Synergistic effect of bovine respiratory syncytial virus

and bovine viral diarrhea virus on functional properties

of bovine alveolar macrophages *in vitro*

by

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GENERAL INTRODUCTION

Bovine respiratory disease, which costs the cattle industry millions of dollars annually, has been extensively investigated and is the subject of numerous reports. In spite of all the efforts, the pathogenesis and etiology of bovine respiratory tract disease is still poorly understood. Bovine respiratory disease has a multifactoral etiology involving complex interactions between viruses and bacteria leading to severe disease. Synergism between viral and bacterial respiratory pathogens is well documented (Bielefield-Ohmann and Babiuk, 1985; Jakab, 1983; Jericho et al., 1982; Reggiardo, 1979; Yates, 1983); less is known regarding synergism among viral respiratory pathogens. Serologic evidence from cases of spontaneous respiratory disease indicates multiple concurrent viral infections are common and thus also present a potential for viral synergism (Bryson et al., 1979; Martin and Bohac, 1986; Richer et al., 1988; Rosenquist, 1974). Although bovine respiratory syncytial virus (BRSV) is only one of many viral agents associated with bovine respiratory tract disease, it is thought to be a major contributor to production losses (Baker and Frey, 1985; Bryson et al., 1979; Gillette and Smith, 1985; Pirie et al., 1981; Stott and Taylor, 1985; Wellemans, 1990). Infection can result in either mild or severe disease (Sharma and Woldehiwet, 1991). Mortality can be as high as 20% of a herd. The severe form has not been reproduced experimentally for evaluation of pathogenesis. Bovine viral diarrhea virus (BVDV) is one of the more frequently isolated viruses from feed lot cattle with pneumonia (Reggiardo, 1979; Richer et al., 1988). BVDV has an immunomodulating effect in vivo (Martin et al., 1990; Muscoplat et al., 1973; Potgieter et al., 1984b; Richer et al., 1988; Roth et al., 1981; Roth et al., 1986) and some strains are pneumotropic (Potgieter et al., 1984a; Potgieter et al., 1985). Alveolar macrophages (AM) play an important role in pulmonary immunity (Khadom et al., 1985; Renolds, 1991). Phagocytosis of opsonized and unopsonized bacteria and viruses enable AM to clear respiratory pathogens directly, while their release of cytokines enables AM to induce a series of

humoral and cell-mediated immune responses (Hearst et al., 1980; Khadom et al., 1985; Sibille and Reynolds, 1990). Respiratory virus infections sometime impair AM functions resulting in severe pneumonia (Khadom et al., 1985; Jakab, 1977).

The objective of this study were to determine the influence of non-cytopathic (ncp) BVDV infection on bovine AM functional properties and to determine if coinfection of bovine AM with BRSV-ncpBVDV produce a synergistic depression on AM functions.

Explanation of thesis format

This thesis consists of a paper preceded by a literature review. The research paper is presented in journal format and will be submitted to the journal "Veterinary Immunology and Immunopathology." References cited in this paper are included with it in the journal format. The literature review is preceded by a general introduction and is about the general knowledge of bovine respiratory syncytial virus, bovine viral diarrhea virus and alveolar macropgage, mainly concentrated on their characteristics and various aspects related to immune responses. Additional literature cited in the general introduction and literature review is listed following the general summary.

LITERATURE REVIEW

Bovine respiratory syncytial virus

History

BRSV has been regarded as an important viral respiratory pathogen in calves. Because of the importance of BRSV in cattle respiratory tract disease, numerous studies have been done and many articles have been written. Many reviews have summarized the work on BRSV (Baker, 1991; Baker and Frey, 1985; Kimman and Westerbrink, 1990; Sharma and Woldehiwet, 1991; Stott and Taylor, 1985; Wellemans, 1990). Isolations of BRSV from cattle in the world were first reported by Inaba et al. (1970), Paccaud and Jacquier (1970) and Wellemans et al. (1970). First initial isolation of BRSV in the United States was reported by Rosenquist (1974) and Smith et al. (1974). The annual winter outbreaks of BRSV infection in the world cause huge economic damage to the beef industry. Bovine RSV along with human RSV and pneumonia virus of mice belong to the genus *Pneumovirus* within the family Paramyxoviridae (Kingsbury et al., 1978). More recently, turkey rhinotracheitis virus has been included with this group of viruses (Collins and Gough, 1988).

Characteristics of BRSV

Bovine RSV was found to be similar to human RSV. Briefly, pneumoviruses are pleomorphic enveloped viruses that contain a single strand of negative sense genomic RNA (Joncas et al., 1969). Respiratory syncytial virus is sensitive to low pH and lipid solvents and can be destroyed by heating at 56 C for 30 min. It lacks both neuraminidase and haemagglutinin. Unlike other members of the Paramyxoviridae family, the nucleocapsid is only 12 to 15 nm in diameter. A roughly spherical form (80 to 500) and a filamentous form (60 to 110 nm in diameter and up to 5 um in length) have been observed in negative-stained preparations. In ultrathin sections, BRSV appears primarily as a filamentous virus,

approximately 95 nm in diameter, budding from the cytoplasmic membrane of infected cells

(Belanger et al., 1988; Sharma and Woldehiwet, 1991). Well defined bridges between virus

particles have been observed in BRSV but not HRSV preparations (Belanger et al., 1988).

The viral RNA has a sedimentation value of 50S, a molecular weight of approximately

5.9×10^6 daltons (Huang and Wertz, 1982; Stott and Taylor, 1985), and directs the synthesis

of 10 virus specific mRNA and 10 virus specific proteins (Lerch et al., 1989; Westenbrink et

al., 1989). Recently, sequence comparisons have been made between the glycoprotein (G),

fusion (F), nucleocapsid (N), phosphoprotein (P), matrix (M), small hydrophobic

(SH/1A), M2/22K, 1B and 1C proteins of HRSV and BRSV (Anderson et al., 1992; Lerch

et al., 1990; Mallipeddi and Samal, 1992; Samal et al., 1991; Samal and Zamora, 1991;

Walravens et al., 1990; Zamora and Samal, 1992). Homologies of 30%, 80%, 93%, 81%,

89%, 38%, 80%, 84% and 71% were observed at the amino acid level between the G,F, N, P,

M, SH/1A, M2/22K, 1B and 1C proteins of BRSV and HRSV respectively. N, P and L

proteins are associated with the nucleocapsid. M, 22K, G and F proteins are associated with

the membrane. Monoclonal antibody to F protein inhibits cell fusion, G protein is an

attachment protein of the virus, L protein is the RNA polymerase of the virion (Huang et al.,

1985), while SH protein may be important in the development of immune responses (Sharma

and Woldehiwet, 1991). BRSV is antigenically related to HRSV (Mohanty et al., 1976;

Paccaud and Jacquier, 1970; Smith et al., 1975; Stott and Taylor, 1985). The large G

glycoproteins of both BRSV and HRSV can be differentiated by using monoclonal antibodies

(Tayor et al., 1984). HRSV can be divided into two subgroups, A and B, based on panels

of monoclonal antibodies to the G protein (Anderson et al., 1985; Mufson et al., 1985).

Recently, Baker et al. (1992) identified two distinct antigenic subgroups among BRSV.

Studies have shown that BRSV belongs to a different antigenic group from HRSV (Baker et

al., 1992; Orvell et al., 1987).

Clinical disease

The high prevalence of serum antibodies to BRSV in the cattle population indicates that cattle are commonly exposed to BRSV (Baker and Frey, 1985). The mortality rates in cattle due to BRSV infection are usually less than 30% (Baker and Frey, 1985; Pirie et al., 1981), but the morbidity rates can be very high (Baker et al., 1985). Outbreaks of BRSV infection occur mainly in early winter (Sharma and Woldehiwet, 1991). The ages of cattle affected by BRSV infection are usually less than 18 months old (Baker et al., 1985). During natural infections, the main clinical signs in cattle are anorexia, mild depression, mucoid nasal discharge, salivation, lachrymation, pyrexia, tachypnoea, coughing and respiratory distress (Baker et al., 1985; Baker and Frey, 1985; Sharma and Woldehiwet, 1991; Stott and Taylor, 1985; Wellemans, 1990).

Pathogenesis and immunopathogenesis

Bovine RSV usually replicates in the cells of the nasal mucosa, pharynx, trachea and lung after experimental exposure of calves to BRSV (McNulty et al., 1983; Thomas et al., 1984). Viral antigens are commonly detected in the cytoplasm of tracheal and bronchial epithelial cells 4 to 10 days after infection (Castleman et al., 1985; McNulty et al., 1983). The common histological lesions are the formation of syncytial epithelial cells and epithelial hyperplasia in small bronchi, bronchioli and alveoli, with the greatest quantity of BRSV antigen in the lungs of calves days 4 to 6 postinfection (Bryson et al., 1982).

In natural BRSV infection, the principal lesions observed in calves are bronchitis and bronchiolitis in the cranioventral (CV) lobes of the lungs, and severe emphysema and edema can be seen throughout the lungs (Van den Ingh et al., 1982). Viral antigen is only present in the CV portion of the lungs, not in the caudodorsal (CD) portion, even though extensive lesions, such as edema and emphysema, may exist in the CD portion (Kimman et al.,

1989b). Kimman et al. (1989b) investigated natural BRSV infections in calves and found that severe respiratory disease may develop when IgM and/or IgG1 are present in serum or lung, while IgA is lacking. In experimental studies, both IgM and IgA were simultaneously detected in bovine serum nasal secretions and lung lavages about 8 to 10 PID (Kimman et al., 1987). BRSV-infected cells can activate complement component 3 (C3). Both IgG1 and IgM, but not IgA and IgG2, enhanced C3 activation (Kimman et al., 1989a). This suggested that C3 activation can play a role in the pathogenesis of BRSV infections. In another study, BRSV-specific IgE serum levels were found to be strongly correlated with the symptomatic response to BRSV infection in calves (Stewart and Gershwin, 1989). This suggests that IgE-mediated hypersensitivity may be involved in the pathogenesis of BRSV infection.

Immunological protection

The leukocyte migration-inhibition test and the delayed hypersensitivity test indicated that cell-mediated immunity was involved in BRSV infection in cattle (Field and Smith, 1984). Virus-specific cytotoxic T cell responses, predominantly CD8+, were demonstrated in lambs experimentally infected with BRSV (Sharma and Woldehiwet, 1991). BRSV-specific cell-mediated response was demonstrated in experimental calves as characterized by lymphocyte blastogenesis which was induced by an inactivated vaccine strain of BRSV (Taylor et al., 1987). An increased titer of serum neutralizing antibodies was detected and was associated with the development of anti-F protein antibodies to BRSV, but the presence and development of anti-F and anti-N protein antibodies were insufficient for protection against or recovery from BRSV infections (Stott and Taylor, 1985). Mucosal and serum antibody memory responses can be developed after priming the respiratory tract with live BRSV virus (Kimman et al., 1989c). Biological factors, such as interferon and tumor necrosis factor, may play an

important role in the recovery from BRSV infections (Bienhoff et al., 1992; Fulton et al., 1986).

Bovine viral diarrhea virus

History

The disease caused by bovine viral diarrhea virus (BVDV) was first reported by Olafson et al. (1946) in association with a transmissable disease of cattle with high morbidity and low mortality. The condition was characterized by fever, diarrhea and coughing. A few years later, a highly fatal disease of cattle with low morbidity rate was described by Ramsey and Chivers (1953). Extensive ulceration of the gastrointestinal tract was the most prominent lesion. The disease became known as mucosal disease because it was thought to be caused by a different agent from BVDV. It is now known that both diseases are caused by the same virus.

Recent work has classified the virus as a member in the family Flaviviridae based on similar genome organization and replication strategies (Collett et al., 1988). Two biotypes of BVDV exist, cytopathic (cp) and non-cytopathic (ncp), based on different viral effects in tissue cultures. BVDV infection during pregnancy may result in abortion, teratogenic defects or birth of persistently infected calves. Superinfection of cpBVDV on persistent ncpBVDV infection in cattle contributes to the generation of fatal mucosal disease (Bolin et al., 1985; Brownlie et al., 1984). BVDV also contributes to the bovine respiratory disease complex (Potgieter et al., 1984; Reggiardo, 1979). Many studies have been done and numerous articles have been written on BVDV because of the economic loss to the cattle industry by this viral infection. Extensive reviews have been written on BVDV (Baker et al., 1987; Collett et al., 1989; Horzinek, 1973; Moennig, 1990; Moennig and Plagemann, 1992).

Characteristics of BVDV

BVDV is a spherical, enveloped virus with diameter about 40 nm (Horzinek, 1973). Irregular spikes project to the outside of the particle. Viral maturation is thought to occur via a condensation process within the smooth membrane vesicles in which virions accumulate and the virus release is thought to be via exocytosis of membrane vesicles. The virus has a single positive-stranded RNA. The infectivity RNA has a sedimentation value of about 40S (Moennig, 1971) and the length of about 12.5 kilobases (Collett et al., 1988). There is no difference in genome sizes between ncp and cytopathic BVDV (cpBVDV) (Collett et al., 1989). It has been hypothesized that cp BVDV arises from ncp BVDV by some mutational events (Howard et al., 1987). Studies on the BVDV genome revealed that cellular gene insertions or duplication in the p125 region leads to cytopathogenicity of BVDV (Meyers et al., 1991; Qi et al., 1992) Molecular studies have demonstrated that duplication and rearrangement of sequences as well as the expression of p28 and p80 proteins are specific for the cytopathogenic BVDV, while ncpBVDV lacks these proteins (Meyers et al., 1992) The number of viral proteins described have ranged from 4 to 12 (Moennig, 1990). The cp biotype of BVDV contains a specific protein, p80, which is derived from the cleavage of the nonstructural protein p125, whereas the ncp biotype has only the p125 protein (Donis and Dubovi, 1987; Pocock et al., 1987). The major glycoprotein of BVDV, gp53, is thought to be the most important protein involved in virus neutralization (Bolin et al., 1988; Donis et al., 1988; Greiser-Wilke et al., 1990; Wensvoort, 1989; Wensvoort et al., 1989).

Clinical disease

The clinical picture has been reviewed by Baker (1987). Briefly, 70% to 90% of the BVDV infections in cattle are subclinical and characterized by mild fever and leukopenia. Clinical disease is usually seen in cattle ranging in age from 6 months to 2 years. The incubation period

is approximately one week. Clinical signs usually are mild depression, inappetence, oculonasal discharge and oral lesions. The cattle may experience diarrhea with high morbidity and low mortality. BVDV has an affinity for cells of the immune system and can result in transient immunosupression (Roth et al., 1981; Roth and Kaeberle, 1983). Thus, there is the potential to facilitate infection by other agents. Infection in pregnant susceptible cattle readily results in fetal infection. Depending on the age and immunologic competence of the fetus the infection may lead to fetal death, malformation or persistent viremia.

Pathogenesis and immunopathogenesis

BVDV causes numerous diseases, but mucosal disease (MD) is the most severe form of BVDV infection. The pathogenesis of MD is due to congenital persistent infection with ncpBVDV and postnatal superinfection with cpBVDV (Bolin et al., 1985; Brownlie et al., 1984). During acute infection, virus replication is usually found in the mucosae around the site of viral entrance and the lymphoid tissue lining the oropharynx, especially the epithelial cells in tonsilar crypts (Moennig and Plagemann, 1992). With intrauterine fetal infection resulting in persistent viral infection, virus is usually present in peripheral blood mononuclear leukocytes, the gastrointestinal tract and central nerverous system almost exclusively in neurons (Moennig and Plagemann, 1992). Immunosupression caused by BVDV infection includes supression of interferon production, supression of lymphocyte transformation, decrease in the absolute number of circulating B and T lymphocytes and percentages of T lymphocytes and impairment of humoral antibody production (Baker, 1987). Recently, Atluru et al. (1992) demonstrated that BVDV inhibited leukotriene B4 synthesis which has a positive effect on lymphocyte proliferation and production of IL-1, IL-2 and interferon. This suggests that some negative immunomoderating factors may be involved in BVDV-induced immunosupression.

Alveolar macrophage

Alveolar macrophages differ from other macrophages by their residency in the lung and by functional and immunological aspects (Hearst et al., 1980; Myrvik et al., 1961; Sibille and Reynolds, 1990). AM play an important role in the clearance of small inhaled particulate materials and microorganisms reaching the periphery of the lung (Reynolds, 1991; Sibille and Reynolds, 1990). AM have many receptors and markers such as the Fc receptor, complement (C3b, C5a) receptors, interleukin-2 (IL-2) receptors and MHC class II antigens on their surface. They also can release a number of mediators such as enzymes, complement components, IL-8, IL-1 and interferon into their surrounding environment (Sibille and Reynolds, 1990). Immunologic stimuli-activated AM have enhanced functional capabilities. Abnormal AM function may result in the loss of local and/or systemic defense abilities (Sibille and Reynolds, 1990). AM can also participate in immune responses to protect the lung from respiratory pathogens.

Characteristics

Bovine AM are derived from peripheral blood monocytes (Khadom et al., 1985). Khadom et al. (1985) reported that AM are not able to proliferate in vivo, but McGuire and Babiuk (1982) demonstrated that bovine AM can proliferate in vitro. The morphology of a typical AM is a round cell with numerous surface folds and numerous inclusions such as phagosomes, lysosomes, Golgi apparatus, mitochondria, endoplasmic reticulum, ribosomes, vacuoles and eccentric nuclei (Fox, 1973 ; Khadom et al., 1985). Freshly isolated AM have a mean diameter of 13 um, while cultured AM are largger with a mean diameter of 22 um (Fox, 1973). Bronchoalveolar lavage can yield 10^5 to 10^6 cells/ml containing 80% to 95% AM (Khadom et al., 1985). Bovine AM have surface receptors for rabbit immunoglubulins, bovine IgG1 and C3b (Khadom et al., 1985) and can also express MHC class II (Ia) antigen

(Ohmann et al., 1986). Recently, Ohmann et al. (1990) demonstrated that bovine AM express tumor necrosis factor-alpha (TNF) receptors.

Ohmann and Babiuk (1986) confirmed that bovine AM are heterogeneous. Four fractions of AM were separated by using density gradient centrifugation. Mature AM are mainly located in the low-density fraction and characterized as having of low Ia-antigen expression, low antibody dependent cell-mediated cytotoxicity (ADCC) activity and high ecto-enzyme and C3b-receptor activity. Immature monocyte-like AM are predominately located in the high density fraction and are characterized by high Ia antigen expression and low expression of the other markers, two fractions of intermediate density overlap in most characteristics. Seidel et al. (1990) identified three subgroups of bovine AM based on the method of carrier-free electrophoresis. These subgroups of AM also correspond to different stages of maturation and the fraction migrating fastest may be the most mature fraction.

Virus replication in AM

Khadom et al.(1985) reviewed replication of BRSV, cpBVDV, parainfluenza-3 (PI-3) virus and bovine rhinotracheitis (IBR) virus in bovine AM culture. Adair et al. (1992) demonstrated that adenoviruses can also replicate in AM culture. Bovine AM have been shown to be nonpermissive for DN 599 herpesvirus (Khadom et al., 1985). Virus infection usually results in dysfunctions of AM. PI-3 virus infection inhibits AM phagocytosis (Khadom et al., 1985), reduces phagosome-lysosome fusion activity (Hesse and Toth, 1985), decreases antibody (Fc)-mediated phagocytic and killing activities (Liggitt et al., 1985) and decreaes AM adherence ability and complement (C3b)-mediated phagocytosis (Brown and Shin, 1990; Liggitt et al., 1985). However Brown and Shin (1990) reported no changes in bovine AM Fc-mediated phagocytosis and binding after PI-3 infection. There are also conflicting results from studies of IBR virus infections. Forman et al. (1982) reported that IBR virus infection reduces Fc-mediated receptor activity and

phagocytosis, complement receptor activity and ADCC activity. Brown and Shin (1990) reported contrasting results that IBR virus infection produced no effect on Fc and C3b-mediated phagocytosis and binding.

Different viral effects on bovine AM may exist due to the differences in strains of virus ultilized. Strain VR-794 of BRSV impaired Fc-mediated phagocytosis, but not lysosome enzyme activity and phagocytosis and killing of *Staphylococcus epidermidis* (Trigo et al., 1985). Compared with a vaccine strain of BRSV, strain AC2 of BRSV decreased AM functions such as complement-mediated phagocytosis, killing activity and C3b and Fc receptor expression more severely (Adair and McNulty, 1992). Adenovirus type-1 infection on bovine AM impaired Fc and C3b receptor expression and phagocytosis and killing activities to a much greater extent than type-8 adenovirus (Adair et al., 1992).

Mechanisms of immune defense

AM participate in a variety of immunologic functions to protect the lung from various respiratory pathogens. The first mechanism involved is phagocytosis. Usually small particles can be cleared by AM directly through phagocytosis. Soluble components, complement and antibody, in alveolar spaces, bind to the AM surface to enhance AM phagocytic ability . Internal or external release of enzymes or oxygen anions during AM phagocytosis enables the AM to kill invading pathogens (Sibille and Reynolds, 1990).

The interaction between AM and polymorphonuclear neutrophils (PMN) in the lung lumen is also a key step in restricting virus or bacterial infections. After being stimulated, AM release IL-8 into the surrounding alveolar capillary lumen to recruit PMN to the inflammatory sites. AM can also inhibit PMN activity by releasing PGE2 after virus infection (Reynolds, 1991).

AM can be involved in humoral and cellular immunity in the alveolar space as described by Reynolds (1991). Usually there are three processes involved: (1) AM digest invading

antigen into segments and present these segments on their cell surface. Appropriate major histocompatible T cells recognize them and then become stimulated to initiate an immune reaction that leads to cellular or humoral immunity, (2) AM can be activated by interferon released by activated T helper cells, and (3) activated AM can release IL-1 to attract T cells to the alveolar space, T cells release IL-2 to stimulate T cell proliferation and activate killer cells and induce B cells to become plasma cells to produce specific antibody to a particular antigen.

PAPER: SYNERGISTIC EFFECT OF BOVINE RESPIRATORY SYNCYTIAL VIRUS
AND NON-CYTOPATHIC BOVINE VIRAL DIARRHEA VIRUS ON
FUNCTIONAL PROPERTIES OF ALVEOLAR MACROPHAGES *IN VITRO*

Synergistic effects of bovine respiratory syncytial virus and non-cytopathic bovine viral

diarrhea virus on functional properties of bovine alveolar macrophages *in vitro*

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ABSTRACT

A study was conducted to investigate the potential synergistic effect of bovine respiratory syncytial virus (BRSV) and non-cytopathic bovine viral diarrhea virus (ncpBVDV) on selected bovine alveolar macrophage (AM) functions. AM were harvested from two to six month-old calves seronegative for BRSV and ncpBVDV and infected with one median cell culture infective dose of virus per AM. The AM functions were evaluated on postinoculation days (PID) 1, 3, 5 and 7. On each interval day, control, BRSV-infected, ncpBVDV-infected and BRSV-ncpBVDV coinfected AMs were evaluated for Fc receptor expression, phagosome-lysosome fusion, superoxide anion (O_2^-) production, and interleukin-8 (IL-8) production. Fc receptor expression was significantly depressed on PID 1 and 3 and on PID 5 and 7 by BRSV and ncpBVDV infection respectively, whereas coinfection resulted in a significant synergistic depression on PID 1, 3 and 5. Coinfection also dramatically depressed the phagosome-lysosome fusion on PID 1, 3 and 5, while infection with either BRSV or ncpBVDV had less effect. Production of O_2^- by AM was not decreased by either BRSV or ncpBVDV infection, but was significantly decreased by coinfection with BRSV-ncpBVDV on PID 5. Coinfection of AM with BRSV-ncpBVDV severely reduced production of IL-8 during each interval day. BRSV and ncpBVDV infection both decreased IL-8 production, BRSV on each day and ncpBVDV on PID 3 and 5, but not to the extent of coinfection. Our results confirm previous reports of BRSV effects on AM functions and indicate that ncpBVDV affects AM functions in vitro. Coinfection with BRSV-ncpBVDV produced a synergistic depression on AM functions.

ABBREVIATIONS

AEC, 3-amino-9-ethylcarbazole; AM, alveolar macrophage; AO, acridine orange; BRSV, bovine respiratory syncytial virus; BT, bovine fetal turbinate; $CCID_{50}$, median cell culture infectious dose; IL-8, interleukin-8; ncpBVDV, non-cytopathic bovine viral diarrhea virus; OFTu, ovine fetal turbinate; PID, postinoculation day; RTD, respiratory tract disease; SOD, superoxide dismutase.

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INTRODUCTION

Respiratory tract disease (RTD) of cattle has been known for years to have a multifactorial

etiology involving a complex interaction between stressors, viruses and bacteria (Horlein and

Marsh, 1957). There is potential for various pathogen synergisms including virus-bacteria

and virus-virus. Virus-bacteria synergisms in RTD are well documented (Bielfield-Ohmann

and Babiuk, 1985; Jakab, 1983; Jerico et al., 1982; Reggiardo, 1979; Yates, 1983). Less is

known regarding the synergism among viral respiratory pathogens. Serological evidence

from cases of spontaneous respiratory disease in cattle indicates that multiple concurrent viral

infections are common (Bryson et al., 1979; Richer et al., 1988; Rosenquist and Dobson,

1974). The high prevalence of serum antibodies to bovine respiratory syncytial virus

(BRSV) indicates that infection in cattle is common and often results in RTD (Baker and

Frey, 1985; Broyson et al., 1979; Gillette and Smith, 1985; Pirie et al., 1981). Although

bovine viral diarrhea virus (BVDV) primarily infects the gastrointestinal tract and lymphoid

tissue, observations indicate that BVDV may be an important respiratory tract pathogen

(Potgieter et al., 1984b; Potgieter et al., 1985; Reggiardo, 1979; Richer et al., 1988). The

immunosuppressive effect of acute BVDV infection may enhance the clinical effects of other

pathogens (Martin et al., 1990; Muscoplat et al., 1973; Potgieter et al., 1984a; Richer et al.,

1988; Roth et al., 1981; Roth et al., 1986), and therefore, may be an important part of the

bovine RTD complex. One study revealed that about 92% of multiple viral infections involved

BVDV (Richer et al., 1988). Recent observations at the National Animal Disease Center

indicate that severe pulmonary lesions can be induced in sheep by infecting with BRSV that

was contaminated by non-cytopathic BVDV (ncpBVDV) (Meehan and Lehmkuhl, personal

observation, 1992).

Alveolar macrophages (AM) play a critical role in the primary response of the lung to

respiratory infections (Khadom et al., 1985). Virus infection may predispose the respiratory tract to the development of secondary bacterial pneumonia by impairing AM functions. Previous studies indicate that BRSV replication is supported in AM in vitro (Adair and McNulty, 1992; Toth and Hesse, 1983a; Trigo et al., 1985) and RSV depresses AM functions (Adair and McNulty, 1992; Trigo et al., 1985). A previous report confirmed that cytopathic BVDV can replicate in AM (Toth and Hesse, 1983a). No studies have been done to determine if ncpBVDV can infect AM and what effect that infection would have on AM functions. The objectives of this study were to determine the influence of ncpBVDV infection on bovine AM functional properties and to determine if coinfection of bovine AM with BRSV-ncpBVDV produced a synergistic depression on AM functional properties.

MATERIALS AND METHODS

Virus preparation

The 375 strain of BRSV (Lehmkuhl et al., 1979) coinfected with a ncpBVDV (obtained

from Dr. James T. Meehan, National Animal Disease Center, Ames, Iowa) was used as the

coinfecting inoculum (BRSV-ncpBVDV). BRSV free from ncpBVDV was prepared from the

coinfectied inoculum by inoculating Vero cells (Toth and Hesse, 1983b) in the presence of

antiserum to BVDV . BRSV was passaged three times in Vero cells and tested for ncpBVDV

using an immunoperoxidase test (see below). Because the titer of the ncpBVDV was higher

than that of the BRSV in the coinfected inoculum, the ncpBVDV was prepared through

terminal dilution using bovine fetal turbinate (BT) cells. The lowest log dilution without

BRSV-induced cytopathic effect (CPE) was subpassaged three times and monitored for the

absence of BRSV induced CPE. Both BRSV-ncpBVDV and purified BRSV stocks were

prepared using ovine fetal turbinate (OFTu) cells. The virus titer determination for both the

BRSV and BRSV-ncpBVDV inoculum was based on the charateristic CPE of BRSV and the

titer for the ncpBVDV was based on the immunoperoxidase test . Aliquots of the virus pool

were frozen at -85 C until they were used to inoculate AM cultures.

Immunoperoxidase test

An immunoperoxidase test was conducted to determine ncpBVDV infection as described by

Bolin et al. (1991) with some modifications. One hundred ul of BT cells were seeded into a

flat bottom 96-well microtiter plate to form a 90% confluent monolayer in 24 hours. The wells

were then inoculated with 50 ul of culture fluid to be analyzed for the presence of ncpBVDV

and incubated for another 24 hours. Positive and negative controls were also set up. After a

24-hour incubation, the cell culture mediums were decanted and the cells were rinsed with 50

ul of warm PBS (0.01 M, pH 7.6). The monolayers were fixed for 10 min at room

temperture by adding 100 ul of fixation buffer (warm PBS + 0.02 % bovine serum albumin + 40% acetone). The fixed monolayers were dried at 37 C in a non-humidified incubator for 30 to 60 min. Fifty ul of pooled bovine antiserum to ncpBVDV diluted in binding buffer PBSTN (PBS with 0.05 ml Tween 20/ml) was applied to each well and the plate was incubated for 30 min at room temperture. The PBSTN was gently poured off and the monolayer was washed 3 times with 50 ul washing buffer (PBST) and incubated for 30 min at room temperture with 50 ul of rec-protein G- peroxidase conjugate (Zymed Laboratories, Inc., San Francisco, CA) diluted 1: 1000 in PBSTN. Then, the medium was poured off and the cells were washed 3 times with 50 ul PBST. Two and one half mg of 3-amino-9-ethylcarbazole (AEC) (Sigma Chemical Co., St. Louis, MO) was dissolved in a polypropylene tube with 0.5 ml N, N-dimethylformamide (Sigma Chemical Co., St. Louis, MO). The AEC solution was slowly added to 10 ml Na acetate buffer (0.05 M, pH 5.0) suplemented with 0.04 ml of 3 % H_2O_2 in acetate buffer. Fifty ul of the AEC substrate solution were added to each well and incubated for 10 to 15 min. Cells with reddish brown cytoplasm were considered positive for ncpBVDV.

Calves

A group of six 2- to 6-month-old Jersey calves seronegative for both BVDV and BRSV was used as the source of alveolar macrophages. No calf was lavaged twice in an interval of less than 14 days. Calves were sedated with xylazine (0.05 mg/kg body weight) administered intravenously (IV) and placed in sternal recumbency for lavage. Following lavage, the calves were given tolazoline (0.5 mg/kg body weight) administered IV to reverse the effect of the xylazine.

Alveolar macrophage cultures

Alveolar macrophages were recovered by bronchoalveolar lavage as described by Trigo et

al. (1984). Briefly, a 0.6 cm diameter polypropylene tube was passed intranasally and trans-

tracheally until gently wedged into a bronchus. Fifty ml of lavage fluid (0.9% NaCl

containing 200 ug/ml gentamicin and 2.5 ug/ml amphotericin B) were infused into the lung

and recovered using a 50-ml glass syringe connected to the end of the tube. The fluid

recovered from the first 50 ml lavage was discarded with the subsequent lavage fluid placed in

a 200-ml glass centrifuge tube kept on ice. The lavage was repeated until 200 ml fluid was

collected. The recovered lavage fluid was filtered through sterile gauze and centrifuged at 450

X g for 40 min at 4 C. The cell pellet was washed 3 times in saline solution by centrifugation.

After the third wash, the cell pellet was resuspended in RPMI-1640 medium supplemented

with 10% bovine fetal serum (BFS), 200 ug/ml gentamicin and 2.5 ug/ml of amphotericin B.

The cells were counted using a hemacytometer, and the concentration was adjusted to $5X10^5$

/ml. The viability of the AM was determined by trypan-blue exclusion. Alveolar macrophages

were cultured in either 8-well tissue culture chamber slides (0.1 ml/well) (Lab Tek Tissue

Culture Chamber, Miller Scientific, Division of Miles Laboratories, Inc., Naperville, IL), or

8-well tissue culture plates (1 ml/well) (LUX Scientific Corporation, Newbury Park, CA)

or 96-well flat-bottom tissue culture plates (0.05 ml/well) (Becton Dickinson Labware,

Lincoln Park, NJ). Cultures were incubated for 30 minutes at 37 C in humidified air

containing 5 % CO_2 to allow AM to settle and adhere. The medium was then removed and the

cells gently washed with warm RPMI 1640 and fresh RPMI-1640 was added.

Virus infection of AM

The AM were inoculated with approximately one median cell culture infective dose

(CCID50) per cell of both BRSV and ncpBVDV. Inoculation of AM with BRSV-ncpBVDV

was approximately 1 CCID50 per AM based on the titer of BRSV in the inoculum. Control

AM cutures were sham-inoculated with virus-free cell culture medium. Following

absorption of virus for 2 hours, the inoculum was removed and the cultures were gently washed 3 times and fresh RPMI-1640 supplemented with 10% BFS was added. Cultures from individual calves were evaluated for Fc receptor expression, phagosome-lysosome fusion, superoxide anion (O_2^-) production, and interleukin-8 (IL-8) production on PID 1, 3, 5 and 7.

Zymosan preparation

Zymosan (Sigma Chemical Co., St. Louis, MO) prepared as described by Nagahata et al. (1986) at a concentration of 10 mg/ml was stored at - 85 C. Each interval day, 10 mg/ml of washed zymosan was incubated with an equal volume of autologous fresh bovine serum for 1 hour at 37 C and resuspended in Hanks' Balanced Salt Solution (HBSS) to a concentration of 4 mg/ml.

Virus replication in AM cultures

AM culture fluids were harvested from 8-well tissue culture chamber slides on PID 1, 3, 5 and 7 and assayed for the presence of virus. For BRSV infection, OFTu cells in 25 cm^2 flasks were inoculated with AM culture fluids and observed for the presence of characteristic CPE. For ncpBVDV infection, BT cells in 96-well microtiter plates were inoculated with AM culture fluids and examined for the presence of ncpBVDV by an immunoperoxidase test. Coinfection cultures were prepared as previously described to separate BRSV and ncpBVDV and to determine virus presence .

Fc receptor assay

The presence of Fc receptors was determined by the method of Bianco and Pytowski (1981). Briefly, sheep red blood cells (SRBC) were collected by venipuncture and diluted 1:1 in Alsever's solution. On each interval day, 5 ml SRBC in Alsever's solution were diluted

in 25 ml ice-cold HBSS and centrifuged at 1800 Xg at 4 C for 10 min. The cells were washed

twice in HBSS and resuspended in HBSS to a 5% (v/v) concentration. The SRBCs were

sensitized with an equal volume of a subagglutinating dilution (1/500) of rabbit anti-SRBC

antiserum (IgG) (Organon Teknika Corporation, Durham, NC) for 15 min at 37 C, washed

three times, and resuspended with RPMI-1640 to a 0.5% (v/v) concentration. Supernatants

were removed on PID 1, 3, 5 and 7 from the wells of virus-infected and uninfected AM

cultures in chamber slides. The AM were washed with HBSS and exposed to sensitized

SRBC (0.2ml/well) for 30 min at 37 C. The chambers were gently rinsed 4 times with HBSS

to remove nonadherent sensitized SRBCs and wet-mounted with cover slides. Fc receptor

expression was determined by the percentage of cells with 3 or more adherent SRBC

(rosettes) in 10 microscope fields per replication. For each viral infection, the mean

percentage of six replications on each interval day was calculated.

Phagosome-lysosome fusion assay

The effect of virus on AM phagosome-lysosome fusion was assayed by a modification of

the acridine orange (AO) method described by Jakab et al. (1980). Candida albican

organisms (from Dr. G.B. Johnston, Iowa State University), grown overnight in 10%

glycerol of 3.7% brain heart infusion solution, were washed twice by centrifugation in

HBSS, counted using a hemacytometer and resuspended in RPMI-1640 to a concentration of

1×10^7 cells/ml. On each interval day, 15 ul of a solution containing 0.8 ug of freshly prepared

AO solution were added to virus-infected and uninfected AM cultures in chamber

slides and incubated for 20 min at 37 C. Excess AO and nonadherent cells were removed by

gently washing the slides and cultures were exposed to 30 ul of yeast suspension

and incubated for 60 min at 37 C. Chambers and gaskets were removed. The slides were

dipped in phosphate buffered saline (0.01 M, pH 7.2) to remove excess yeast, then cover

slipped, and observed with an epifluorescent microscope at 400 X magnification. Phagosome-lysosome fusion occurred when yeast stained yellow, orange or red within phagososmes. For each viral infection on each interval day, the mean percentage of six replications of phagosome-lysosome fusion in 200 AMs / replication were calculated.

Respiratory burst assay

Both virus-infected and uninfected AM were monitored for O_2^- production by the method described by Pick and Mizel (1981). The first four columns of AM cultures in 96-well flat-bottom tissue culture plates contained no virus, whereas subsequent columns were inoculated with BRSV, ncpBVDV and BRSV-ncpBVDV, respectively. During each interval day, 300U/ml of superxide dismutase (SOD) (Calbiochem Corporation, La Jolla, CA) in 5 ul was added to columns 2 and 3 and incubated for 5 minutes at 37 C. Then, 100 ul of phenol red-free Earle's balanced salt solution (EBSS) (Life Technologies Inc., Grand Island, NY) and 40 ul of HBSS was added to the first column. One hundred ul EBSS containing 160 uM ferricytochrome c (Sigma Chemical Co., St. Louis, MO) plus 40 ul of opsonized zymosan (4 mg/ml) were added to the second and fourth through seventh columns and the plates were incubated for 1 hr at 37 C in a humidified atmosphere with 5 % CO_2. Cytochrome c reduction was determined using microplate reader (Molecular Devices, Woodbury, MN) at 550 nm. The amount of O_2^- produced per well was expressed by the formula: nanomoles O_2^- per well = (absorbance at 550 nm X 100) / 6.3. For each viral infection on each interval day, the mean of superoxide production in six replications was calculated.

IL-8 assay

Chemotactic activity was assayed by using the procedure of Nelson et al. (1975) with some modifications. During each interval day, virus-infected and uninfected culture fluids

from eight-well tissue culture plates were discarded and the AM were washed twice with RPMI-1640. Then 1 ml of either opsonized or unopsonized zymosan (4 mg/ml) was added to each well followed by incubation for 3 hours at 37 C in a humidified atmosphere with 5 % CO_2. The culture fluids were harvested separately and centrifuged, and the supernatants were stored at -85 C until tested. Neutrophils were recovered from freshly prepared heparinised normal calf blood by centrifugation on Ficoll-Diatrizoate (Sigma Chemical Co., St. Louis, MO). The supernatant was aspirated and the pellet of cells was washed once with HBSS. The erythrocytes were lysed with two cycles of ice-cold 0.2% NaCl followed by the addition of 1.6 % NaCl and centrifugation at 1000 X g for 10 min at 4 C. The leukocytes were washed two additional times with HBSS. Neutrophils were counted in a hemacytometer, and the concentration adjusted to $1X10^7$ /ml in RPMI-1640 supplemented with 0.1 % gelatin and 5 mM EDTA. Five ml of warm agarose solution (0.8% in RPMI-1640 supplemented with 10% BFS) were pipetted into each 60 x 15-mm tissue culture dish (Becton Dickinson Labware, Lincoln Park, NJ) and allowed to solidify. Four series of three wells 3.0 mm in diameter and spaced 3.0 mm apart were cut in each plate using a plexiglass template and stainless steel punch. The agarose plugs were removed and the center well of each three-well series received a 10 ul volume of purified neutrophils. The outer well received 10 ul of culture fluid from unopsonized zymosan stimulated AM cultures, while the inner well received 10 ul of culture fluid from opsonized-zymosan stimulated AM cultures. After a 2-hour incubation, the cells were fixed with the agarose in place by the addition of 3 ml absolute methanol for 30 min followed by 3 ml of 37% buffered formalin for 30 min. After fixation, the gel was removed intact and the plates were stained with Giemsa solution and air dried. Quantitation of migration was conducted by measuring the linear distance (in cm) the cells had migrated from the margin of the well toward the chemotactic factor (A: chemotaxis) and the linear distance (in cm) the cells had migrated from the margin of the well toward the unopsonized medium (B:

spontaneous migration). The chemotatic differential A - B was calculated for each replication. The mean value and standard deviation of six replications for each viral-infection on each interval day were determined.

Statistical analysis

Student's t-test was utilized to test the significant differences between virus-infected and uninfected AM cultures, and the differences were considered significant when P< 0.05.

RESULTS

Alveolar macrophage culture and infection

The viability of AM was 87.27+/-1.41% (mean+/-SEM) on the day the cultures were established. After 7 days, the viability of normal AM was greater than 95%, but the total number of AM in culture over 7 days decreased about 30-40%. Assay results of AM culture for BRSV, ncpBVDV and BRSV-ncpBVDV on PID 1, 3, 5 and 7 are presented in Table 1. Cultures positive for virus declined over the 7 day observation period. In BRSV inoculated cultures, BRSV isolation decreased from 6 on PID 1 to 1 on PID 7. For BRSV-ncpBVDV inoculated cultures, 6 samples were positive on PID 1, whereas 2 were positive on PID 7. Recovery of ncpBVDV from cultures inoculated with ncpBVDV decreased from 6 to 3 over the 7 day observation period.

Fc receptor assay

Tests for Fc receptors were conducted on infected and uninfected AM cultures. The results are presented in Fig.1. Compared with sham-inoculated control cultures BRSV infection significantly depressed Fc receptor expression starting on PID 1 (45.85+/-4.46%, P< 0.01) and reached the lowest point on PID 3 (41.73+/-1.62%, P< 0.001). After PID 5 (61.96+/-6.04, P< 0.05), Fc receptor expression began to recover from BRSV infection and returned to normal on PID 7. Non-cytopathic BVDV infection induced a significant decrease of Fc receptor expression starting on PID 5 PI (61.04+/-4.97%, P < 0.05) and persisting on PID 7 (57.99+/-5.27%, P< 0.01). The response to BRSV-ncpBVDV infection was similar to that of BRSV alone but the decrease in Fc receptor expression was more severe. The percentage of AM with Fc receptors was 39.82+/-1.98 (P< 0.001) on PID 1, reached the lowest point on PID 3 (30.72+/-3.27%, P< 0.001) and then returned to normal by PID 7.

Phagosome-lysosome fusion assay

Phagosome-lysosome fusion activity in control and virus-infected AM cultures was determined by counting the number of cells with yellow, orange or red staining yeast cells. The percentage of control and virus-infected AM with phagosome-lysosome fusion are shown in Figure 2. A smaller decrease in phagosome-lysosome fusion was noted with either BRSV or ncpBVDV infection on PID 3 (75.16+/-1.73%, P< 0.05 and 74.88+/-1.92%, P< 0.05 respectively) and PID 5 (72.90+/-3.31%, P< 0.05 and 72.78+/-1.87%, P< 0.01, respectively). Phagosome-lysosome fusion in cultures inoculated with BRSV-ncpBVDV was depressed starting on PID 1 (72.25+/-3.70%, P< 0.05) with the greatest reduction on PID 5 (60.58+/-3.88%, P< 0.01). By PID 7, the phagosome-lysosome fusion of AM recovered from both single and combined viral infections.

Respiratory burst assay

Superoxide anion responses to virus infection were quantitated by the reduction of cytochrome c and the specifity of the response was determined by the stimulation of AM in the presence of SOD. After stimulation with opsonized zymosan, neither BRSV nor ncpBVDV infected AM cultures resulted in significant changes in O_2^- production by AM, compared with the sham-inoculated control (Fig.3). However, BRSV-ncpBVDV infection significantly depressed O_2^- release by AM on PID 5 (10.45+/-0.32 nmol/well, P< 0.05). Furthermore, when cultures were stimulated in the presence of SOD almost all O_2^- release was eliminated indicating that O_2^- contributed to the specific reduction of cytochrome c (data not shown).

IL-8 production

Production of IL-8 by virus-infected and uninfected AM cultures was determined by measuring chemotactic differentials. Table 2 presents the results. Interleukin-8 production

by AM was depressed by ncpBVDV infection on PID 3 (0.1400+/-0.0052 cm, P< 0.05)
and on PID 5 (0.1383+/-0.0060 cm, P< 0.05). By day 7, IL-8 production was back to the
normal level. IL-8 production was significantly depressed by BRSV infection over the 7-day
observation period with the greatest effect on PID 1 and 3. By PID 7, IL-8 production was
returning to normal. For BRSV-ncpBVDV coinfected cultures, IL-8 production was
severely depressed for at least 7 days.

Table 1. Detection of bovine respiratory syncytial virus (BRSV), non-cytopathic bovine viral diarrhea virus (ncpBVDV) or both (BRSV-ncpBVDV) in alveolar macrophage cultures prepared from 6 calves.

PID*	BRSV	BRSV-ncpBVDV		ncpBVDV
		BRSV	ncpBVDV	
1	6/6**	6/6	6/6	6/6
3	3/6	4/6	5/6	6/6
5	3/6	4/6	4/6	5/6
7	1/6	2/6	2/6	3/6

* Postinoculation Day
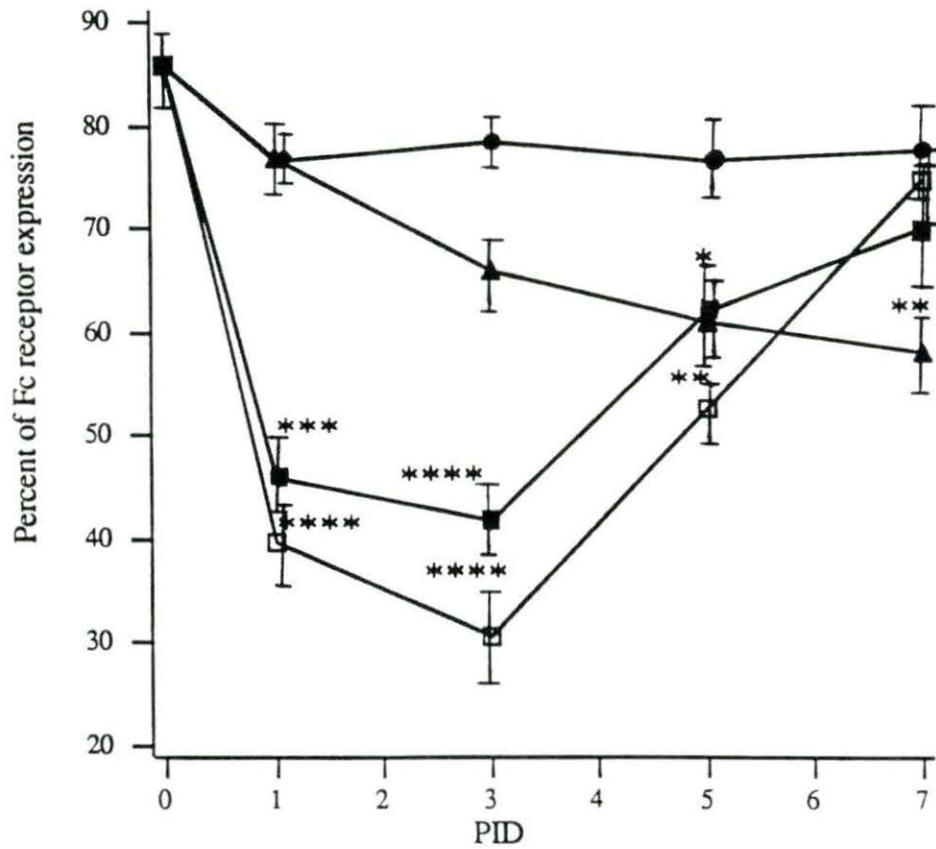
** Number Positive/Number Sampled

Fig.1. Percentage of AM expressing Fc receptors on PID 1, 3, 5, and 7 following infection with BRSV(■), ncpBVDV (▲), BRSV-ncpBVDV (▫) or left as sham-inoculated controls (●). Results expressed as mean +/- SEM (from 6 calves). * P< 0.05, ** P< 0.02, *** P< 0.01, **** P< 0.001.
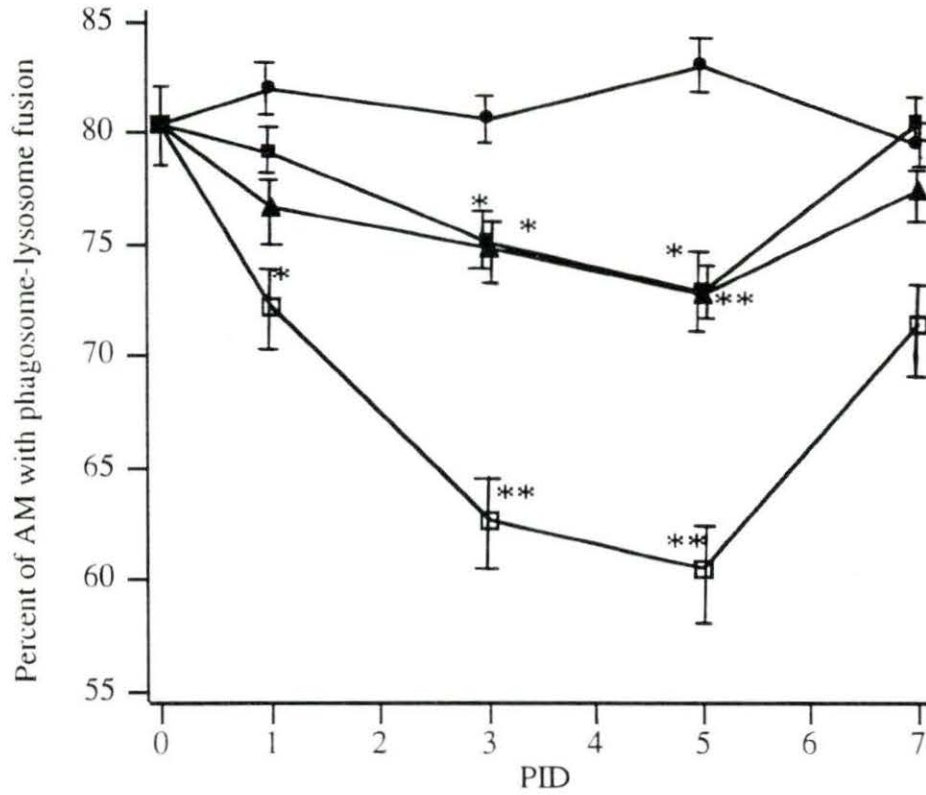
Fig.2. Percentage of alveolar macrophages demonstrating phagosome-lysosome fusion on PID 1, 3, 5 and 7 following infection with BRSV (■), ncpBVDV (▲), BRSV-ncpBVDV (❏) as compared with sham-inoculated control (●). Results expressed as mean+/- SEM (from 6 calves). *P< 0.05, **P< 0.01.
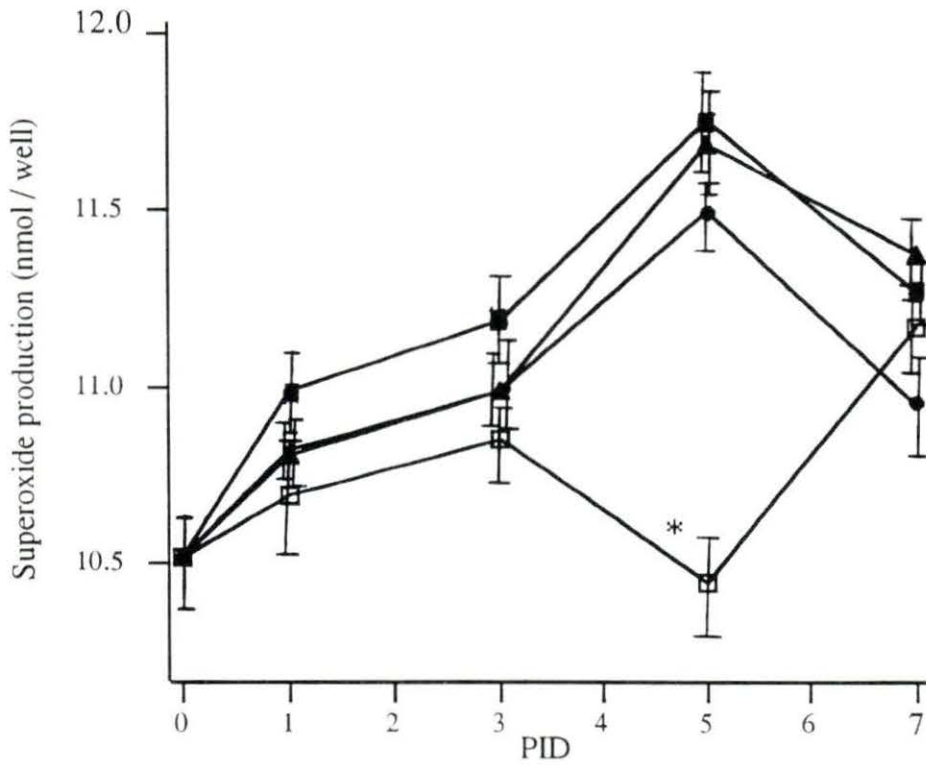
Fig.3. The production of superoxide anion on PID 1, 3, 5 and 7 following
infection with BRSV (■), ncpBVDV (▲), BRSV-ncpBVDV (▢) as
compared with sham-inoculated control (●). Results expressed as the mean
+/- SEM (from 6 calves). *P< 0.05.

Table 2. IL-8 production by bovine alveolar macrophage infected with BRSV, BRSV-ncpBVDV, and ncpBVDV or left as sham-inoculated control. (1)

PID	Control	BRSV	BRSV-ncpBVDV	ncpBVDV
0	(2) 0.1550+/-0.0103	0.1550+/-0.0103	0.1550+/-0.0103	0.1550+/-0.0103
1	0.1400+/-0.0073	* 0.1050+/-0.0115	***0.0583+/-0.0091	0.1383+/-0.0031
3	0.1550+/-0.0085	** 0.1033+/-0.0092	***0.0483+/-0.0060	* 0.1400+/-0.0052
5	0.1567+/-0.0067	** 0.1183+/-0.0079	***0.0767+/-0.0051	* 0.1383+/-0.0060
7	0.1583+/-0.0070	* 0.1433+/-0.0049	***0.0833+/-0.0084	0.1417+/-0.0070

(1) Expressed as chemotactic differential (cm) = chemotaxis - random migration
(2) Mean +/- SEM (n = 6)
 * P< 0.05,   ** P< 0.01,   *** P< 0.01 (compared with control values)

DISCUSSION

Synergistic depression of Fc receptor, phagosome-lysosome fusion, O_2^- production and IL-8 production were observed following coinfection with BRSV-ncpBVDV, while the effect was more limited in AM cultures infected by either BRSV or ncpBVDV alone. These results indicate that BRSV-ncpBVDV coinfection in vivo may be important because coinfection could severely prevent normal clearance of bacteria leading to bacterial pneumonia in young calves.

Virus recovery from infected AM cultures decreased over the 7-day period in this study (Table 1). Our observations were based on a qualitative test (cytopathic effect and immunoperoxidase test) rather than quantitative determination of virus. More sophisticated monitoring techniques might have demonstrated presence of live virus for a longer period. Previous reports (Toth and Hesse, 1983a; Trigo et al., 1985) suggested that BRSV infection in AM culture is abortive, but more recently Adair and McNulty (1992) demonstrated increased numbers of BRSV-infected AM in cultures prepared from 2 of 10 calves over the 7- to 10-day observation period of their study. Becker et al. (1991) found that human AM were susceptible to infection with RSV but the infection was abortive after the initial cycles of virus replication. Panuska and his coworkers (1990) showed that AM cultures infected with human RSV produced very low levels of infectious virus for up to 25 days. Although cpBVDV can replicate in AM cultures (Toth and Hesse, 1983a), no studies have been conducted on ncpBVDV. The virus recovered from AM cultures infected with ncpBVDV indicated that ncpBVDV can replicate in AM.

Fc receptor expression is an important indicator of AM function because it reflects the phagocytic ability of AM. Trigo et al. (1985) and Adair and McNulty (1992) showed that BRSV-infected bovine AM had a significantly impaired ability to phagocytize antibody-coated erthrocytes over 3- to 11-day observation period. Significant depression of Fc receptor expression by BRSV occurred beginning on PID 1 and lasted through PID 5 in our study. A

steady decrease in Fc receptors was observed in the ncpBVDV-infected cultures over the 7-day observation period. Interestingly, the effect of BRSV-ncpBVDV coinfection on Fc receptor expression mirrored the effect produced by BRSV but produced a synergistic depression. This indicates that BRSV may play the dominating role in this combined virus infection.

After being ingested by AM, foreign pathogens are usually enclosed within phagosomes which are promptly fused with lysosome membranes. These ingested pathogens are then acted upon by digestive enzymes (Axline and Cohn, 1970; Jensen and Bainton, 1973). Viral infection can either impair phagocytic ability or prevent phagosome-lysosome fusion or both in AM (Jakab et al., 1980; Jakab, 1982; Warr et al., 1979). Thus, phagosome-lysosme fusion has become a valuable index in measuring virus-induced macrophage dysfunction. Hesse and Toth (1983) showed that although parainfluenza-3 (PI-3) virus did not impair phagocytosis by AM, it prevented efficient processing of ingested yeast particles. In the present study, AM infected with either BRSV or ncpBVDV produced a limited effect on phagosome-lysosome fusion on PID 3 and PID 5, while BRSV-ncpBVDV coinfection resulted in severe depression on PID 1, 3 and 5. In normal macrophages, fusion of lysosomes with phagocytic vacuoles is controlled by intracellular levels of cyclic AMP (cAMP). Prostaglandin E2 (PGE2), one of the prostaglandins produced by AM, competes with cAMP to inhibit macrophage function (Samuelsson et al., 1978; Schnyder et al., 1981). Virus infection has been shown to enhance PGE2 production by macrophages (Samuelsson et al., 1978). The mild down-regulation of phagosome-lysosome fusion in BRSV and ncpBVDV-infected AM may be due to the low levels of PGE2 production, whereas severe depression of BRSV-ncpBVDV coinfected AM may result from two mechanisms, the high level of PGE2 production or the inhibition of phagocytosis.

Our studies show that neither BRSV nor ncpBVDV infection in AM depressed O2- release

from AM, but that BRSV-ncpBVDV coinfection significantly depressed O_2^- production on PID 5. Roth et al. (1981) previously detected no significant depression of NBT reduction or chemiluminescence by polymorphonuclear (PMN) leukocyte after BVDV infection, whereas significant depressions of the iodination reaction of PMN was seen after infection with either cpBVDV or ncpBVDV. The iodination reaction is influenced by several variables including ingestion, oxidative metabolism, degranulation and myeloperoxidase. Depressed iodination indicates a defect in one or more of these variables. The mechanism involved in our study of AM is probably similar. Thus, it would be interesting to investigate further the effect on iodination reactions for each virus group in our study to see whether there is any depression. The normal production values for O_2^- by AM after exposure to either BRSV or ncpBVDV indicated that the virus-infection had not depleted their energy. A study has shown that phagocytosis and degranulation by AM were not affected by PI-3 virus infection, though AM phagosome-lysosome fusion was depressed (Hesse and Toth, 1983). The synergistic depression of O_2^- production on PID 5 by AM could have been due to severe reduction of Fc receptor expression by BRSV-ncpBVDV coinfection. Kobzik et al. (1990) verified that both membrane-derived and mitochondrial components of the AM respiratory burst have important roles in the response to opsonized particles. Thus, O_2^- production by AM can be maintained at normal levels unless the numbers of Fc receptors expressed by virus-infected AM are reduced. The coinfected AMs probably responded to extremly low numbers of opsonized zymosan to decrease markedly the generation of both mitochondrial and membrane-derived oxygen metabolites.

Infection of human AM by human RSV was shown in a previous study to not only express IL-8 mRNA but also to release IL-8 protein (Becker et al., 1991). The secretion IL-8 levels had a tendency to be lower in RSV-infected AM than in AM exposed to inactivated RSV (Becker et al., 1991). The low levels of IL-8 production induced by RSV infection were

shown to have some degree of inhibition of AM function. McGuire and Babiuk (1984) infected calves with infectious bovine rhinotracheitis (IBR) virus followed five days later by aerosol exposure to *P. haemolytica* and found that AM were not able to produce normal levels of IL-8. Results of our study indicate that BRSV, ncpBVDV or BRSV-ncpBVDV infection also depressed IL-8 production. Noncytopathic BVDV infection induced a mild but significant depression of IL-8 on PID 3 and 5, while a significant depression that lasted for at least 7 days was produced by BRSV . On PID 7, IL-8 production was returning to normal. The mechanisms involved in the severe depression of either IL-8 mRNA expresion or IL-8 protein or both synthesis due to BRSV-ncpBVDV coinfection is not known. These results contrast with those reported by other researchers who demonstrated that RSV stimulated increased production of IL-8 in cultures of infected macrophages (Adair and McNulty, 1992). The differences could be due to the variation in procedures. In our experimentation, before challenging with opsonized zymosan, the virus-infected AM culture fluids were discarded and the adherent AMs were washed eliminating the IL-8 produced by virus stimulation. The test we used then measured the ability of virus-infected AM to produce IL-8 following stimulation with opsonized zymosan. Virus may stimulate IL-8 production during the early stage of virus infection, followed by a decrease due to viral inhibition on either IL-8 mRNA expression or IL-8 protein synthesis.

The synergistic effects of BRSV and ncpBVDV on AM functions were clearly demonstrated. The mechanisms involved in this process are probably different from that involved in viral-bacterial synergism. The interaction of viruses and the immune status of the host may be the key factors in determining virus-virus synergism. The synergistic decrease in AM function produced by BRSV-ncpBVDV coinfection may partially explain the pathogenesis leading to severe clinical respiratory disease.

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GENERAL SUMMARY

BRSV and BVDV are the two most prevalent viruses present in bovine respiratory disease. Bovine respiratory disease experimentally induced by either BRSV or BVDV usually results in milder clinical syndromes as compared to natural outbreaks. This implies that synergism between or among viruses may contribute to natural viral infections induced by respiratory viruses. AM is the first defensive guard of the body to respiratory pathogens. Impairment in AM functions can decrease the defensive ability of the host to bacterial infection.

This study revealed that BRSV-ncpBVDV coinfection can severely depress AM functions, therefore presenting direct evidence for the synergism between viruses. This finding may partially explain the pathogenesis of respiratory virus infections leading to severe clinical diseases and may provide new ideas for clinical therapy in natural outbreaks.

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