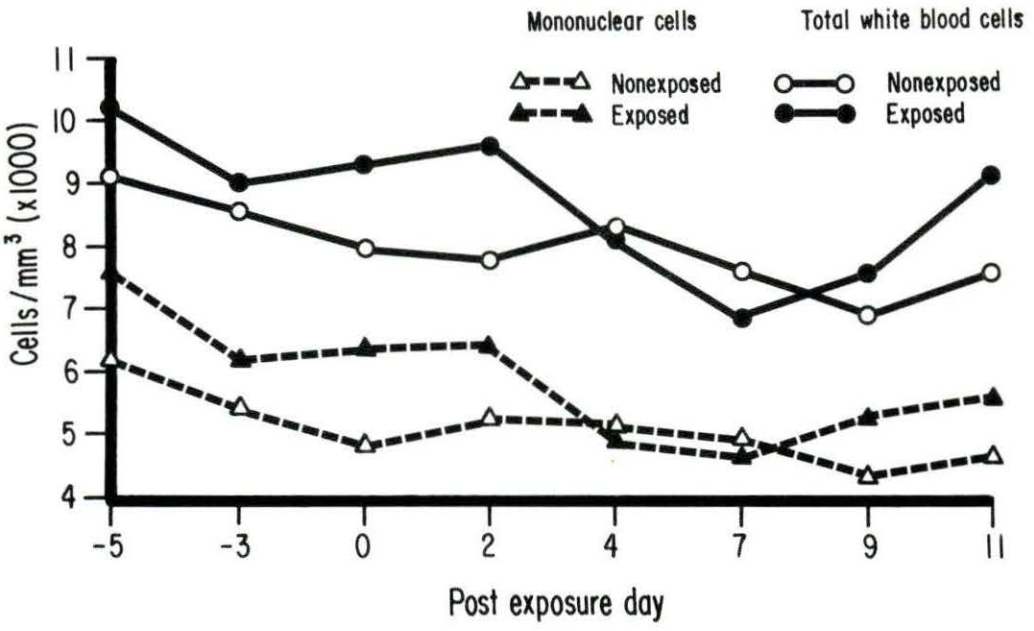

FIGURE 3. Total WBC and mononuclear cell counts before and after exposure to infectious bovine rhinotracheitis virus

TABLE 1. Mean values for neutrophil function factors and WBC count for control calves (n=6) during experiments with infectious bovine rhinotracheitis (IBR) virus and parainfluenza-3 (PI-3) virus

	PI-3		IBR		
Factor	Mean	SEM	Mean	SEM	Units
Iodination	23.8	0.77	42.0	1.7	-[a]
Cytochrome-c reduction	0.40	0.01	0.35	0.01	OD_{550}
Antibody-dependent cell-mediated cytotoxicity	93.4	3.0	90.0	4.0	-[b]
Native chemiluminescence	2263	66	2218	82	-[c]
S aureus ingestion	47.9	1.6	34.6	1.1	% ingestion
Random migration	10.9	0.2	13.1	0.3	mm
Total WBC count	8202	240	7850	138	$cells/mm^3$
Mononuclear cells	6039	198	4932	116	$cells/mm^3$
Neutrophils	2082	119	2797	113	$cells/mm^3$
Eosinophils	81	14	121	25	$cells/mm^3$

[a] nmoles NaI/10^7 PMN/hr.

[b] Percent chromium release.

[c] Sum of 10 counts over 70 minutes in liquid scintillation counter in out of coincidence mode.

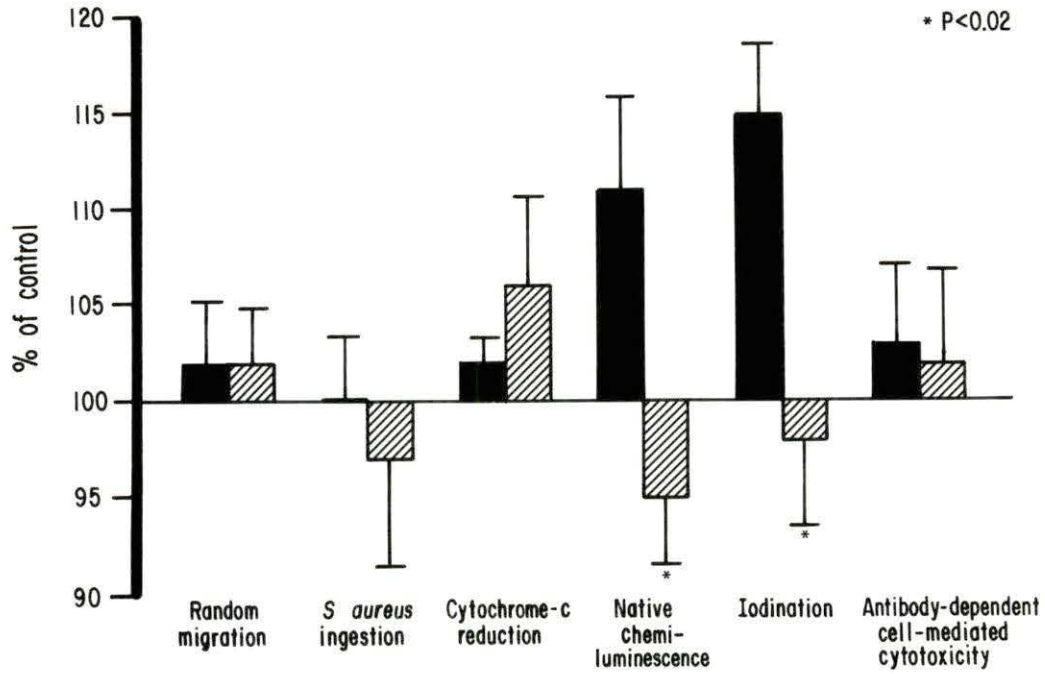

FIGURE 4. Neutrophil functions before and after infection with parainfluenza-3 virus infection. Days before exposure through postexposure day 2 (solid) and postexposure days 4 through 11 (diagonal lines) are shown as percentage of control. Statistical significance was assessed by use of treatment group by time interaction

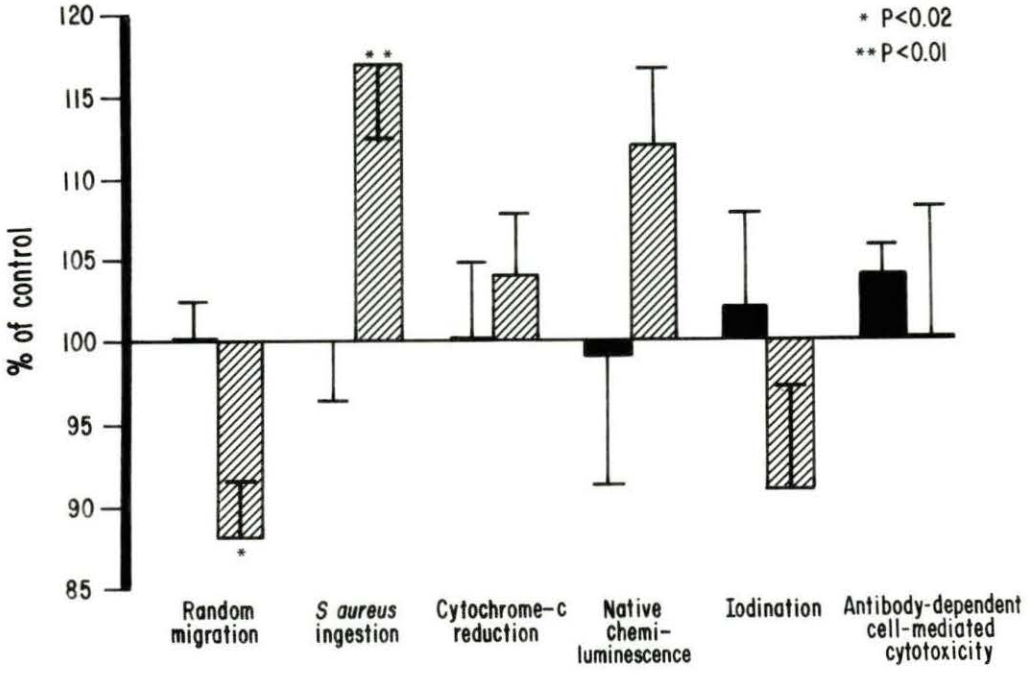

FIGURE 5. Neutrophil functions before and after infection with infectious bovine rhinotracheitis virus. Days before exposure through postexposure day 2 (solid) and postexposure days 4 through 11 (diagonal lines) are shown as percentage of control. Statistical significance was assessed by use of treatment group by time interaction

DISCUSSION

Results of our study indicate that infection with PI-3 virus or IBR virus can directly or indirectly alter blood neutrophil function in calves. Viral infection might cause immunosuppression by various mechanisms. Direct viral effects might be caused by the action of soluble or insoluble viral products, or by viral infection of immune cells, resulting in functional impairment or cellular destruction. Indirect effects might result from fever, high plasma cortisol concentration, interferon production, immune complexes, immune hypersensitivity, or by activation of latent infections with other agents. In addition, there is evidence that blood neutrophils are not functionally homogeneous.[14] A population shift might result in alteration of measured functional capacity.

Parainfluenza-3 virus is ubiquitous in much of the world. Cattle in field situations are seldom seronegative.[15] Partly because of this, most uncomplicated natural infections are mild or without clinical signs. Immunity after vaccination or natural exposure is partial rather than complete, and it is possible to experimentally re-infect calves after prior exposure to PI-3 virus.[15] The calves in our study had become seropositive to PI-3 virus before experimental exposure, as evidenced by seronegative (SN) status at 1 month of age, and low HI titers just before experimental exposure. The mild fevers, minimal clinical signs, and the sixteen-fold rise in serum HI titers to PI-3 virus after PI-3 virus exposure were compatible with other experimental PI-3 virus re-infections in calves.[16,17] Despite the mild clinical course of PI-3 viral

infection, iodination and chemiluminescence by neutrophils were reduced from amounts before infection. Reduced iodination activity by neutrophils has been noticed also by Roth and Kaeberle[18] in cattle given a modified live vaccine strain of bovine viral diarrhea virus.

In both virus-exposure experiments, we used three measures of neutrophil oxidative metabolism: native chemiluminescence, cytochrome-c reduction, and iodination. Native chemiluminescence measures several oxidative reactions during the oxidative burst of the neutrophil, thus reflecting overall neutrophil oxidative activity. Cytochrome-c reduction measures superoxide anion production by the neutrophil. The iodination reaction depends on phagocytic activity, oxidative metabolism, degranulation, and the capacity of myeloperoxidase to iodinate tyrosine residues.[10] Because we did not observe a change in cytochrome-c reduction, superoxide anion production appears normal. It has been reported that the chemiluminescent response depends somewhat on myeloperoxidase activity; hence, chemiluminescence might correlate with iodination.[19,20] The rate-limiting step for the iodination reaction appears to be associated with myeloperoxidase activity subsequent to degranulation.[10] The observed reductions in chemiluminescence and iodination subsequent to PI-3 viral infection then might have been caused by a loss of myeloperoxidase activity. This loss of activity might be caused by direct effects on myeloperoxidase, by partial inhibition of degranulation, or by a reduction in amount of myeloperoxidase available in primary granules. Reduction of phagosome-lysosome fusion has been reported in PI-3 virus-infected bovine alveolar macrophages in vitro.[21]

Ultrastructural analysis or fluorometric neutrophil function assays might clarify the reason for the apparent loss of myeloperoxidase activity. In any case, it appears that infection with PI-3 virus directly or indirectly reduced one of the neutrophil's more important bactericidal mechanisms.

In retrospect, the initial exposure to IBR virus was adequate. On the fifth day after exposure, all exposed calves were febrile. Reported incubation periods for IBR virus range from 2 to 6 days.[22] The initial IBR viral exposure in our experiment was lower than that used in our previous experiments.[23] Because the incubation period for IBR virus has been shown to be inversely related to the magnitude of viral exposure,[24] the longer-than-expected incubation period is not unreasonable. Re-exposure to IBR virus 4 days after the initial exposure apparently was unnecessary, but probably did no harm because the calves were likely beginning to shed virus by this time,[25] although no virus isolation procedures were done in these experiments.

It is improbable that prior exposure to PI-3 virus altered the effects of IBR viral infection, because effects of PI-3 virus are not known to be latent. However, the ubiquity of PI-3 virus in the field suggests that persistent infections might develop, so PI-3 virus could potentially exert some effect on the response of cattle to other agents. Also considering the ubiquity of PI-3 virus, such effects would be commonplace. In our experiment, we could not demonstrate functional alterations of neutrophils beyond 2 weeks after exposure to PI-3 virus. Values of infected calves returned to the baseline values of control

calves within 2 weeks after exposure to PI-3 virus and remained there until exposure to IBR virus 3 months later.

Infection with IBR virus was associated with reduced random migration and enhanced *S aureus* ingestion, whereas neutrophil oxidative capacity was largely unaffected. The reduction in neutrophil random migration supports the observation by McGuire and Babiuk,[9] who noticed a reduction in random migration and directed migration toward a chemotactic stimulus. They postulated that delay of neutrophil recruitment into infected lungs is responsible for enhanced susceptibility to pneumonia. Bielefeldt Ohmann and Babiuk[26] noticed a transient increase in neutrophil superoxide anion production from day 4 through day 12 after exposure to IBR virus in calves given low or moderate doses of virus. We did not observe a change in superoxide anion production measured by cytochrome-c reduction. In our experiments,the neutrophil's oxidative killing mechanisms appeared unimpaired, and their ability to ingest opsonized particles actually was enhanced in IBR virus-infected calves. The alterations of neutrophil function observed during IBR virus infection are consistent with alterations due to gamma interferon exposure. Gamma interferon has been shown to activate bovine neutrophils, consistently resulting in reduced random and directed migration, and often resulting in enhanced *S aureus* ingestion, ADCC, and certain measures of oxidative metabolism (Roth JA, Iowa State University, Ames, Iowa: Personal communication, 1988).[27,28] This suggests that if neutrophils get into the lungs, they should be effective at clearing bacteria, assuming that those bacteria are opsonized. A delay in recruitment of neutrophils to

lung tissue invaded by bacteria, however, might allow the bacteria to become established and replicate rapidly. In addition, the delay might allow *Pasteurella haemolytica* to elaborate sufficient quantities of leukotoxin to kill the neutrophils that finally arrive.[29,30]

Infection with IBR virus or PI-3 virus has been shown to result in the proliferation of *P. haemolytica* in the upper respiratory tract of calves.[23] *Pasteurella haemolytica* has been isolated from droplets in inspired tracheal air.[31,32] The lungs, therefore, tend to be exposed to greater amounts of *P. haemolytica* during viral infection. Normally the lungs can clear *P. haemolytica* rapidly.[33,34] Virus-infected calves, however, are often unable to clear *P. haemolytica* from the lungs.[24,35-37] Because the neutrophil is one of the predominant cells in inflamed lungs,[38] and influxes of neutrophils into the lungs correspond with clearing of challenge bacteria,[39] there is reason to believe it is an important cell in pulmonary defense. If virus-induced defects in neutrophil function account for the enhanced susceptibility of calves to bacterial invaders, the mechanisms appear to differ between IBR virus and PI-3 virus. Whereas neutrophils of PI-3 virus-infected calves apparently are recruited in normal numbers,[6] their oxidative bactericidal activity might be reduced. Neutrophils of IBR virus-infected calves apparently are recruited in less than normal numbers.[9] Their oxidative bactericidal activity, however, appears to be adequate.

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SECTION III. PLASMIDS OF *PASTEURELLA HAEMOLYTICA*

Plasmids of *Pasteurella haemolytica*

Robert E. Briggs, D.V.M.

Robert A. Schneider

Glynn H. Frank, D.V.M., Ph.D.

From the National Animal Disease Center, Agricultural Research Service, United States Department of Agriculture, Ames, IA 50010

ABSTRACT

Plasmids from 518 isolates of *Pasteurella haemolytica* from pneumonic bovine lungs and bovine nasal passages ranged in size from 2 to 7 megadaltons. The plasmid profiles were limited in number and were associated with serotype. None were exclusive to lungs or to nasal passages. There was no association of plasmids with virulence although ampicillin resistance was found to be plasmid mediated. To assess whether plasmid content might prove useful in epidemiologic studies of *P. haemolytica*, 200 calves were sampled during shipment to the feedlot. Plasmid profiles of isolates obtained show that calves can simultaneously shed multiple strains of *P. haemolytica*, and that shedding may shift from one strain to another over time. The small number of plasmid profiles found in *P. haemolytica* limits their value in epidemiologic studies.

INTRODUCTION

Pasteurella haemolytica is commonly carried in the nasal passages of healthy cattle and sheep, and is frequently associated with pneumonia in both species.[1,2] Over 12 serotypes have been recovered.[1,3,4] Serotypes 1 and 2 are frequently isolated from the nasal passages of healthy cattle.[1,5] Serotype 1 is recovered from nasal passages with increased frequency and in greater quantity from stressed cattle.[1,5] Isolates from pneumonic bovine lung are primarily serotype 1.[1] Other serotypes are infrequently isolated.[1]

Since bovine respiratory disease (BRD) involving *P. haemolytica* is associated primarily with isolates of serotype 1, serological surveys are of limited use in epidemiologic studies of *P. haemolytica* in BRD. A similar situation exists with human *Salmonella* and staphylococcal infections in which isolates of common serotype are encountered with common antibiotic sensitivity but with different genetic characteristics.[6,7] In these studies, rapid techniques were utilized to identify plasmid profiles of the organisms under study. The plasmid profiles differed among isolates which otherwise appeared identical and thus proved useful in epidemiologic consideration of those isolates. The objective of the present study was to utilize a rapid plasmid identification technique to determine the number and size of plasmids carried by bovine isolates of *P. haemolytica*, to search for plasmids carried by lung isolates, or associated with antibiotic resistance, and to examine the suitability of plasmid screening for epidemiological studies of *P. haemolytica* in BRD.

MATERIALS AND METHODS

Plasmid isolation and resolution

A series of *P. haemolytica* isolates from naturally occurring infections in cattle from several areas of the United States were examined for their plasmid content by a modification of the method of Kado and Liu.[8] Briefly, a small sample of bacteria was suspended in 40ul TE buffer (10mM Tris, 1mM EDTA, pH 8.0) and lysed for 30 minutes at 57C in 100ul of a solution containing 3% SDS, 30mM NaOH, and 10mM Tris (pH 12.5). The lysate was extracted with 50:50 chloroform:distilled-phenol, chilled on ice, and centrifuged at 12,000G for 10 minutes. The aqueous supernatant then could be aspirated (taking care to avoid the material at the interface) and loaded onto 1% agarose vertical gels. The gels were electrophoresed at approximately 8 volts/cm in tris-borate buffer, pH 8.25. Molecular weight of plasmids were estimated by comparison with plasmids of known molecular weight. Serotypes were determined by plate agglutination against known antisera.[9] One hundred ten isolates of serotype 1, 2 isolates of serotype 2, 3 isolates of serotype 6, and 2 isolates of serotype 11 were analyzed from pneumonic lung. Three hundred twenty five isolates of serotype 1, 75 isolates of serotype 2, and one isolate of serotype 11 were analyzed from nasal passages.

Antibiotic resistance

One hundred and five isolates of serotype 1 were tested for antibiotic sensitivity by the disc diffusion technique. Antibiotics tested were: ampicillin, penicillin, gentamicin, neomycin, streptomycin,

spectinomycin, erythromycin, lincomycin, triple sulfa, and tetracycline. Since a high degree of correlation was noted between plasmid carriage and ampicillin resistance, manipulations were performed to see whether ampicillin resistance is plasmid mediated. Plasmid DNA was used from an ampicillin resistant isolate of *P. haemolytica* serotype 1 to transform *E. coli* K-12 strain HB101 by a calcium chloride technique.[10] Ampicillin resistant colonies were selected. In order to show that *P. haemolytica* DNA had in fact been transferred, radiolabeled plasmid DNA from the donor *P. haemolytica* strain was used to hybridize with transformed and untransformed *E. coli* by a colony hybridization technique.[11]

Epidemiologic study

For epidemiologic study, 100 calves in each of two studies were sampled at two or more times during shipment. In the first study, calves were sampled by nasal swabs (one per nostril) upon arrival at the order-buyer barn, and subsequently upon arrival at the feedlot. In a later study, calves were sampled at the farm of origin, upon arrival at the order-buyer barn, upon arrival at the feedlot, and on 3 subsequent occasions at the feedlot. Isolation of *P. haemolytica* was performed on blood agar plates, and serotypes were confirmed by plate agglutination.[9] Five isolates of serotype 1 from each calf, on each day in which shedding was noted, were analyzed for plasmid content.

RESULTS

Serotype 1 isolates displayed 3 plasmid profiles with 2 predominating, as well as a large number of strains with no plasmids (Fig 1). The predominant profiles (a and b) were observed in 24% and 53% of the serotype 1 isolates respectively. These isolates, and plasmidless strains (23%), were widely distributed in the United States and were found in both nasal passages and pneumonic lungs in approximately equal proportions. Profile c was obtained from a single lung isolate. The profiles included a total of 6 bands ranging in apparent MW from 2.5 to 2.9 Mdal. Several plasmids of identical size were common to more than 1 profile.

Serotype 2 isolates included 3 profiles plus strains with no plasmids (Fig 1). One of these profiles, plus plasmidless strains, were found to be widely distributed in the United States. The profiles included 2 plasmids of 2.7 and 2.4 Mdal.

One of the 3 serotype 6 isolates contained 3 plasmids of 3.6, 3.0, and 2.6 Mdal (Fig 1). The other two isolates contained no plasmids.

Each of the 3 serotype 11 isolates contained a different profile with only one plasmid size shared among 2 of the isolates (Fig 1). These profiles included 5 plasmids, one at 2.3 Mdal and the others ranging form 5.3 to 7.1 Mdal.

No two isolates of different serotype carried common plasmid profiles, although plasmidless strains are common in serotype 1 and present in many of the other recognized serotypes (unpublished data). Nasal isolates of *P. haemolytica* from a single herd at a given time often

contained varied plasmid profiles (Fig 2).

Of 105 isolates tested for antibiotic sensitivity by the disc diffusion technique, 70 were resistant to ampicillin and 76 contained plasmids. All resistant isolates contained plasmids.

Screening of *E. coli*, transformed with *P. haemolytica* plasmid DNA, on ampicillin selective media demonstrated that some transformants carried plasmids of sizes (2.5 and 2.7 Mdal) similar to the donor *P. haemolytica* strain. Other transformants were plasmidless. When the transformants were hybridized to radiolabeled plasmid DNA isolated from the *P. haemolytica* donor strain, strong hybridization was observed with the plasmid containing transformants, weak hybridization was observed with the plasmidless transformants, and no hybridization was observed with untransformed HB101 (data not shown).

In the first epidemiologic study, three plasmid profiles were identified (Table 1). It was possible to isolate *P. haemolytica* from 79% of the calves, but only 12% were identified as carriers on both dates. Of the 12 %, 4 changed from one strain to another, judged by plasmid content, and one calf shed 2 strains simultaneously. In eleven cases where shedding occurred on a single date, it was possible to recover 2 strains of *P. haemolytica* on that same date.

In the second epidemiologic study only 2 plasmid profiles were identified (Table 2). It was possible to recover *P. haemolytica* from 75% of the calves, of which 59% shed on 2 or more dates of sampling. Only in 4 cases was simultaneous shedding of 2 strains noted. Only one calf changed from shedding one strain to another.

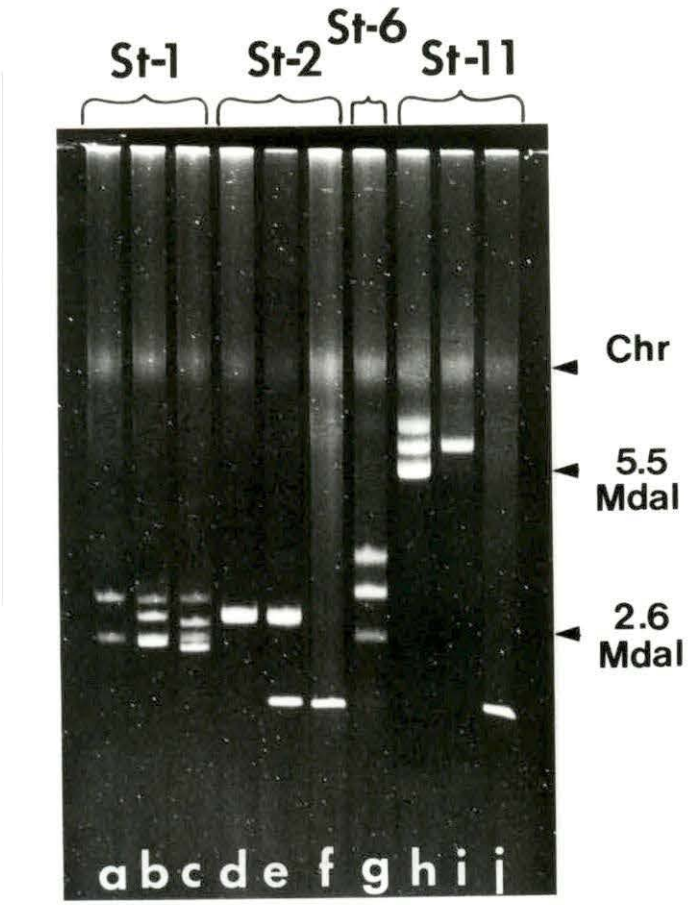

Figure 1. Plasmid profiles obtained from bovine isolates of *Pasteurella haemolytica* serotypes 1,2,6, and 11. Markers correspond to chromosomal DNA and plasmids of known molecular weight (5.5 and 2.6 Mdal)

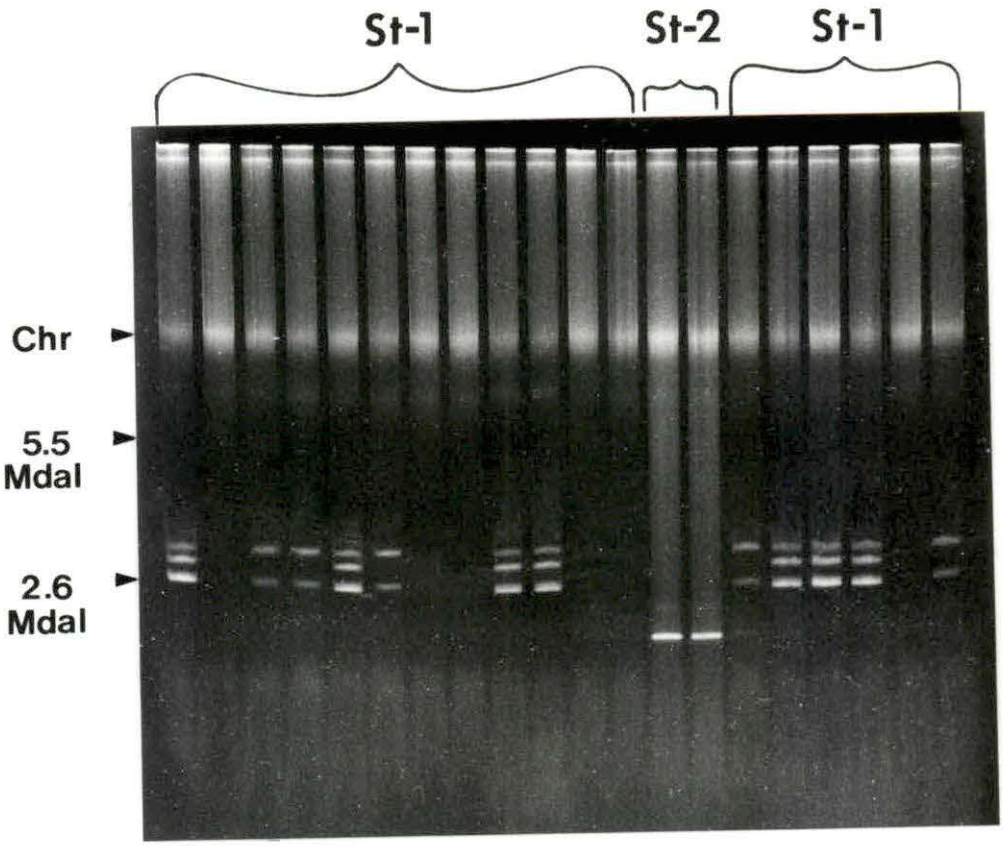

Figure 2. Typical plasmid profiles of nasal isolates of *Pasteurella haemolytica* serotypes 1 and 2 obtained from a single herd of cattle during shipment to the feedlot. Markers correspond to chromosomal DNA and plasmids of known molecular weight (5.5 and 2.6 Mdal)

Table 1. Summary of plasmid profiles of *Pasteurella haemolytica* isolated from 100 calves sampled on 2 dates during shipment to the feedlot

Plasmidless	Profile a	Profile b
8%	38%	54%

Isolations	Calves	Mixed Profiles	Changed Profiles
None	21	-	-
Once	67	11	-
Twice	12	1	4[a]

[a]Between dates, three calves changed from shedding isolates containing plasmid profile 1 to isolates containing profile 2, one calf changed from profile 2 to plasmidless.

Table 2. Summary of plasmid profiles of *Pasteurella haemolytica* isolated from 100 calves sampled on 6 dates during and after shipment to the feedlot

Plasmidless	Profile a	Profile b
0%	82%	18%

Isolations	Calves	Mixed Profiles	Changed Profiles
None	25	-	-
Once	16	2	-
Twice	22	1	1[a]
Three	27	1	0
Four	10	0	0

[a]Between dates, one calf changed from shedding isolates containing plasmid profile 1 to isolates containing profile 2.

DISCUSSION

The results of this study do not support a major role for plasmid-borne virulence factors of *P. haemolytica* in bovine respiratory disease. The frequency of recovery of a given plasmid profile from lung isolates was no greater than from nasal isolates. This suggests these plasmids offer no great selective advantage in ability to colonize lung. Plasmidless strains comprised 25% of the isolates recovered from pneumonic lung, demonstrating plasmids are not necessary for virulence.

Zimmerman and Hirsh passed ampicillin resistance from a biotype T isolate of *P. haemolytica* to *E. coli* by transformation with plasmid DNA, but their transformed ampicillin resistant *E. coli* failed to demonstrate plasmid DNA.[12] They speculate the resistance gene may be coded on a transposable element. Since we were able to pass ampicillin resistance to *E. coli* with *P. haemolytica* plasmid DNA, it is evident that the ampicillin resistance gene is plasmid borne in *P. haemolytica* serotype 1, as it was in the biotype T isolate which Zimmerman and Hirsh utilized. Hybridization data show that *P. haemolytica* DNA was passed to all *E. coli* transformants, since untransformed *E. coli* did not hybridize while all transformants did. Since plasmidless transformants hybridized with radiolabeled *P. haemolytica* plasmid DNA, it is likely that the ampicillin resistance gene is encoded on a transposable element which incorporates into the *E. coli* genome. In contrast to the data of Zimmerman and Hirsh, however, we were able to recover intact plasmids from some transformants, demonstrating that *P. haemolytica* plasmids can exist in *E. coli* K-12 as independent replicons.

The recovery of identical plasmid profiles within a given serotype from widely separated parts of the United States may indicate that only a few plasmids can be stably maintained in a given serotype of *P. haemolytica*. It may, however, be a reflection of the wide dissemination of a few strains of *P. haemolytica* through transportation of cattle. Since both serotypes 1 and 2 of *P. haemolytica* may be isolated from a single herd, and since a given animal may simultaneously shed 2 strains of *P. haemolytica* serotype 1, it is evident that a good deal of mixing occurs among strains of *P. haemolytica*. If transfer of plasmids were possible between serotypes it would be expected that serotype would not be inferred by plasmid profile. The fact that plasmidless strains are found among many serotypes of *P. haemolytica*, and that plasmids of serotype 1 isolates do not hybridize with genomic DNA largely precludes the possibility that plasmids specify serotype. The obvious conclusion is that either *P. haemolytica* transfers plasmid DNA to other *P. haemolytica* with great difficulty, or that certain strains of *P. haemolytica* are incompatible with particular plasmids. By either freeze-thaw techniques or calcium-chloride techniques, plasmid transfer from *P. haemolytica* serotype 1 into plasmidless strains has not been successful (unpublished results). The evidence therefore points to incompatibility of plasmids as the basis of serotype specificity of plasmid profiles. However, among isolates obtained from sheep (which commonly carry any serotype contained in biotype A *P. haemolytica*), a given plasmid profile may be found in a subset of *P. haemolytica* serotypes (except serotype 1, unpublished results). If profiles carried

by multiple serotypes have identical plasmids in common, it may be possible to develop effective protocols to transfer plasmids among a limited number of *P. haemolytica* serotypes.

At the present time, the infectious spread of *P. haemolytica* in outbreaks of BRD is subject only to speculation. Serotyping of *P. haemolytica* isolates has shown that BRD involving *P. haemolytica* is primarily associated with serotype 1.[3] Many healthy calves carry *P. haemolytica* in low numbers in their upper respiratory tract.[3,4] It is possible that calves undergoing viral infection or stress may develop pneumonia caused by their own flora. Alternatively, these same calves may be susceptible to *P. haemolytica* shed by other calves. Since several plasmid profiles exist in isolates of *P. haemolytica*, and these different isolates often coexist in the same herd of cattle, further epidemiologic studies may be possible. Evidence obtained in the present experiment neither supports nor disproves animal to animal spread of *P. haemolytica*, although simultaneous carriage of two strains suggests that superinfection might occur by environmental exposure to *P. haemolytica*. The small number of plasmid profiles carried by *P. haemolytica* limits their usefulness as epidemiologic markers. Large numbers of cattle sampled at frequent intervals may be required for satisfactory epidemiological analysis. Techniques involving *in situ* colony hybridization could make such studies possible.

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SUMMARY AND CONCLUSIONS

It is well known that several factors acting in combination are necessary to produce bacterial pneumonia in the BRD complex. The association of respiratory viral infection with development of bacterial pneumonia has led to the concept that viral-bacterial synergism might play an important role in BRD. In support of this concept is considerable evidence that bovine immunologic function is often altered or impaired by common respiratory viral agents. It is not clear, however, exactly which immunologic functions must change, nor how much change is necessary to permit bacteria to successfully colonize bovine lungs. The answer to these questions will likely be as complicated as the disease itself. Many immunologic functions lead to immunity, and bacteria vary in their ability to colonize bovine lungs. Effective preventive measures against BRD will likely depend upon more complete understanding of host-viral-bacterial interactions at the cellular and molecular level.

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