Evaluation of the cell-mediated immune response to bovine respiratory syncytial virus infection in cattle
by Emery Waine Field

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Veterinary Science
Montana State University

Abstract:
Six Holstein calves were experimentally infected by intranasal instillation with 7.5 X 10^7 plaque forming units (PFU) of bovine respiratory syncytial virus (BRSV), strain VC-494. One calf was maintained as a noninfected control. The calves were evaluated for the development of a cell-mediated immune (CMI) response using the leukocyte migration inhibition (LMI) test under agarose and the delayed hypersensitivity skin test (DHST). Serological evaluations were conducted prior to infection and at the conclusion of the study using the indirect fluorescent antibody test (IFAT).

Results of the LMI test indicate that the infected calves did develop a CMI response which was detected as early as five days postinfection. The response, measured as migration inhibition, reached a maximum value of 34%, 21 days after the experimental infection. An enhanced inhibition, which developed more rapidly, was observed with leukocytes from one calf reinfected with BRSV.

DHS tests were conducted following the LMI evaluations. Infected calves developed intradermal reactions which reached maximal intensity between 48 and 72 hours. The control calf did not develop a delayed type of reaction. Results of the IFAT showed that serum antibody levels also increased in the calves following the experimental BRSV infection.

Results of this study indicated that both humoral and cell-mediated immune responses were elicited in the calves infected with BRSV, strain VC-494.

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Date August 22, 1979

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EMERY WAINE FIELD

A thesis submitted in partial fulfillment
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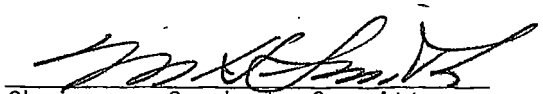
Approved:



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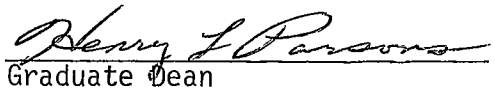

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ABSTRACT

Six Holstein calves were experimentally infected by intranasal instillation with 7.5×10^7 plaque forming units (PFU) of bovine respiratory syncytial virus (BRSV), strain VC-494. One calf was maintained as a noninfected control. The calves were evaluated for the development of a cell-mediated immune (CMI) response using the leukocyte migration inhibition (LMI) test under agarose and the delayed hypersensitivity skin test (DHST). Serological evaluations were conducted prior to infection and at the conclusion of the study using the indirect fluorescent antibody test (IFAT).

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CHAPTER 1

INTRODUCTION

Bovine respiratory disease (BRD) continues to constitute a major economic loss for cattle producers in the United States (71). The widespread use of vaccines against viral and bacterial pathogens currently associated with the disease has failed to prevent outbreaks of respiratory infection in cattle. This ineffectiveness of the vaccines suggests that other etiologic agents may be involved in the pathogenesis of the disease. The failure to prevent outbreaks of BRD also indicates that a complex interrelationship exists between environmental factors, the infectious agent, and the immune response of the host animal in determining the outcome of respiratory tract infection.

Bovine respiratory syncytial virus (BRSV) is widespread in cattle and is recognized as a primary etiologic agent of BRD (9, 94, 97, 104). Recent evidence suggests that the virus can also occur as a concurrent infection with other viral pathogens including parainfluenza-3 (PI-3), infectious bovine rhinotracheitis (IBR), and bovine viral diarrhea (BVD) viruses (9, 62). Vaccines have been developed against PI-3, IBR, and BVD viral infections but an effective vaccine against BRSV is unavailable.

The type of viral disease manifested and the severity, duration and distribution of the disease is in part a result of the inter-

action of the infectious agent and the host's defense mechanisms. These mechanisms are comprised of both specific and nonspecific factors. The nonspecific factors, usually active against a variety of viruses, constitute "native immunity", and are for the most part a genetic property of an animal species (89). The specific immune mechanisms, on the other hand, are dependent upon the host animal's exposure to a particular virus. There are two general types of effector mechanisms which mediate specifically acquired immunity; those mediated by cell products of sensitized lymphocytes (humoral immunity) and those mediated by the lymphocytes themselves and the cells recruited by these sensitized lymphocytes (cell-mediated immunity).

The humoral immune response of cattle to BRSV infection has been investigated using serological techniques (77). The relative importance of this response in counteracting the infection however, remains undefined. Cell-mediated immunity has not been demonstrated to participate in the immune response of cattle to BRSV infection. A better understanding of the importance of the immune response of cattle to BRSV infection will depend upon defining the mechanisms involved and how various events interphase to combat the infection.

Statement of Purpose

The purpose of this study was: (1) to determine if cell-

mediated immunity (CMI) is elicited following experimental infection of calves with BRSV and (2) if CMI is elicited, to determine the minimal time between exposure to the virus and detection of sensitized lymphocytes.

CHAPTER 2

LITERATURE REVIEW

Respiratory syncytial virus (RSV) is recognized as a respiratory tract pathogen in man and cattle. Since its initial isolation from a chimpanzee in 1956, the virus has been shown to be the major cause of severe respiratory disease in human infants (5, 16). The virus produces a type of infection and disease unlike that of any other known respiratory tract pathogen (14).

The first reported isolation of a bovine respiratory syncytial virus (BRSV) was in Switzerland in 1970 from cattle with acute respiratory disease (86). The virus was subsequently shown to be associated with bovine respiratory disease in several countries and is now recognized as one of the primary etiologic agents of the bovine respiratory disease complex (43).

Characteristics of the Virus

The human and bovine respiratory syncytial (RS) viruses together with the pneumonia virus of mice (PVM) are classified as pneumoviruses, members of the paramyxoviridae family (72, 111). Spherical forms of both the human and bovine RS viruses range from 80 to 400 nm in diameter (6, 48, 49, 54, 84). Filamentous forms, exceeding 2 um in length, have been observed budding from cytoplasmic membranes (84). Although there is still some controversy concerning the dimensions of the RSV helical nucleocapsid, it appears to be approximately 14 nm

in diameter with a helical pitch of 6.5 nm (6, 54). Virus assembly occurs by budding through virus modified cell membranes where the virion acquires its envelope containing glycoprotein surface projections (6, 49, 84). The lack of inhibition by halogenated deoxyribosides suggests that the viral genome consists of a single stranded RNA (39, 48).

Buoyant densities for both the human and bovine RS viruses have been determined and range from 1.22 to 1.24 g/ml (21, 48). Sensitivity to low pH has been demonstrated with complete loss of activity at pH 3.0 (39, 48, 86, 104). The virus also appears to be relatively thermolabile, losing 90% of its infectivity when maintained at 55 C for 5 minutes (38, 48).

Unlike other members of the paramyxoviridae family, neither hemagglutination nor neuraminidase activity have been demonstrated with either virus (90). Strains of the virus are not serologically identical but antigenic variation is limited and does not appear to be progressive (20, 22).

The RS viruses can be grown in cell culture using conventional tissue culture techniques. Human RS virus has been grown in human cancer cells including HeLa, Hep 2, and Chang liver cells (55). The bovine RS virus replicates in a variety of bovine cells including kidney, lung, testicle, aorta, and rectum cells (86, 66, 104).

In cell culture, the primary cytopathic effect is the forma-

tion of multinucleated epithelial giant cells termed syncytia. These syncytia, developing within 5 to 6 days postinfection, often contain numerous eosinophilic cytoplasmic inclusion bodies (55, 86, 104).

Human RSV

In 1956, Morris and associates (81) isolated an agent from a chimpanzee with an upper respiratory tract infection. This newly discovered agent, an RNA virus, was shown to be serologically distinct from previously identified paramyxoviruses and was designated chimpanzee coryza agent (CCA). An etiologic association was established between this isolate and a respiratory illness observed in a laboratory worker who had been in close contact with the chimpanzee. In the following year Chanock and co-workers, attempting to recover new agents from infants with severe lower respiratory tract illness, reported two isolations of a similar virus from children in Baltimore (18). Using neutralization tests, the virus was shown to be serologically indistinquishable from the CCA. Cell cultures infected with the virus developed a syncytial type of cytopathic effect. Owing to its association with respiratory illness and the fact that it induced the formation of syncytia in cell culture, the virus was designated respiratory syncytial virus (RSV).

Serological surveys have shown that RSV infections are quite common in the human population. Over 70% of the persons tested possessed neutralizing antibodies for the virus by five years of age and

over 90% by 15 years of age (12, 15, 37). The lowest incidence of neutralizing antibody was found in the 6-month to one-year age group which is temporally associated with the disappearance of passively acquired maternal antibody.

Annual epidemics of RSV infections occur primarily from late fall to early spring, with the peak months being January and February. These epidemics are associated with a dramatic increase in the number of infants and young children requiring hospitalization for lower respiratory tract disease (15, 57). This is in contrast to the virtual absence of confirmed RSV infections during the summer months, May through August.

Since its initial isolation, RSV has emerged as the major respiratory tract pathogen of infancy and early childhood (5, 10, 16, 25, 110). The virus is also considered to be a major cause of fatal respiratory tract disease in the first year of life. A study in Britain has shown that 19 of 22 children with fatal respiratory tract infections were under one year of age and RSV was isolated from eight of these patients postmortem (34).

A variety of clinical patterns of respiratory illness have been associated with the isolation of RSV but the most common are bronchiolitis and bronchopneumonia (15). The virus was shown to be associated with respiratory illness in a large proportion of infants admitted to a children's hospital during the first three months of 1962.

The RSV illnesses ranged from mild upper respiratory tract infection to severe bronchiolitis and bronchopneumonia. A ten month old boy, from whom RSV was isolated, died 24 hours after admission to the hospital with severe lower respiratory tract disease. Microscopic examination of respiratory tissues revealed cellular necrosis of the tracheobronchial mucosa, focal consolidation of both lungs, and severe emphysema, suggesting an incomplete bronchial or bronchiolar obstruction (45).

The severe disease occurs most often in children one month to six months of age, the frequency of disease then decreases with increasing age (16, 17). Older children and adults usually develop a less severe disease with predominately an upper respiratory involvement (52). This age-illness relationship of RSV disease is still not completely understood but investigators have advanced several theories.

Infants with the severe disease were found to possess moderate to high levels of maternally derived antibody specific for the virus (87). The decreasing levels of these passively acquired antibodies with increasing age appeared to correlate with the decreasing frequency of disease. This apparent correlation indicated that serum antibody did not provide effective protection against the lower respiratory tract disease. Furthermore, these observations provided the basis for Chanock et al. (13) to speculate that serum antibody might actually

contribute to the development of the serious lung damage. They proposed that a type 2 or possibly a type 3 immune hypersensitivity reaction might result from the interaction of pre-existing antibody and viral antigen in the lungs.

Vaccination studies using a formalin-inactivated, alum-precipitated RSV vaccine appeared to provide support for this theory (56, 58). Children receiving this killed vaccine developed substantial levels of serum neutralizing antibody to the virus. Upon exposure to a wild-type RSV however, 80% of the children in the vaccinated group required hospitalization and two of the children died. This unexpected result was interpreted as clearly establishing the involvement of serum antibody in the pathogenesis of the severe RSV disease (13). Recent reports have failed to support this theory and it now appears that serum antibody does provide a protective effect against the severe disease (8, 61). Data from a 10-year study of respiratory illness in normal children indicate that gradually accumulating levels of humoral antibody parallel the acquired resistance to RSV infections (40). There is substantial reduction in the attack rate in susceptible individuals and also in the severity of illness produced by a third re-infection with RSV. These findings indicate that both age and immunity, although not necessarily serum antibody, act to ameliorate the illness associated with infection due to RSV.

If serum antibody does not provide adequate protection against the severe RSV disease, other mechanisms must be involved since older children and adults do show some resistance. Cell-mediated immunity (CMI) could conceivably play a significant role in limiting the cell to cell spread of the viral infection. Results obtained from vaccination trials and natural RSV infections indicate that a CMI response does occur but that it does not prevent the development of the serious disease (59, 98, 99).

The role of interferon in recovery from RSV infection is still unclear. Conflicting results have been obtained regarding the induction of interferon by RSV and its sensitivity to the antiviral protein (33, 41, 75). Although infants appear to be capable of producing significant levels of interferon, McIntosh (67) detected only low levels in children with RSV infections. Hall et al. (36) found that the quantity of interferon produced by children with RSV infections was significantly less than those with influenza and parainfluenza virus infections. Whether these results suggest that interferon is not involved in recovery from RSV infection or that low levels are adequate in limiting infection will require further investigation.

Local secretory antibody has been shown to be a major determinant in resistance to viral respiratory tract infections. Mills et al. (74) observed that high levels of specific IgA in nasal secretions prevented RSV infection in adult volunteers regardless of the

level of serum antibody. Other studies have indicated that local secretory antibody may also provide a curative effect in RSV infections. McIntosh et al. (68) found that the disappearance of virus from nasal secretions correlated with the appearance of secretory antibody. Nasopharyngeal epithelial cells from infants with confirmed RSV infections have been shown to be coated with an anti-RSV IgA (32, 69). This coating of the infected cell with specific antibody could neutralize viral particles as they bud from the surface of the infected cell. Another possibility is that the antibody prevents the successful maturation of virus at the cell surface, thereby preventing the release of infective viral progeny. This type of action has been demonstrated in influenza A infections where the antibody prevented the release of infective viral particles (24).

These studies coupled with the ineffectiveness of vaccine-induced serum antibody, have encouraged the development of an attenuated strain of RSV which can induce resistance without producing significant illness (11). A chemical mutagen, 5-fluorouridine, has been used to produce a temperature-sensitive (ts) mutant which is designated ts-1. When administered into the nasopharynx of adult volunteers, the mutant induced resistance without producing significant illness. It appeared genetically stable under laboratory conditions but when children were infected experimentally with the ts-1 they shed a genetically altered virus with a wild-type temperature

sensitivity (42). Current research efforts are directed at producing a more genetically stable ts mutant of the RSV. It will hopefully provide a means of preventing the severe disease which occurs in infants less than one year of age.

Bovine RSV

In 1968, Doggett et al. (23) found neutralizing activity to human RSV in bovine sera suggesting the presence of an antigenically similar agent in cattle. Then in 1970, Paccaud and Jacquier (86) reported the isolation of a viral agent from cattle in Switzerland. They isolated the virus from a cow and a calf during an outbreak of respiratory disease in February of 1967. The disease was characterized primarily by an upper respiratory tract infection with nasal discharge and a mild cough. In approximately one third of the cases however, a high fever developed with discrete signs of bronchopneumonia. The seroconversion of these cattle, in neutralization and complement-fixation tests, clearly established that the isolate was of bovine origin.

Neutralization tests using human sera indicated that the virus, or an antigenically related agent, was also present in the human population. This fact together with characteristics of the isolate such as acid-lability, chloroform sensitivity, and a syncytial type of cytopathic effect in cell culture, suggested that the virus might be a

bovine representative of the RSV. Cross-neutralization and complement fixation tests clearly established the close antigenic relationship between the isolate and human RSV. With this evidence the authors concluded that the new isolate was indeed a bovine respiratory syncytial virus (BRSV).

Following this initial isolation Inaba and associates (46, 47) reported the isolation of a BRSV from cattle during an outbreak of respiratory disease in Japan. The disease swept across Japan during the months from October 1968 to April 1969. Clinically ill cattle exhibited anorexia, depression, pyrexia, respiratory distress, and a mild cough. In fatal cases, emphysema and consolidation of the lungs were noted. The close antigenic relationship between this virus and human RSV was established using neutralization tests. Serological data indicated that the virus was widespread in Japan. Over 60% of the cattle tested possessed detectable levels of neutralizing antibodies to the virus. Experimental infection of a calf with the new isolate produced pyrexia, anorexia, depression, serous nasal discharge, and a leukopenia. The calf developed neutralizing antibody against the virus which were detectable two weeks postinoculation.

In 1971, Wellemans reported a BRSV isolate from cattle with respiratory disease in Belgium (112, 113). The virus was described as the principle viral etiologic agent associated with bovine respiratory disease in that country. Efforts were initiated to develop an

attenuated strain of BRSV which could be used in a vaccination program against the viral related disease.

Jacobs and Edington (50, 51) reported the isolation of a BRSV from a group of 14 calves in Dorset, England. The calves had a serous nasal discharge, pyrexia, increased respiration rate, and a mild cough. The isolation confirmed serological data that indicated the virus was prevalent throughout Britain. Experimental infection of gnotobiotic, colostrum-deprived, and conventional calves produced a temperature response with a serous nasal discharge in all three experimental groups. The virus was re-isolated from nasal secretions of infected calves four to ten days postinoculation.

The first isolation of BRSV in the United States was reported by Smith, Frey, and Dierks in 1974 (103). They isolated the virus from Iowa feedlot cattle with an acute respiratory disease. Biological and physical characteristics together with cross-neutralization tests indicated a close but non-identical relationship to human RSV (104). Serological data indicated that 81% of the cattle from 43 herds possessed serum antibody to the virus. Experimental infection of five calves with the isolate produced clinical illness characterized by anorexia, pyrexia, leukopenia, nasal discharge, and malaise. The virus appeared capable of producing illness in calves possessing maternal antibody for the virus.

Rosenquist, in 1974, reported the isolation of two strains of BRSV from nasal secretions of calves with acute respiratory disease in Missouri (94). The calves exhibited clinical signs of respiratory illness, including pyrexia, increased respiration rate, and nasal discharge. Three calves, out of a group of nine, developed significant levels of neutralizing antibody to the virus isolate.

Since its initial isolation in Iowa, the virus has been shown to be prevalent in several states. Potgieter and Aldridge (88), using the indirect fluorescent antibody test (IFAT) to detect BRSV specific antibody, reported that 73% of the cows from 160 herds in Oklahoma were seropositive for BRSV. In three herds in which respiratory disease developed, the incidence of BRSV seroconversion approached 100%. Mohanty et al. (76) conducted a serological survey of neutralizing antibody to BRSV in Maryland. Thirty-eight of 100 cattle surveyed possessed antibody to the virus. In a recent serological survey of Montana cattle at Montana State University, Smith (personal communication) demonstrated widespread exposure to the virus. These studies indicate that BRSV may be as prevalent in cattle as human RSV has been shown to be in the human population.

In the fall when calves are weaned, they are exposed to physical stress and are frequently placed in feedlots with calves from other geographical regions. These factors often contribute to the development of respiratory disease. The disease has been referred to

as "shipping fever" but new terminology designates the illness as "bovine respiratory disease complex". The present concept of the etiology of respiratory tract disease of cattle is that it is a multifactorial syndrome involving stress, viral, and bacterial infections (43). Parainfluenza-3 (PI-3), infectious bovine rhinotracheitis (IBR), and bovine viral diarrhea (BVD) viruses have been given considerable significance in the etiology of the disease but little attention has been given to the involvement of BRSV.

Lemkuhl and Gough (62) collected serum samples from early weaned Iowa fall calves shortly after the onset of respiratory tract disease. Seroconversion rate of cattle to IBR virus was 4.3%, PI-3 - 16.3%, BVD - 9.6%, and BRSV - 45.4%. They also observed an increased rate of seroconversion for IBR, PI-3, and BVD viruses in the presence of BRSV seroconversion. These results appear to implicate BRSV in this disease of calves with the possibility of BRSV infection facilitating infection by other viral pathogens.

In a recent report, Bryson and associates (9) described four outbreaks of calf pneumonia in which there was serological evidence of concurrent infections with PI-3 and BRS viruses. Calves exhibited reduced appetites, nasal discharge, coughing, dyspnea, and pyrexia. An emphysematous crackling was also noted over the diaphramatic lobes of the lungs. Three calves out of a group of six failed to respond to antibiotic treatment and died within five days of the onset of disease.

Postmortem examination of these calves revealed pneumonia involving the cranial lobes and severe pulmonary emphysema. Purulent exudate was evident in the bronchioles and there were areas of intralobular hemorrhage. Microscopic examination revealed the most severe lesions were those in the bronchioles, respiratory acini, and small blood vessels. Bronchitis and bronchiolitis were evident, with affected airways containing an exudate of polymorphs, mononuclear cells, and desquamated epithelial cells. Alveolar lesions consisted of overinflated alveoli, areas of alveolar collapse, focal necrosis, alveolar edema, and congestion of the alveolar blood vessels. Multinucleated epithelial syncytia were prominent in bronchiolar and alveolar walls with intracytoplasmic inclusion bodies within these syncytia.

Whether BRSV was totally responsible for the lesions or acted in concert with PI-3 cannot be determined but the involvement of BRSV in the development of these lesions is most certainly implicated.

Immunological Response to BRSV Infection

Natural and experimental BRSV infections in cattle have been shown to elicit a humoral response with the development of detectable levels of both nasal secretory and serum antibody (47, 51, 77, 82, 94, 104, 113). The relative importance of these antibodies in the resistance to the viral infection, or recovery from it, has not been clearly established.

Serum antibody does not appear to play a prominent role in

resistance to viral respiratory tract infections. Mohanty et al. (77) observed that calves challenged intranasally with BRSV developped clinical illness in the presence or absence of circulating antibody. Smith et al. (104) also reported that calves with pre-existing serum antibodies for BRSV developed clinical illness upon intranasal challenge with the virus. Similar results have also been reported in studies with PI-3 virus infection of calves. Levels of serum antibody did not correlate with resistance to infection but did appear to be associated with reducing the severity of disease (27).

These studies in cattle are in agreement with the results of human RSV studies where the level of serum antibody failed to correlate with resistance to respiratory infection (13, 87). These studies also indicate that serum antibody appears to be involved in reducing the severity of respiratory illness.

Serum antibody plays a major role in preventing those diseases in which the virus must travel through the blood stream to reach its target organ. In respiratory tract infections where the target organ is also the portal of entry, local factors including interferon, secretory antibody, and local cell-mediated immunity appear to be the most important defense mechanisms (93).

A secretory antibody system has been identified in the bovine species with IgA being the predominant immunoglobulin in nasal and lacrimal secretions (64). This secretory antibody system can function

independently from the systemic antibody system and can be activated by local application of antigen (96, 109). This fact is of extreme importance where the primary site of virus replication is the respiratory tract. Studies of viral respiratory infection in man have shown that the presence of antibody in nasal secretions correlates with protection against influenza, parainfluenza, and RSV infections (1, 21, 65, 74, 95, 102).

Studies with PI-3 virus infection in cattle also indicate that local secretory antibody (IgA) confers better protection against respiratory tract infection than circulating antibody (27, 35). Mohanty et al. (77) reported that four of five calves inoculated intranasally with BRSV developed detectable levels of nasal secretory antibody. When these calves were re-challenged with the virus five weeks after the primary challenge, they were found to be solidly immune. This was interpreted as evidence of the importance of secretory antibody in resistance to BRSV infection. It should be noted however, that a non-specific factor, possibly interferon, may have been responsible for the observed immunity in these calves. These studies, while not clearly establishing secretory antibody as the protective mechanism in resistance to respiratory viral infection, strongly implicate the participation of antibody in the process.

In focusing our attention on these specifically acquired immune responses, ie., the serum and secretory antibody responses, the

importance of nonspecific mechanisms of local protection should not be overlooked. Factors such as mucocilliary barriers, temperature, and nonspecific phagocytosis by alveolar macrophages play a significant role in resistance to viral infection. In addition, nonspecific effects of infection, such as cellular resistance due to interferon production, may often determine the final outcome of the disease (73).

Once a viral infection is established, with virus replication occurring in a fully susceptible host, active defense mechanisms must be elicited to combat the infection. As described earlier in this review, viruses that promote systemic infections with viremia are controlled primarily by circulating antibody. On the other hand, viruses which spread from cell to cell via intercellular bridges and which usually elaborate specific antigens on the cell surface, are more likely to elicit a CMI response (85, 115). Examples of this type of virus include members of the orthomyxo- and paramyxoviridae families. Most of the members of these families have been shown to elicit both cell-mediated and humoral immune responses (28).

The CMI response can occur locally in the respiratory tract after intranasal infection without a generalized response, or at least with a diminished one (78, 112). T-lymphocytes, stimulated by contact with viral antigen on the surface of infected cells, may become directly cytotoxic to the infected cells. These cytotoxic reactions are very likely to be important in the control of noncytolytic viral

infections (115). The interaction between these T-lymphocytes and the infected cells can also result in the release of soluble mediators called lymphokines. The biological activity of lymphokines affect the behavior of macrophages, polymorphonuclear leukocytes, lymphocytes, and other cell types in vitro. A list of these factors published in a report of a World Health Organization scientific group (115) is found in table 1.

The following is an excerpt from R.E. Rocklin's chapter on mediators of cellular immunity (29). It is a qualitative interpretation of the events that may occur in certain cell-mediated reactions based upon the existence of these lymphokines.

> ...." Antigen-sensitive lymphocytes, when stimulated by the appropriate antigen, become activated and start synthesizing the various lymphocyte mediators. Chemotactic factors for monocytes and polymorphonuclear leukocytes recruit inflammatory cells to the reaction site. Once there, the macrophages and polymorphonuclear leukocytes might be held at the site by MIF and LIF. Macrophages might be activated to an enhanced state by the action of the activating factor. Other lymphocytes are recruited to participate in the reaction by the mitogenic factor. Lymphocyte mitogenic factor nonspecifically activates the other lymphocytes in the area which would perhaps, in turn, start producing lymphocyte mediators. These events have the effect of amplifying an initially small reaction. Once activated, the inflammatory cells

Table 1. Biological activities of products of activated lymphocytes[a]

A. Affecting macrophages		
Migration inhibition factor	(MIF)	inhibits the migration of normal macrophages
Macrophage aggregation factor	(MAF)	agglutinates macrophages in suspension
Macrophage chemotactic factor	(MCF)	cause macrophages to migrate through micropore filter along gradient
Macrophage resistance factor (postulated)		renders macrophages non-specifically resistent to infection with certain bacteria and viruses
Cytophilic antibodies		confer on macrophages specific reactivity with antigen
B. Affecting lymphocytes		
Blastogenic or mitogenic factor	(BF or MF)	induces blast cell transformation and tritiated thymidine incorporation in normal lymphocytes
Potentiating Factor	(PF)	augments or enhances ongoing transformation in mixed lymphocyte culture or antigen-stimulated cultures
Cell cooperation or helper factor		produced by T cells, increases the number or rate of formation of Ab-producing cells *in vitro*
Suppressor factor (postulated)		inhibits activation of, and/or antibody production by B cells

Table 1. (Continued)

C. Affecting granulocytes		
Inhibition factor	(LIF)	inhibits the migration of human buffy coat cells or peripheral blood leukocytes from capillary tubes or wells in agar plates
Chemotactic factor		causes granulocytes to migrate through micropore filter along a gradient
D. Affecting cultured cells		
Lymphotoxin	(LT)	cytotoxic for certain cultured cells, e.g., mouse L cells or HeLa cells
Proliferation inhibition factor and cloning inhibition factor	(PIF, CLIF)	inhibit proliferation of cultured cells without lysing them
Interferon		protects cells against virus infection
E. Producing effects in vivo		
Skin reactive factor (possibly a combination of several of the above activities)	(SRF)	in normal guinea pig skin induces indurated skin reactions that are similar histologically to delayed hypersensitivity reactions
Macrophage disappearance factor		injected intraperitoneally, causes macrophages to adhere to peritoneal wall

[a]From: Cell-mediated immunity and resistance to infection. World Health Organization Technical Report Series No. 519. 1973. WHO, Geneva.

> become bactericidal or tumoricidal. Furthermore, the vasoactive properties of some of the mediators may account for part of the inflammation. Other protein systems, including the complement system, the kinin system, and the clotting system are also called into play. If antigen remained at the site, such a reaction would continue, abating only as the antigen supply was exhausted."

This interpretation is purely speculative since it has not as yet been possible to confirm in vivo.

The apparent correlation between the interaction, in vitro, of sensitized lymphocytes and appropriate antigens, and delayed hypersensitivity skin reactions, led to the establishment of a series of in vitro correlates of cell-mediated immunity. These in vitro correlates have been extensively detailed and reviewed by Bloom and Glade (7), the World Health Organization (115), and McCluskey and Cohen (70). Therefore, consideration of this subject will be limited, with emphasis on the two techniques that relate directly to the experimentation described in this thesis.

Delayed Hypersensitivity Skin Test

When certain antigens are injected into the skin of sensitized animals, an inflammatory response may develop at the injection site. Erythema and swelling gradually appear, reaching maximal intensity from 24 to 72 hours after the injection. Histologically the reaction is

characterized by the accumulation of large numbers of macrophages and lymphocytes.

This response to the antigen can be transferred from sensitized to normal animals through the use of lymphocytes, but not by means of serum, indicating that the response is cell-mediated. The reaction is the result of the interaction of sensitized lymphocytes and appropriate antigen. These circulating, antigen-sensitive T-lymphocytes, upon encountering the injected antigen respond by both recruiting other lymphocytes and by dividing, differentiating, and releasing lymphokines. Some of these lymphokines, chemotactic factors and migration inhibition factors, are thought to be responsible for the accumulation of the large number of macrophages and lymphocytes. Vascular changes are mediated through the release of "skin-reactive factors" as well as by the release of lysosomal enzymes from the macrophages. As the macrophages ingest and eventually destroy the antigen, the stimulus for further lymphokine production diminishes, permitting the tissues to return to normal (108).

Skin testing with the appropriate antigen is the most important diagnostic test for CMI (115). Perhaps the best known example of this test is the Mantoux reaction, obtained by injecting tuberculin antigen into the skin of a sensitized animal. In cattle, the caudal fold is usually used as the injection site, with positive animals developing the characteristic erythema and indurated swelling

from 24 to 48 hours later. Although a neck site has been shown to be more sensitive the difficulty in restraining the animal make the caudal fold the most commonly used injection site in cattle. Other antigens including mumps, PI-3, histoplasmosis, and toxoplasmosis have been used for assessing delayed hypersensitivity to infectious diseases in man and animals (79, 115).

Leukocyte Migration Inhibition (LMI) Test

In 1967 Soborg and Bendixen (106) described an *in vitro* test based upon the inhibition of the migration of human peripheral leukocytes from glass capillary tubes. They found that this specific inhibition correlated with the presence of delayed hypersensitivity to brucella antigen. In recent years, this or modifications of the test, have been applied in a number of studies of cellular immunity. The method is now recognized as an *in vitro* correlate of cellular immunity in man and animals (105, 106).

In 1971 Clausen (19) developed a leukocyte migration inhibition (LMI) test in which the leukocytes migrate out from wells punched in an agarose gel. The leukocytes, primarily polymorphonuclear neutrophils (PMN), migrate out from the wells between the agarose and the surface of the petri dish, forming a circular area of migrated cells. The migration is inhibited if appropriate antigen is preincubated with the antigen-sensitive lymphocytes. These lymphocytes interact with the antigen and produce lymphokines, including a leukocyte inhibitory

factor (LIF). This LIF has been shown to be chemically distinct from the migration inhibitory factor (MIF) that inhibits the migration of macrophages in the macrophage migration inhibition test (91). The presence of cytophilic antibody, antigen-antibody complexes, and antigen alone do not affect the migration patterns in the LMI test (44).

Erard (26) demonstrated the suitability of the LMI test in agarose for routine detection of CMI in man. He found a good correlation between the PPD skin test and the inhibition of leukocyte migration using this antigen. Other investigators have employed the test to evaluate cellular immunity to numerous antigens including toxoplasmosis (31), hepatitis B (26), and candida antigen (4). Anders and Natvig (71) used the LMI test to measure CMI to mumps, herpes simplex, adenovirus, and cytomegalovirus antigen. They found a good correlation between the results of the LMI test and skin reactivity to the antigen.

In 1977 Moreno-Lopez evaluated the CMI response of cattle to PI-3 virus infection. Test systems included the skin-hypersensitivity (SH) test, lymphocyte stimulation (LS) test, and the leukocyte migration inhibition (LMI) test. In four calves vaccinated intranasally with PI-3 virus, inhibition of leukocyte migration was observed two weeks after vaccination. Maximal inhibition reached 50 to 60% of control values, and occurred ten weeks postinfection. Results of the LMI test correlated with those obtained with both the

SH and LS tests. The LMI test was described as being more rapid to perform and more reproducible when compared to the LS test (80).

In a recent review of in vitro methods in CMI, the LMI test in agarose was characterized as being easy to perform, rapid, and a reliable correlate of cell-mediated immunity in man and animals (107). The test requires only a minimal amount of antigen which can be very crucial when only small amounts of purified antigen are available (26). The test offers an excellent tool in evaluating the cellular immune response to a large number of agents in both man and animals.

CHAPTER 3

MATERIALS AND METHODS

Experimental Animals

Seven Holstein calves, three to eight months of age, were purchased from local farms for use in this study. Two of the calves, approximately three months of age, were seronegative for BRSV. The remaining five calves possessed low levels of serum antibody for the virus as determined by the indirect fluorescent antibody test (IFAT) (88).

Six calves were inoculated intranasally with 7.5×10^7 plaque forming units (PFU) of BRSV/cell suspension. One of the calves was also inoculated intranasally with four milliliters of IBR/PI-3 modified live virus vaccine. The remaining calf was housed separately and was kept as a noninfected contol.

Virus and Viral Antigen

Bovine RSV, strain VC-494, was propagated in a continuous cell line of Georgia bovine kidney (GBK) cells. Media for growth and maintenance of cell cultures consisted of Dulbecco's medium [1] supplemented with 10% fetal calf serum [1] (FCS) and adjusted to pH 7.4.

Infected cell cultures were incubated at 37 C for 72 - 96 hours and harvested when 70 - 80% of the monolayer showed a cytopathic

[1]Grand Island Biological Co., Grand Island, New York.

effect (CPE). The cultures were then subjected to three cycles of freeze-thawing followed by sonication for 20 seconds at a setting of 35 kc/second.

Cellular debris was partially removed by low speed centrifugation (1000 X g) for 20 minutes. PFU titrations were then performed, the amount of virus present ranging from 1.5×10^6 to 5.0×10^6 PFU/ ml. The viral suspension was aliquoted into appropriate amounts and frozen at - 70 C.

The BRSV test antigen was prepared from GBK cell cultures infected with strain VC-494. The infected cell cultures were frozen at - 70 C when 70 - 80% of the cell monolayer showed CPE. The virus/cell suspension was then allowed to thaw at 37 C and partially purified and concentrated according to the procedure described by Senterfit and Baldridge (100). Briefly, cell debris was partially removed by low speed centrifugation (1000 X g) for 20 minutes. The virus in the resulting supernatant fluid was then concentrated by precipitation with 6% (w/v) polyethylene glycol-6000 [1] and partially purified by sucrose-gradient centrifugation for 16 hours at 105,000 X g. The resulting band of viral antigen was recovered and dialyzed against five changes of 50 volumes of phosphate-buffered saline solution (PBSS; pH 7.4) at 4 C. The concentration of viral protein was determined according to the method of Lowry et al. (63). A control antigen

[1]Sigma Chemical Co., St. Louis, Missouri.

was prepared from noninfected cell cultures according to the procedure described above.

An IBR/PI-3 modified live virus vaccine [1] was used to inoculate one of the BRSV infected calves in this study. A dilution of this vaccine was also used as a test antigen in the LMI test.

Selection of Optimum Protein Concentration

The optimum protein concentration of the BRSV test antigen preparation was established by a dose-response study. A sample of the test antigen was adjusted to 10, 25, 50, 100, 150, 200, and 250 ug/ml of protein. The dilution of the antigen which inhibited sensitized leukocytes with minimal effect upon nonsensitized leukocytes was determined. The antigen preparation was diluted to a protein concentration of 50 ug/ml and stored at - 70 C in 0.1 ml aliquots until needed. The control antigen was treated similarly and stored until used in the LMI test.

Indirect Fluorescent Antibody Test (IFAT)

The IFAT was performed according to the procedure of Potgieter and Aldridge (88). Cells were grown in four-well chamber slides [2] and infected with BRSV, strain VC-494. After 20 hours of incubation, the

[1] Jensen-Salsbery Lab., Kansas City, Missouri.

[2] Lab-Tek Products, Division Miles Lab. Inc., Kankakee, Il.

cells were washed with PBSS and fixed in acetone for ten minutes. The slides were stored at - 70 C until needed.

The test was performed by rehydrating the slides in a moist chamber at room temperature. Ten-fold dilutions of serum samples, including known negative and positive samples, were placed onto the infected cells. The slides were then incubated for 30 minutes at 37 C. Following three washings in PBSS (pH 7.5) the slides were air dried. An anti-bovine IgG conjugate [1], diluted 1:16 in PBSS, was placed onto the slides and allowed to incubate for 30 minutes. This incubation was followed by three washings in PBSS, after which the slides were allowed to air dry. Coverslips were mounted using Wellcome's medium (pH 8.9).

The slides were examined using a microscope equipped with a darkfield condenser and a 200-W mercury vapor lamp. The intensity of the fluorescence was rated subjectively from 0 (negative) to a +4 (very intense).

Delayed Hypersensitivity Skin Test

Calves were injected intradermally into a shaved area on the lateral portion of the neck with 0.2 ml of the BRSV antigen and with 0.2 ml of the virus-free control antigen. The distance between the injection sites was approximately 10 cm. The thickness of the skinfold at the site of each injection was measured using a micrometer

[1]Miles Lab., Inc., Research division, Elkhart, In.

gauge shortly before and at 24, 48, and 72 hours after the injection of antigen. The injection sites were also checked at six to eight hours to insure that an Arthus type of reaction was not interfering with the latter evaluations.

Preparation of Peripheral Blood Leukocytes

Peripheral blood leukocytes were isolated using a modification of the method described by Naylor and Little (83). Blood (20 ml) was collected in a 20 cc syringe containing preservative free heparin [1] (10 IU/ml). The blood was divided equally into two centrifuge tubes, each containing 20 ml of distilled water. After shaking gently for 45 to 60 seconds, 10 ml of 0.0132 M phosphate buffer in 2.7% NaCl solution (pH 6.8) was added to restore isotonicity. The tubes were then centrifuged for ten minutes at 450 X g. The supernatant fluid was discarded and the pellets of white cells were washed once in Hank's BSS [1] containing heparin (5 IU/ml) followed by two washings in the same medium but without heparin. The cells were then resuspended in TC medium Parker 199 [1] supplemented with 10% FCS, penicillin (60 IU/ml) and streptomycin (60 ug/ml) [1] to a concentration of 2 - 2.5 X 10^8 cells per milliliter. The viability of the cells was found to be greater than 90% by trypan blue exclusion. Average differential count was 34% neutrophilic granulocytes, 56% lymphocytes, 7% monocytes, and

[1]Grand Island Biological Co., Grand Island, New York.

3% eosinophilic granulocytes.

Preparation of Agar Medium for the LMI Test

The agarose plates were prepared using a modification of the procedure described by Gaines et al. (31). A 1.6% agarose suspension was mixed with FCS and TC medium Parker 199 supplemented with penicillin (60 IU/ml) and streptomycin (60 ug/ml). The final concentration was 0.8% agar and 10% FCS. Six milliliters of the agar medium was poured into disposable plastic petri dishes [1] (60 X 15 mm). The agar medium was allowed to cool at room temperature and was then placed in a cooler at 4 C for 15 to 30 minutes. Six wells, 3 mm in diameter, were punched in the agarose in each dish and the plugs were gently removed by suction. The dishes were then incubated at 37 C in an atmosphere of 2% CO_2 in air until use. The pH of the agarose medium was between 7.2 and 7.5 .

Leukocyte Migration Inhibition Test

A modification of the procedure described by Clausen (19) was used. The BRSV antigen was prepared as described above. Aliquots (0.2 ml) of the leukocyte suspension were incubated for 45 minutes at 37 C with aliquots (0.1 ml) of the BRSV test antigen and the control antigen. The cells were resuspended at ten minute intervals during

[1] Lux Scientific Corporation, Newbury Park, California.

incubation to facilitate maximal contact with the antigen. Following this incubation period 10 ul of the mixture of cells and BRSV antigen or control antigen were placed into each well. Each dish contained three wells with cells and BRSV antigen and three wells with cells plus control antigen. The plates were incubated at 37 C in an atmosphere of 2% CO_2 in air enclosed within a moist chamber.

After 18 hours of incubation the cells were fixed in the agarose dish with 8.0% glutaraldehyde [1] for 30 minutes. The agar was then removed and the cells stained with crystal violet. The cells were rinsed twice with distilled water and then allowed to air dry.

The degree of cell migration was determined by measuring the linear distance of migration from the edge of the well to the perimeter of the area covered by the migrating cells. For this purpose a micrometer disc was used and the values expressed as the number of grids covered by migrating cells. The migration inhibition in percent was calculated for each test well according to the formula:

$$1 - \frac{\text{Mean linear migration of antigen treated cells}}{\text{Mean linear migration of control cells}} \times 100$$

An average of three replicates of each test was used in the final computations.

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[1] Sigma Chemical Co., St. Louis, Missouri.

Cytological Examination

Characterization of the migrated cells was accomplished by staining the cells after 18 to 20 hours of incubation. A coverslip (22 X 40 mm) was placed in the bottom of the plate before the addition of the agarose medium. The plate was then treated as previously described. At the end of the incubation period the plate was floated on water (45 C) for 15 minutes. The agarose medium was then carefully removed and the coverslip placed in absolute methanol at room temperature. The cells were stained in Wright's stain according to standard staining procedures.

CHAPTER 4

RESULTS

Clinical Observations

Calves experimentally infected with BRSV developed clinical illness characterized by nasal and lacrimal discharge, increased respiration rate, depression, and a transient pyrexia. Nasal discharge was first observed on the fifth day postinfection and persisted for four to five days. The maximum postinfection temperatures of individual calves are shown in table 1.

Table 1. Maximum Postinfection temperatures

Calf No.	Maximum temperature	Day Postinfection
1	104.6	8
2	105.6	8
3	107.2	8
4	104.8	9
5	102.8	8
6	105.6	8

Average preinfection temperatures ranged from 101.2 to 102.0 F.

Leukocyte Migration Inhibition Test

The migration of bovine peripheral leukocytes from wells punched in an agarose gel is shown in figure 1. The leukocytes migrated between the agarose layer and the surface of the dish to form a cir-