THE EFFECT OF VACCINATION ON THE RESPONSE TO EXPERIMENTAL INFECTION WITH BOVINE RESPIRATORY SYNCYTIAL VIRUS INFECTION IN CALVES

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SUMMARY OF DISSERTATION

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The Effect of Vaccination on the Response to Experimental Infection with Bovine Respiratory Syncytial Virus Infection in Calves

Bovine respiratory syncytial virus (BRSV) is an important respiratory pathogen of cattle. This thesis investigated vaccine efficacy, the significance of qualitative differences in antibody responses, and the correlates of immunity to BRSV infection, using a virulent experimental challenge.

The functional properties of antibodies induced by a MLV and 3 inactivated BRSV vaccines were compared in feedlot calves (n=10 per group)(trial 1). A challenge model that induces severe clinical disease and pulmonary pathology was developed by serially passing a field isolate of BRSV in newborn calves, using lung washing as the challenge inoculum. The effect of vaccination on BRSV infection was investigated. In trial 2, BRSV naïve calves were vaccinated twice with a formalin inactivated (FI) vaccine, 10^3 pfu MLV or sham vaccinated (n=4 per group). In trial 3, calves were vaccinated with either 2 doses of MLV intramuscularly (IM) or intradermally, with a single dose IM of MLV or MLV with an adjuvant (four groups, n = 6 per group), or were unvaccinated (n = 9). Calves were challenged 34 days (trial 2) or 21 days (trial 3) after the second or only vaccination.

MLV BRSV vaccines preferentially induced antibodies with potentially protective, fusion inhibiting, properties (trial 1). Significant reductions in clinical disease, pulmonary pathology, and in trial 3, reduced virus shedding, were observed in calves vaccinated with the MLV or the FI BRSV vaccines. Decreased protection in calves that received a single dose of unadjuvanted MLV vaccine was associated with decreased lymphoproliferative and IFNγ responses and delayed post challenge serum IgG. In trial 3,

prechallenge serum antibody was not indicative of protection. Post challenge serum and mucosal antibody responses had a low but significant negative predictive value for virus shedding and pulmonary pathology. Virus clearance in unvaccinated calves was independent of antibody and coincident with detection of BRSV specific cytotoxic cells, a response marginally accelerated by vaccination with MLV. Pulmonary emphysema and edema were independent of examined immune responses, but all vaccines were associated with an earlier clinical response to challenge.

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ABSTRACT

Bovine respiratory syncytial virus (BRSV) is an important respiratory pathogen of cattle. Both inactivated and modified live virus (MLV) BRSV vaccines are currently in use. Vaccine efficacy, the significance of qualitative differences in antibody responses, and the correlates of immunity to BRSV infection, remain unexamined by a virulent experimental challenge. This thesis addressed these issues.

The functional properties of antibodies induced by a MLV and 3 inactivated BRSV vaccines were compared in feedlot calves (n=10 per group)(trial 1). A challenge model that induces severe clinical disease and pulmonary pathology was developed by serially passing a field isolate of BRSV in newborn calves, using lung washing as the challenge inoculum. The effect of vaccination on BRSV infection was investigated. In trial 2, BRSV naïve calves were vaccinated twice with a formalin inactivated (FI) vaccine, 10^3 pfu MLV or sham vaccinated (n=4 per group). In trial 3, calves were vaccinated with either 2 doses of MLV intramuscularly (IM) or intradermally, with a single dose IM of MLV or MLV with an adjuvant (four groups, n = 6 per group), or were unvaccinated (n = 9). Calves were challenged 34 days (trial 2) or 3 weeks (trial 3) after the second or only vaccination.

MLV BRSV vaccines preferrentially induced antibodies with potentially protective, fusion inhibiting, properties (trial 1). Significant reductions in clinical disease, pulmonary pathology, and in trial 3, reduced virus shedding, were observed in calves vaccinated with the MLV or the FI BRSV vaccines. Decreased protection in calves that received a single dose of unadjuvanted MLV vaccine was associated with delayed post challenge serum IgG and decreased lymphoproliferative and IFNγ responses. In trial 3, prechallenge serum antibody was not indicative of protection, but anamnestic serum and mucosal antibody responses had a low but significant negative predictive value for virus shedding and pulmonary pathology. Virus clearance in

unvaccinated calves was independent of antibody and coincident with detection of BRSV specific cytotoxic cells, a response marginally accelerated by vaccination with MLV. Pulmonary emphysema and edema were independent of examined immune responses, but all vaccines were associated with an earlier clinical response to challenge.

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ADCMC	antibody dependent complement mediated cytotoxicity
AHR	airway hyperresponsivenesss
AIP	atypical interstitial pneumonia
ANOVA	analysis of variance
BAL	bronchoalveolar lavage
BHV-1	bovine Herpesvirus-1
BRD	bovine respiratory disease (complex)
BRSV	Bovine Respiratory Syncytial Virus
BT	bovine turbinate (cell line)
BVDV	Bovine Virus Diarrhea Virus
C3	complement factor 3
CCS	cumulative clinical score
CD4+	cluster of differentiation antigen 4 (positive T cells)
CD8+	cluster of differentiation antigen 8 (positive T cells)
CPE	cytopathic Effect
cpm	counts per minute
CTL	cytotoxic T lymphocytes
DMEM	Dulbeccos' modified Eagles medium
DMF	dimethy formamide
DTH	delayed type hypersensitivity
EBTR	embryonic bovine tracheal (cell line)
F	fusion surface gycoprotein of RSV
FBS	fetal bovine serum
Fc	fragment crytaline (of immunoglobulin)
FI	formalin-inactivated (vaccine) or fusion inhibiting (antibody)
G	attachment surface glycoprotein of RSV
HEPES	N-2- hydroxyethylpiperazine-N-2-ethanesulfonic acid
HIC	heat inactivated complement
HRSV	Human Respiratory Syncytial Virus
HSD	Honest Significant Difference (Tukey's)
ID	intradermal
IFNγ	interferon gamma
IL-11	interleukin-11
IL-6	interleukin-6
IL-8	interleukin-8
IM	intramuscular
L	polymerase (large) protein of RSV
LP	lymphoproliferative (blastogenesis)

LRT	lower respiratory tract
M	inner envelope protein of RSV
M2	envelope associated protein of RSV
mAb	monoclonal antibody
MDBK	Madin-Darby bovine kidney (cell line)
MHC	major histocompatability complex
MLV	modified-live virus (vaccine)
MOI	multiplicity of infection
MTT	3(4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide
N	structural nucleoprotein of RSV
NF-κB	nuclear binding transcription factor Kappa B
NP ELISA	nucleoprotein (of RSV) enzyme-linked immunosorbent assay
NS1	non-structural protein 1 of RSV
NS2	non-structural protein 2 of RSV
OD	optical density
P	nucleocapsid phosphoprotein of RSV
PaO_2	arterial partial pressure of oxygen
PBL	peripheral blood leukocytes
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming units
PI_3	Parainfluenza virus 3
PMSF	phenyl-methyl-sulphonyflouride
PVC	polyvinyl chloride
RANTES	regulated upon activation, normal T cell-expressed and-excreted
RR	respiratory rate
RSV	Respiratory Syncytial Virus
rVV	recombinant Vaccinia Virus
SDS	sodium dodecyl sulphate
SE	standard error
SH	small hydrophobic surface protein of RSV
SI	stimulation index
SS	sickness score
$TCID_{50}$	tissue culture infective dose (in half the wells)
Th	T-helper (lymphocyte)
TNF	tumor necrosis factor
URT	upper respiratory tract
VI	virus isolation
VN	virus neutralization

1 INTRODUCTION

1.1 Brief Introduction to Bovine Respiratory Syncytial Virus (BRSV)

Bovine respiratory disease (BRD) has a major economic impact on cattle production, with estimated losses approaching $1 billion annually in North American beef cattle alone (Griffin, 1997).BRSV is a pneumovirus in the family Paramyxoviridae (Smith 1975) and was first isolated in 1970 (Paccaud 1970). Since that time BRSV has been established as a ubiquitous pathogen and important viral etiology of BRD. It affects young dairy calves in repeated seasonal outbreaks (Van der Poel 1994), with mortality rates up to 20 % (Holzhauer 1979) and is associated with BRD of feedlot cattle (Martin 1986). BRSV often predisposes to secondary bacterial pneumonia caused by Pasteurella spp. As well, fatal infections occur without identification of synergistic pathogens (Kimman 1989c, Bryson 1993). BRSV infection and clinical disease occur in the face of maternal antibody (Kimman 1988) and mild or subclinical reinfections occur repeatedly (Van der Poel 1993, 1994).

Several aspects of BRSV infection remain poorly understood and are currently areas of research and speculation, including, the mechanism of viral persistence in closed populations (De Jong 1996), the lack of solid long term immunity (Van der Poel 1993), the pathogenesis of the pulmonary pathology associated with BRSV infection (Kimman 1989c, 1989d), the potential for immunopotentiation of disease by vaccination (Stewart 1989c), the correlates of immunity associated with reduced disease

upon reinfection or subsequent to vaccination (Kimman 1987a, 1989e) and the impact of the qualitatively different immune responses induced by inactivated and MLV vaccines on protection or disease enhancement (Ellis, 1995b). Most discussions of BRSV are inextricably entwined with those of human respiratory syncytial virus (HRSV), an antigenically similar pneumovirus that causes disease in children with a remarkably similar epidemiology, pathology and clinical presentation (Kimman, 1990), and which begs many of the unanswered questions previously listed. It is generally hoped that insight gained by research in one species can further understanding of the disease in the other, and this thesis is no exception.

1.2 Thesis Outline

A general review of BRSV infection, with emphasis on the effects of vaccination and the correlates of immunity, which is the main thrust of this thesis, is presented in chapter 2. The formulation of the hypotheses and statement of experimental objectives follows this review.

The ability of MLV or inactivated vaccines to induce antibodies with potentially protective functional properties, primarily the ability to inhibit synctia formation in BRSV infected cells, was investigated in chapter 3.

In chapter 4, the potential of vaccine associated enhancement of disease in cattle was investigated using a formalin inactivated BRSV vaccine prepared in a manner similar to the disease enhancing vaccine used in children in the 1960s (Kim, 1969). A low dose MLV vaccine group and sham vaccinated groups were comparatively included. Clinical parameters, virus shedding, pulmonary pathology, serum antibody and peripheral and pulmonary cellular cytotoxic responses were investigated. The effect

of treatment on disease due to BRSV infection was assessed using a BRSV challenge model that induced severe clinical disease, the development of which is described. Pathology typical of field BRSV infections is described with comments on possible pathogenic mechanisms.

The ability of MLV vaccines to protect from clinical disease and pulmonary pathology was more extensively investigated in chapter 5. Serum and mucosal antibody and pulmonary cellular cytotoxic responses were investigated as potential correlates of immunity in vaccinated and unvaccinated animals.

Chapter 6 includes a general discussion, the conclusions of the studies described, and prospects for future research.

2 BOVINE RESPIRATORY SYNCYTIAL VIRUS; A REVIEW OF INFECTION, IMMUNITY AND THE EFFECT OF VACCINATION

2.1 Introduction

Bovine respiratory syncytial virus (BRSV) and human respiratory syncytial virus (HRSV) are antigenically similar pneumoviruses in the family Paramyxoviradae (Smith, 1975). Both viruses are ubiquitous respiratory pathogens with similar epidemiology, clinical disease and pathology (Kimman, 1990; Van der Poel, 1993; 1994; Baker, 1997), making a review of BRSV incomplete without some comparative discussion of HRSV. BRSV is an important respiratory pathogen of cattle, affecting primarily calves in both beef and dairy settings, but severe disease can occur in naive animals of all ages (Elazhary, 1982; Harrison, 1985; Baker, 1986a; 1986b; 1986c; Martin, 1986; Ellis, 1996c; Elvander, 1996). HRSV is the leading cause of severe lower respiratory tract disease in infants < 1 year of age (Heilman, 1990), but is also prevalent in the elderly (Falsey, 1992; 1995), in immunocompromised individuals (Fishaut, 1980; Hall, 1986), and as a predisposing factor to subsequent hyperactive airway disease (Sly, 1989; Sigurs, 1995). In spite of its' importance as a human pathogen, there is currently no vaccine for HRSV, delayed in part due to immunoenhancement of disease observed in children vaccinated with a formalin

inactivated (FI) alum adjuvanted vaccine (Chin, 1969; Kim, 1969). Most subsequent HRSV work has been done using rodent models, however the similarity of human and bovine RSV infections suggests additional understanding can best be obtained by a comparison of the diseases in these species. However, the difficulty in experimentally reproducing BRSV associated disease has hindered research in cattle (Belknap, 1995) and the relationship between pathogenesis and the immune response, the effect of vaccination in RSV infections and correlates of immunity are still incompletely understood. This paper will briefly review the properties of BRSV, the epidemiology, clinical disease, and pathology associated with infection, and concentrate on the correlates of immunity in naive and vaccinated calves, discussing potential immunopathogenic mechanisms, with reference to HRSV where appropriate.

2.2 Classification and Molecular Aspects of BRSV

BRSV was first isolated in 1970 (Paccaud, 1970) following the isolation of HRSV in 1956 (Morris, 1956). Respiratory syncytial viruses are enveloped, with a negative sense, single stranded, non-segmented RNA genome, encoding the following 10 proteins: 3' NS1 (1C), NS2 (1B), N, P, M, SH, G, F, M2 (22K, containing 2 ORF), L 5,' which are preferentially transcribed in that order (McIntosch, 1990). The nucleocapsid protein (N), phosphoprotein (P) and the polymerase (L) are required for replication (Mazumder, 1994; Yu, 1995) but the non-structural protein 1C (NS1) and M22 proteins appear to have regulatory roles (Atreya, 1998; Hardy, 1998). The attachment (G), fusion (F), and small hydrophobic (SH) membrane proteins are involved in syncytia formation, a hallmark of RSV infection (Heminway, 1994; Pastey, 1997). In vitro syncytia formation is much reduced but not eliminated in the absence of

either the SH or G protein (Pastey, 1997). Neither the SH or the G protein are required for in vitro replication (Bukreyev, 1997; Karron, 1997), but in vivo replication of SH deletion mutants is altered in mice (Bukreyev, 1997). SH and G double deletion mutants are overly attenuated for vaccine use in children (Karron, 1997), suggesting impaired in vivo replication . The ligand for the attachment (G) protein of HRSV appears to be heparin, but this has not been confirmed for BRSV (Krusat, 1997).

2.3 Antigenic Variation

Knowledge of antigenic variation is particularly important when contemplating vaccine development. Based on reactivity to panels of monoclonal antibodies, two major antigenic subgroups of HRSV have been defined: A and B with a further subdivision into B1 and B2 (Akerlind, 1988). These differences are primarily in the attachment glycoprotein (G) and are accompanied by up to 47% gene sequence variability (Johnson, 1987). Group-specific differences in the molecular weights of the P protein and the F_1 and F_2 cleavage products have also been identified (Gimenez, 1986; Norrby, 1986). Infections due to subgroup A strains are associated with more severe disease, indicating a biological significance to these differences (Hall, 1990; McConnochie, 1990; Walsh, 1997). Both subgroups can be identified concurrently in the same outbreak (Hendry, 1986) and antigenic variation between groups and strains within groups does not appear to be the mechanism responsible for the high reinfection rates associated with annual outbreaks (Mufson, 1987; Hall, 1991; Faverio, 1997).

The G protein of BRSV does not crossreact with antisera to HRSV glycoprotein, indicating the two viruses are antigenically distinct, although a high degree of crossreactivity exists between other proteins (Lerch, 1990). BRSV has until

recently been considered monotypic, primarily by default due to the lack of investigative research (Kimman, 1990). On the basis of reactivity to panels of monoclonal antibodies to the G protein, and molecular weight differences of the F protein and its cleavage products, at least two antigenic subgroups have been proposed (Furze, 1994; Prozzi, 1997). At least two of these subgroups appear to circulate concurrently (Schrijver, 1996a), but biological differences between groups have not been described. These antigenic differences in the G protein are accompanied by relatively minor genetic variations, relative to HRSV, with 85% identity at the amino acid level between all strains examined to date (Mallipeddi, 1993; Furze, 1997; Prozzi, 1997). These differences appear to cluster at the immunodominant ectodomain, with considerable variability within antigenic subgroups, as opposed to the intragroup conservation observed in this region for HRSV, suggesting BRSV strains may form a continuum rather than distinct subgroups (Prozzi, 1997). BRSV strains can be separated into two groups based on differences in the electrophoretic mobility of the P protein, but there does not appear to be a consistent group relationship between these differences and those observed for the F protein (Shadomy, 1997). The antibody response to recombinant G protein of HRSV is subgroup specific (Sullender, 1990; Sullender, 1996), but the importance of these differences for BRSV vaccine development, particularly when combined with other proteins such as the F protein which induces cross-reactive antibody (Hendry, 1986; Oien, 1993) or when modified-live vaccines are used, remains to be investigated.

2.4 Epidemiology

The epidemiology of RSV infections has been well-reviewed (Van der Poel, 1994; Baker, 1997). BRSV and HRSV infections occur worldwide, usually in the form of seasonal outbreaks, occasionally coinciding with sudden environmental stressors (Baker, 1986b; Van der Poel, 1994). Seroprevalence increases with age in both cattle and humans, often exceeding 80%, with most infants and calves becoming infected in their first year of life (Baker, 1985a; 1985b; Brussow, 1991). Severe respiratory disease due to infection is usually confined to this period (Van der Poel, 1994), although naive older animals may also succumb to infection (Harrison, 1985; Ellis, 1996c; Elvander, 1996). BRSV has been serologically associated with respiratory disease in feedlots (Martin, 1986; Caldow, 1988) and is the most commonly identified viral etiology in epizootics of dairy calf pneumonia (Baker, 1986a; 1986c; Uttenthal, 1996). Reinfections with BRSV and HRSV are common and can occur more than twice in one year, but disease is usually mild or subclinical (Hall, 1991; Van der Poel, 1993; 1994), with only slight reductions in milk yield associated with reinfections in dairy herds (van der Poel, 1995). There may be partial group-specific protection from HRSV reinfection (Mufson, 1987), but experimental reinfection with the same strain can occur in adult volunteers after 2 months (Hall, 1991). Calves were reported to be protected from experimental reinfection with the same strain for at least 2 months although the challenge used was of low virulence (Kimman, 1989e).

The mechanism of persistence of BRSV in closed cattle populations between outbreaks is still unknown, as the virus is extremely labile and shedding from acute primary infections appears to last, at most, up to 12 days post infection (Elazhary, 1980;

Van der Poel, 1994). The ability of RSV to repeatedly reinfect seropositive subjects suggests that asymptomatic viral cycling may maintain the virus in these populations. In one study, persistent infection was a more plausible explanation for herd maintenance of BRSV than cyclical reinfections of seropositive animals (Van der Poel, 1993; De Jong, 1996), particularly over the summer months when few infections or reinfections could be detected. The reinfection rate was determined by increases in serum neutralizing antibody titer, which may not necessarily accompany all reinfections, but lack of seroconversion in seronegative sentinel animals in the summer suggests periodic absence of cycling. Persistently infected cell cultures have been established with BRSV and HRSV (Pringle, 1978; Stott, 1984a) and persistence of viral protein and nucleic acid has been described in guinea pigs 60 days after infection (Hegele, 1994). A persistent carrier state has been suspected, but not yet identified in cattle (Thomas, 1980; Van der Poel, 1993; De Jong, 1996). Infectious virus or PCR product has not been recovered from corticosteriod treated seropositive animals (Thomas, 1980; Van der Poel, 1995) but in some treated animals, a rise in BRSV specific serum antibody was detected, and interpreted as indirect evidence of viral replication and persistent infection (Van der Poel, 1995). The ability of RSV to repeatedly reinfect both cattle and humans does not suggest persistent infection, but rather complete clearance of infection (antigen) and a return of the immune response to the memory state.

2.5 Clinical Disease

The severity of clinical disease varies considerably. Pyrexia, hyperpnea and frequent spontaneous coughing are the clinical signs most frequently reported in natural BRSV infections and in the limited experimental infections that have resulted in clinical

disease (Pirie, 1981; Elazhary, 1982; Baker, 1986b; Kimman, 1989c; Belknap, 1995). This may be followed by a relatively sudden onset of severe dyspnea with grunting expiration, with occasional development of pneumothorax and subcutaneous emphysema (Pirie, 1981; Bryson, 1983; Baker, 1986b; Kimman, 1989c). Hypoxemia with sporadic occurrence of associated hypercapnea has been reported in experimental (Belknap, 1991) and natural BRSV infections (Lekeux, 1985; Verhoeff, 1985a; Verhoeff, 1985b) and with RSV associated bronchiolitis in children (Hall, 1979). Mortality rates between outbreaks vary considerably and may approach 20% (Holzhauer, 1979; Baker, 1997).

Experimental infections of cattle with BRSV have generally failed to reproduce the clinical disease observed in natural infections even when low passage field isolates have been used (Belknap, 1995). There are three reports of experimental infection producing significant clinical disease using low passage field isolates, all using combined intranasal, intratracheal inoculation of ~ 10^6 PFU for 4 consecutive days (Bryson, 1983; Belknap, 1991; Ciszewski, 1991). There is one report of mild clinical disease occurring following a single intranasal inoculation of a lung wash from a BRSV infected calf containing ~ 10^3 PFU of BRSV (van der Poel, 1996). The factor(s) responsible for the decreased virulence of in vitro passaged BRSV are unknown, but it is thought there is a rapid selective loss of virulence on tissue culture passage (Stewart, 1990; Belknap, 1995). The molecular weight of the attachment glycoprotein (G) of in vitro cultured RSV has been shown to be dependent on the cell type in which it is propagated (Garcia Beato, 1996), suggesting the possibility of host cell factors in bovine

respiratory epithelium being required for the production of highly infectious and virulent virus.

2.6 Pathology

The pathology associated with BRSV infection has been well-described (Bryson, 1983; 1993; Kimman, 1989c; Baker, 1997). Areas of atelectasis and consolidation are present in the cranioventral lung lobes and in mildly affected animals and most experimentally infected animals these are the only gross lesions observed (Bryson, 1993; Belknap, 1995). In animals with severe or fatal disease there is severe, diffuse, pulmonary edema and emphysema that is most obvious in the caudal lobes which are voluminous and fail to collapse (Bryson, 1983; 1993; Kimman, 1989c; Baker, 1997). BRSV is often associated with Pasteurella spp and other respiratory pathogens, resulting in the superimposition of a suppurative pneumonia (Bryson, 1993), but severe fatal disease in natural infections occurs in the absence of other identified pathogens (Van Den Ingh, 1982; Kimman, 1989c). Enhancement of disease or lesions was not demonstrated by combined infection of calves with BRSV and Mycoplasma bovis (Thomas, 1986) or infection of calves persistently infected with bovine virus diarrhea virus (BVDV) (van der Poel, 1996).

Virus replication is limited to ciliated and non-ciliated respiratory epithelium, primarily in bronchi, bronchioles and alveoli, resulting in a necrotizing bronchitis/bronchiolitis and/or interstitial pneumonia (Bryson, 1993; Viuff, 1996; Baker, 1997). Antigen is most often detected in collapsed lesions in cranial lung lobes but has been demonstrated by immunohistochemistry in the caudal lobes of severely affected animals (Ellis, 1996c; Viuff, 1996). Replication does not occur in all sites in

all animals, being restricted to the bronchi or bronchioles in some animals, which may partially account for differences in clinical presentations (Viuff, 1996). In the cranioventral regions, bronchiolar lumens are often occluded with desquamated epithelial cells, syncytial cells and inflammatory cells, which are primarily neutrophils (Bryson, 1983; 1993; Kimman, 1989c; Baker, 1997). Re-epithelialization, and in severely affected bronchioles, organization of exudate, begins rapidly and is present in many cases dying of severe infection (Bryson, 1983; 1993; Kimman, 1989c). There is interstitial infiltration of inflammatory cells in the alveolar septae. These histopathologic changes occur, but are less common in the caudal lobes (Kimman, 1989c; Ellis, 1996c), where the primary lesions in severe cases are emphysema, alveolar edema, hyaline membranes and in the reparative phase, alveolar epithelialization (Kimman, 1989c; Bryson, 1993; Baker, 1997).

2.7 Pathogenesis of Pulmonary Lesions

2.7.1 Cranioventral Atelectasis and Consolidation

The pathogenesis of the airway epithelial lesions in cranioventral regions appears to be a direct effect of viral replication (Baker, 1997). The collapse of affected lobules can be ascribed to complete blockage by exudate in some, but not all cases (Viuff, 1996). Markedly decreased surfactant production has been noted in children with severe RSV induced bronchiolitis, and surfactant therapy appears beneficial (Dargaville, 1996; Vos, 1996). This suggests that decreased surfactant production, possibly resulting from infection of alveolar type II cells, may also be responsible for alveolar collapse (Dargaville, 1996; Viuff, 1996; Vos, 1996). The occluding inflammatory infiltrate, consisting of predominantly neutrophils, may be a result of virally induced

chemokine or cytokine production. HRSV infection has been shown to induce interleukin-8 (IL-8), a neutrophil chemotractant, as well as IL-6 and TNF release from respiratory epithelium, and IL-8 and IL-6 release from granulocytes (Noah, 1993; Arnold, 1994; 1995; Noah, 1995). Interleukin-8 has been demonstrated in the lungs of calves recovering from a severe experimental BRSV infection (Caswell, 1998).

2.7.2 Emphysema and Edema

The emphysema observed in severe cases may be the result of mechanical damage due to forced expiration through partially blocked airways (Baker, 1997), however airway involvement cannot always be identified in the caudal lobes where the emphysema is most severe (Kimman, 1989c). Increased pulmonary resistance and airway reactivity have been described in calves experimentally infected with BRSV(LeBlanc, 1991). Severe bronchoconstriction resulting in a physiologic rather than a static physical blockage may be induced by RSV infection, and there is some evidence supporting several different hypotheses in both children and calves.

2.7.2.1 Type 1 Hypersensitivity

A type I hypersensitivity with subsequent bronchospasm and inflammation has been proposed to contribute to disease in RSV induced bronchiolitis in children, as cell bound IgE, RSV specific IgE titers, and histamine levels in nasopharyngeal secretions of infected children, were found to be significantly correlated with wheezing (Welliver, 1980; Welliver, 1981; 1985; 1986). Other workers have failed to substantiate these results by finding less clear correlations (Everard, 1995), or undetectable RSV specific IgE in severely affected children (Toms, 1996). RSV infection does appear to be a risk factor in the development of subsequent persistent wheezing, asthma and atopy,

particularly in children with a hereditary predisposition (Sly, 1989; Sigurs, 1995), indicating some long-term effects on either pulmonary physiology or immunology. It has been suggested that severe RSV associated disease may also have a hereditary component (Baker, 1991) but atopic children are not more prone to HRSV induced bronchiolitis (Sims, 1981). A correlation between disease expression and BRSV specific IgE and histamine was reported following repeated experimental infection, particularly in vaccinated calves (Stewart, 1989b; 1989c; 1990). Other repeat challenge studies, and epidemiological evidence that repeated infections result in less, rather than more severe symptoms, indicate a type I hypersensitivity is not a general phenomenon of natural RSV or BRSV infections (Fernald, 1983; Ciszewski, 1991; Ames, 1993). In occasional epizootics, BRSV can present as a bi-phasic disease, with the second, more severe phase occurring days to weeks after apparent improvement (Baker, 1986b; 1991). This lead to speculation that the second phase results from the development of a type I hypersensitivity (Baker, 1991). The relatively rapid clearance of virus in even severe infections (Van der Poel, 1994), would not support development of a hypersensitivity to BRSV weeks later. However, it is possible BRSV infection may predispose calves to the development of airway hyperresponsiveness to other antigens or stimuli, as does HRSV infection in children (Sly, 1989; LeBlanc, 1991; Gershwin, 1994; Sigurs, 1995). The role of IgE in the uncommon presentation of severe disease in children or delayed disease in natural infection in calves awaits further investigation.

2.7.2.2 Induction of Inflammatory Mediators by RSV

Various proinflammatory mediators ((Histamine, leukotriene C-4, eosinophil cationic protein, IL-6, IL-8, IL-11 and TNF-alpha, RANTES (regulated upon activation, normal T cell-expressed and-excreted)) capable of altering local inflammation or airway physiology, have been described in the nasal secretions of RSV infected children (Welliver, 1981; Volovitz, 1988; Garofalo, 1992; Colocho Zelaya, 1994; Noah, 1995; Einarsson, 1996; Becker, 1997). Histamine, leukotriene C-4, eosinophil cationic protein and IL-11 have been correlated to the degree of suspected bronchiolitis (Welliver, 1981; Volovitz, 1988; Garofalo, 1992; Colocho Zelaya, 1994; Einarsson, 1996)(Welliver 1981, Volovitz 1988, Garafalo 1992, Colocho 1994, Einarsson 1996). IL-6, IL-8, IL-11 and RANTES, are induced by HRSV infection of epithelial cells (Noah, 1993; Arnold, 1994; Einarsson, 1996; Saito, 1997), and leukotriene C-4 is induced by RSV infection of a human macrophage cell line (Ananaba, 1991; Osiowy, 1994), indicating the viral infection may have a direct effect on the inflammatory response by inducing the elaboration of various chemokines. Some of these such as IL-6 and IL-8 may be induced by various viral infections (Noah, 1995), but others, such as IL-11, are selectively induced in human cell lines by the respiratory viruses which cause viral induced asthma; HRSV, parainfluenza and rhinovirus (Einarsson, 1996). IL-11 induces airway hyperresponsiveness in mice, and it has been suggested that the IL-6 family of cytokines may contribute to the increased cholinergic sensitivity and tachykinnin excess demonstrated in rodent models of virally induced airway hyperresponsiveness (AHR) (Einarsson, 1996). RANTES is a potent eosinophil and basophil chemotractant and activator that is selectively expressed by HRSV infected human nasal epithelial cells in

vitro (Saito, 1997) and is found in nasal secretions from children with HRSV, but not other respiratory infections (Becker, 1997). It is possible this chemokine may regulate pulmonary eosinophil infiltration and activation seen in RSV infected children with bronchiolitis (Becker, 1997; Saito, 1997). While most children with RSV infections have an eosinopenia, those with severe bronchiolitis tend to have higher eosinophil counts and significantly higher nasopharyngeal secretion of eosinophil basic protein than those with mild disease (Garofalo, 1994). In most pathologic descriptions of BRSV infection in cattle, eosinophil infiltration is not noted, or is mild (Van Den Ingh, 1982; Bryson, 1983; Kimman, 1989c). BRSV induced chemokine production in cattle has been insufficiently investigated to comment, but is a plausible explanation for the emphysema observed in the dorsal lung of calves and warrants further investigation.

2.7.2.3 Pulmonary Arthus Reaction

Kimman (Kimman, 1989a; 1989c; 1989d) proposed a pulmonary Arthus reaction to explain both the emphysema and edema seen in severe natural BRSV infections, as intravenous or pulmonary infusion of activated complement factors have been shown to induce pulmonary edema and emphysema in small animal models (Stimler, 1980; Mulligan, 1996). The appearance of IgG1 and IgM in the later stages of disease was often noted to coincide with onset of severe symptoms. In calves dying of BRSV, mast cell degranulation, low pulmonary histamine content and deposition of C3 on epithelial cells suggested the activation of complement with complement anaphylotoxin mediated activation of mast cells (Kimman, 1989d). A similar hypothesis, implicating the high titers of vaccine induced non-neutralizing antibodies,

has also been used to explain the enhanced disease observed in children that received formalin inactivated vaccine (Kim, 1969; Murphy, 1986). At variance with this hypothesis is the protective rather than disease enhancing effects of passive antibody in both children and calves (Kimman, 1988; Baker, 1991; Belknap, 1991; Groothuis, 1993). It is possible that the passive antibody, consisting primarily of IgG1, is less likely to induce an Arthus reaction than the IgM induced in calves early in a primary response (Kimman, 1987a) or that the maternal antibody limits replication in the lung and prevents accumulation of sufficient antigen. However, some naturally infected calves die of BRSV with no, or only maternally derived antibody (Kimman, 1989c). BRSV infected cells directly activate the classical pathway of complement, albeit less efficiently than in the presence of antibody (Kimman, 1989a). Fc receptor (FcR) deficient mice have markedly diminished Arthus reactions (Sylvestre, 1994; 1996a; 1996b), suggesting the presence of complement anaphylotoxins without immune complex stimulation of $Fc\gamma R^{+}$ cells may be insufficient to initiate this reaction, at least in mice.

The extensive edema observed in calves is the main departure in similarity between pathology associated human and bovine RSV infection. This is not a feature unique to BRSV infection, but has been described under the generic term of atypical interstitial pneumonia (AIP) (Baker, 1991; 1997). Several different etiologies, with different pathogenic mechanisms, appear to induce this pathology in cattle (Baker, 1991; 1997), and the mechanism operative in severe BRSV infections remains unresolved.

2.8 Immunity to Infection

2.8.1 Passive Antibody

2.8.1.1 Prophylaxis

Passive antibody has not been shown to prevent infection, and severe disease occurs in calves and children with maternal antibody, but there is an inverse relationship between the level of passive RSV specific antibody and the severity of disease in calves (Kimman, 1988; Belknap, 1991), children (Groothuis, 1993; 1994; 1995; Meissner, 1993) cotton rats and mice (Siber, 1992; Siber, 1994; Graham, 1993a; Sami, 1995). There is amelioration of BRSV and HRSV induced lower respiratory tract (LRT) disease with passive serum antibody, but no or marginal effect on infection or upper respiratory tract (URT) virus shedding except at very high antibody titers (Graham, 1993a; 1993; 1994; 1995; Sami, 1995). This may be due to the close association of blood and alveoli in the LRT relative to the URT mucosa (Kimman, 1990). The protection was correlated to titer as determined by a virus neutralizing, but not ELISA assays (Groothuis, 1993; Siber, 1994). Further work in mice and in calves, using monoclonal antibodies, demonstrated protection was associated with the ability to inhibit syncytia formation (Taylor, 1992; Thomas, 1998). All fusion inhibiting antibodies were neutralizing as well, but the reverse was not always so (Taylor, 1992). The ability to fix complement may be a correlate of protection in mice infected with HRSV (Corbeil, 1996), but does not appear to be in calves (Thomas, 1998).

2.8.1.2 Therapy

In cotton rats, systemic administration of monoclonal antibodies to the fusion protein has therapeutic value when administered up to 4 days after infection (Wyde, 1995) and some benefit was seen when immune globulin was given intravenously to children with acute HRSV infections (Hemming, 1987; Rodriguez, 1997), or to HRSV infected owl monkeys (Hemming, 1990). Protection correlated with virus neutralizing titer. Local administration of antibody is more effective than systemic administration (Hemming, 1988; 1990), suggesting mucosal delivery of anti-RSV antibodies is a potential therapeutic option for children (Tempest, 1991; Taylor, 1995a), but results of preliminary trials have shown no benefits (Rimensberger, 1996). Immunoglobulin therapy has not been investigated in calves.

2.8.1.3 Effect of Maternal Antibody on the Immune Response to Infection or Vaccination

In calves, both mucosal and systemic antibody responses to challenge are suppressed by passive antibody, although a weak IgM response may be detected (Kimman, 1989c; 1989e). Infection primed for a rapid secondary serum and mucosal antibody response and protection from a subsequent challenge was not altered (Kimman, 1989e). Calves vaccinated intramuscularly with live virus or inactivated virus in the face of maternal antibody had suppressed memory antibody responses and reduced protection from shedding at rechallenge (Kimman, 1989e). Protection from disease could not be assessed, as the challenge did not induce clinical disease. In another study, lymphoproliferative response could be detected in calves vaccinated with modified live virus (MLV) in the face of maternal antibody (Ellis, 1996b), indicating

activation of cellular immune responses in the absence of detectable antibody, but protection was not assessed. In field studies, some protection was seen in calves vaccinated with MLV intramuscularly or intranasaly despite the presence of maternal antibody (Thomson, 1986; Kubota, 1992). In children, maternal antibodies also suppress an active antibody response to infection, limiting the usefulness of serologic assays in the diagnosis of infection (Brandenburg, 1997). The presence of RSV specific IgM detects only 3% of infections (Brandenburg, 1997). Children less than 6 months of age that received a FI vaccine had a diminished G, but not F, specific response (Murphy, 1986) and children less than 12 months of age demonstrated a similar depressed antibody response to a live virus vaccine (Belshe, 1982). An effect on protection could not be assessed, as neither vaccine was associated with significxant protection. In mice, passive antibody depressed primary antibody responses (Graham, 1993a), as well as impaired primary and secondary cytotoxic T-cell responses (Bangham, 1986) and protection from subsequent challenge was decreased. The effect of maternal antibody on cytotoxic responses has not been investigated in cattle.

2.8.2 Primary Infection

2.8.2.1 Primary Antibody Response and Protection

Calves develop a detectable antibody response to all structural proteins of BRSV (Duncan, 1993), but the role of antibody in clearing a primary RSV infection in children or calves is not clear. BRSV-specific IgM and IgA could be detected simultaneously in the serum and at mucosal sites 8-10 days following experimental infection, with serum IgG1 and IgG2 following at 13-17 days and 1-3 months post infection, respectively (Kimman, 1987a). Virus could be detected from days 3-8 post infection indicating

clearance occurred at or before the time of detectable BRSV-specific antibody. Responses to the F and N proteins predominate in maternal antibodies, convalescent samples, and in calves dying of BRSV, implying differences in protein specificity of the antibody response are not responsible for variations in clinical outcome (Westenbrink, 1989). Children and calves can experience severe disease when they still have maternal antibody, which although apparently non-protective, does suppress the primary antibody response, implying the infection is subsequently cleared largely independent of the antibody response (Kimman, 1988; Brandenburg, 1997). Serum and mucosal antibody responses do not appear to be correlated to the severity of disease in calves and normal children (Kimman, 1987a; 1989c; Toms, 1989), however, in children with leukemia, the lack of mucosal antibody was associated with persistent infection, but the effect of concurrent deficits in other immune responses is unknown (Taylor, 1990). Calves that were depleted of CD4+ T-cells eliminate the virus at the same time as undepleted calves, but most fail to mount an antibody response (Taylor, 1995b). In these calves there was increased lung pathology relative to undepleted control calves, which may indicate antibody has a disease sparing effect but this could be due to the absence of other undetermined Th functions. Similar findings are reported for anti-mu treated (B cell depleted) mice infected with HRSV; illness was increased but virus clearance was unaffected (Graham, 1991a). Mice depleted of CD4+ and CD8+ T-cells have persistent HRSV infection, but illness is eliminated (Graham, 1991c). In mice, which are only semi-permissive hosts of HRSV, T cell mediated immunity is a major determinant of illness and pulmonary pathology, whereas antibody is protective and not disease enhancing (Graham, 1991a; 1991b; 1991c). In cattle and humans, virus cytocidal effect

appears to be a major illness determinant as immunosuppression or T cell subset depletion (in calves) enhances illness and pathology (Fishaut, 1980; Thomas, 1984; Hall, 1986; Taylor, 1995b). These differences should be kept in mind when extrapolating results from the rodent models to calves and humans.

2.8.2.2 Primary Antibody Response and Disease

It has been suggested that the primary antibody response of infants to HRSV may be disease enhancing as it is directed toward linear epitopes of surface proteins, is poorly neutralizing (Forster, 1995) and may preferentially enhance viral infection of Fcγ receptor positive cells (Osiowy, 1994). HRSV antiserum has been shown in vitro to enhance the rapid release of leukotriene C4, a potent bronchoconstrictor, from macrophage cell lines (Ananaba, 1991). Bovine BRSV antiserum has also been shown to enhance replication of BRSV in ovine peripheral monocytes (Keles, 1998). Lambs experimentally challenged with an admixture of BRSV and antiserum had significantly prolonged viral shedding compared to lambs challenged with virus mixed with fetal bovine serum, although clinical disease was unaltered (Keles, 1998). Possible enhancement of infection by low levels of passive or naturally acquired serum antibody awaits further investigation.

2.8.2.3 Primary Cell Mediated Responses

In calves depleted of CD8+ T-cells there is persistence of BRSV infection and increased pulmonary consolidation despite a normal antibody response, confirming the importance of cell mediated immunity in a primary immune response against BRSV (Taylor, 1995b). There is an increase in the CD8+ to CD4+ T-cell ratio in the lung of

BRSV infected calves (Thomas, 1996), and MHC I restricted, CD8+ cytotoxic T lymphocytes (CTLs) were demonstrated ex-vivo in the peripheral blood on days 7-10 and in the lung on day 10 after infection (Gaddum, 1996a). Specific lysis of BRSV infected targets by unstimulated peripheral blood leukocytes has been described in lambs as early as 6 days post infection (Sharma, 1991; 1996b) at the time of virus clearance. Few studies have investigated cytotoxic responses in children, perhaps due to the difficulty in performing the assays and the transient nature of the response, but significant cytotoxicity has been described in 18-67% of children during acute HRSV infection (Isaacs, 1987; Chiba, 1989). In some children older than 6 months, cytotoxic responses were detected in the absence of neutralizing antibodies (Chiba, 1989).

2.8.3 Reinfection

2.8.3.1 Possible Suppression of Memory Responses

Both cattle and humans can be repeatedly reinfected with RSV, although clinical disease is usually absent in calves, and of decreased severity in children (Denny, 1979; Mufson, 1987; Van der Poel, 1993; 1994; 1995). The in vitro suppressive effects of live HRSV on lymphocyte proliferative responses, IL-1 release and leukocyte clustering, has led to speculation that there is a viral suppression of memory responses allowing reinfection (Preston, 1992; 1995; Salkind, 1992). The factor responsible for this suppression was determined to be virus-induced release of interferon α (Preston, 1995). The significance of these findings is unknown, as other respiratory viruses induce interferon α in vivo and in vitro, and the comparatively small amounts induced by HRSV infection have no correlation with disease severity (Hall, 1978; McIntosh, 1978;

Roberts, 1992). [Similar suppressive effects of live BRSV on the lymphoproliferative response of bovine peripheral leukocytes has been noted, West unpublished observation].

2.8.3.2 Correlates of Immunity to Reinfection

Complete resistance to reinfection has been described in calves, humans, and in rodent models (Kimman, 1987a; 1989e; Hall, 1991; Oien, 1994; Johnson, 1996). The route of primary infection or immunization was mucosal, but resistance was not dependent on mucosal or serum antibody at challenge, and was thought to involve unidentified, probably cellular mechanisms (Kimman, 1987a; 1989e; Hall, 1991; Oien, 1994; Johnson, 1996). In calves, resistance was correlated with the ability to mount a strong mucosal response by day 6, rather than mucosal or serum antibody at challenge (Kimman, 1989e). High serum neutralizing and F and G specific antibody, but not mucosal IgA, has been correlated with reduced reinfection with HRSV (Kasel, 1987; Hall, 1991), but even in the subjects with the highest titers, the reinfection rate was 25% (Hall, 1991).

2.8.3.3 Correlates of Immunity to Clinical Disease on Reinfection

Reduced illness in children on reinfection has been reported to have an inverse relationship with HRSV specific serum antibody titer (Fernald, 1983), but lymphoproliferative responses of naturally infected children are reported to have no correlation with reinfection risk or illness severity (Kim, 1976; Fernald, 1983). Detection of a memory cytotoxic response was associated with reduced illness in adults

(Isaacs, 1990). Similar studies of cell mediated responses have not been reported in cattle naturally reinfected with BRSV.

2.8.3.4 Summary of Correlates of Immunity in Primary Infections and Reinfections

The role of induced antibody responses in controlling a primary and secondary RSV infection is less apparent than the effect observed with passive antibody in naive animals. In a primary infection, virus clearance may be accomplished by other mechanisms before significant antibody is present, precluding any relationship. Reinfection is generally established using samples from the upper respiratory tract (URT). The protective effect of passive serum antibody is primarily confined to the lower respiratory tract (LRT) (Graham, 1993a; Groothuis, 1993; 1994; 1995; Sami, 1995), and as few reinfections result in LRT disease, serum antibody is unlikely to have a demonstrable effect. The lack of correlation between URT infection and mucosal IgA is unexpected, but in cattle these antibodies have been shown to be non-neutralizing (Kimman, 1989e). Resistance to URT infection appears to be short lived, as would be expected if dependent on the presence of cellular effector mechanisms which would require time to be reactivated from memory state(Kulkarni, 1993; Gaddum, 1996b), and if so, obviates the need for suppression of memory responses as an explanation for reinfection. The long-term reduction in disease severity may be mediated by existing or anamnestic antibody responses or a rapid restoration of cell mediated mechanisms, and should be an achievable goal for vaccination.

2.9 Vaccination and Vaccine Induced Correlates of Immunity

2.9.1 Route of Vaccine Administration and Effect on Virus Shedding

Kimman (Kimman, 1989e) concluded that the most important requirement for protection was mucosal MLV priming, as opposed to systemic MLV or killed virus vaccination, to limit nasal viral recovery. This is supported by work in other systems where a decrease in pulmonary (LRT), but not URT virus recovery has been shown following parenteral live virus vaccination in cotton rats and chimpanzees (Murphy, 1989; Crowe, 1993) and following parenteral FI HRSV or subunit vaccination in cotton rats and mice (Murphy, 1989; Oien, 1993). Intranasal live RSV or subunit vaccines in cholera toxin adjuvant have induced both URT and pulmonary protection in rodent models of HRSV (Kanesaki, 1991; Oien, 1994). The protection of the URT in these studies depended on mucosal priming and appeared to correlate with the ability to mount a mucosal immune response including local IgA production, but other cell mediated mechanisms may also play a role (Kimman, 1987a; 1989e; Oien, 1994; Johnson, 1996). Several other studies in calves have shown variable reductions in post challenge virus recovery from nasal secretions following systemic vaccination with various inactivated vaccines (Mohanty, 1976; Taylor, 1989; Stewart, 1990), as has an HRSV study in green monkeys using a FI alum precipitated HRSV vaccine (Kakuk, 1993). An F protein recombinant BRSV vaccine administered systemically with a Quill A adjuvant reduced nasal shedding in lambs (Sharma, 1996a). Some reduction in nasal shedding in calves was observed due to vaccination by intradermal scarification with vaccinia virus recombinants (rVV) expressing the F, G and N proteins, but a greater reduction was present in the lung (Taylor, 1997). Parenteral vaccination of calves with

live BRSV results in anamnestic mucosal antibody responses which are only slightly delayed compared to mucosally primed animals (Kimman, 1989e). This indicates priming for mucosal antibody responses occurs with systemic immunization and may help reduce shedding, but other mucosal responses may rely on mucosal priming (Kimman, 1989e). Mucosal vaccination may decrease URT infection shortly after vaccination, but a long term advantage may be less apparent as protection appears short lived even after natural infection (Van der Poel, 1994). The significance of mucosal vaccination in preventing disease has not been established as the experimental challenges used failed to induce significant clinical disease (Kimman, 1987a; 1989e); a caveat that must be applied in some degree to all conclusions reached regarding the protective effects of vaccination in cattle using experimental infection.

2.9.2 The Effect of Vaccine Induced Serum Antibody

The effect of vaccinal serum antibody on protection from disease or shedding in calves has varied depending on the study. Several studies using MLV and inactivated vaccines have failed to show an association between serum IgG or neutralizing antibody titers, and disease upon subsequent challenge (Kimman, 1987a; 1989e; Stewart, 1990; Kubota, 1992), whereas others have associated the presence of neutralizing antibody with protection (Mohanty, 1976; Sharma, 1996b). Other studies, investigating a glutaraldehyde- inactivated and two MLV vaccines, have shown that protection correlated with BRSV specific IgG1 as measured by a radioimmunoassay, but not virus neutralizing titer, indicating protection was associated with non-neutralizing antibodies that were induced primarily by the inactivated vaccine (Stott, 1984b; Taylor, 1987). The well established requirement for antibodies with neutralizing or fusion inhibiting

properties for passive immunity (Taylor, 1992; Groothuis, 1993; Siber, 1994; Thomas, 1998), would suggest protection was only associated with the non-neutralizing antibodies, with other mechanisms responsible for the observed protection. In calves, rVV vaccines expressing the F, G or N proteins of BRSV all induced similar protection (Taylor, 1997), but the ability to induce neutralizing antibody or lymphoproliferative responses varied between antigens and the protective mechanism(s) could not be identified. Several commercial inactivated BRSV vaccines induce antibodies with poor neutralizing and fusion inhibiting properties relative to MLV preparations, most likely due to alteration of critical epitopes during the inactivation process, (Ellis, 1992b; West, 1997a), but this evidence alone appears insufficient to assume they are poorly protective. Vaccination of calves with either modified live or inactivated vaccines induces antibodies that primarily recognize the F protein (Ellis, 1992b; 1995b) and there does not appear to be a significant difference in the IgG1/ IgG2 ratio (Schrijver, 1996b; West, 1997a)

2.9.3 Vaccine Induced Cell Mediated Responses

2.9.3.1 Cellular Cytotoxicity

The requirement for $CD8^+$ T-cells for virus clearance in naive calves, and the influx of these CD8+ cytotoxic cells, into the lungs following infection, suggests this is a critical immune effector mechanism in calves (Taylor, 1995b; Gaddum, 1996a; Thomas, 1996), but reports on the effect of vaccination on this response in calves are limited. It is anticipated that MLV vaccines would prime for a more effective CTL response than inactivated virus by virtue of cell infection, presentation of antigen on MHC I and stimulation of $CD8^+$ T cells (Nicholas, 1990). There is some evidence that

vaccination of calves with MLV vaccines can prime for genetically restricted cytotoxicity in peripheral blood leukocytes after in vitro stimulation (Ellis, 1996a). An anamnestic BRSV specific CTL response was detected ex vivo in peripheral blood of lambs primed by vaccination with recombinant F protein in a Quil-A adjuvant, by day 3 post challenge (Sharma, 1995). This is similar to the recall response observed in lambs rechallenged 2 months later after a primary infection (Sharma, 1991). These studies, in a natural host of RSV, indicate vaccination can induce CTL recall responses similar to natural infection, and that with the appropriate adjuvant, MHC class I presentation of inactivated antigens can be achieved.

In experimental models of HRSV infection, the effectiveness of CTL activity in clearing HRSV infection is well documented (Cannon, 1988; Kulkarni, 1993; 1995; Tang, 1994;), and priming with live HRSV or F and M protein vaccinia recombinants induces CTL activity, whereas inactivated virus or F-G glycoprotein vaccines in an alum adjuvant do not (Nicholas, 1990). In mice, the protective effect of vaccination, in which the sole mechanism of protection is CTL activity, is of short duration (Kulkarni, 1995; Gaddum, 1996b). The effectiveness or importance of CTL recall responses in limiting disease upon reinfection, and the conditions for stimulating precursor or memory cytotoxic lymphocytes by vaccination in cattle are unknown.

2.9.3.2 Lymphoproliferative Responses

There are few reports correlating vaccine induced lymphoproliferative responses with protection from BRSV infection in calves. In one study, more consistent responses, of higher magnitude, were induced by an inactivated vaccine containing a Quil-a adjuvant, than by a MLV or a live temperature sensitive mutant vaccine (Taylor, 1987).

These increased responses were associated with higher serum antibody and better protection from challenge. The low and inconsistent responses induced by the MLV vaccines, while apparently less protective, were similar to the responses seen in either naturally exposed or experimentally infected calves (Taylor, 1987) in which reinfection rarely causes disease. In other studies, calves that received commercial vaccines containing either modified live or inactivated BRSV have had similar in vitro lymphoproliferative responses (Ellis, 1992a; 1995b), but protection was not assessed. The phenotype of the proliferating cells was primarily CD4+ T-cells in both cases (Ellis, 1992a). The established importance of $CD8^+$ rather than $CD4^+$ T cells for viral clearance in cattle (Taylor, 1995b), may partially account for the inconsistent association of lymphoproliferative responses, but protection from disease on reinfection, seen in naturally exposed calves. Similarly, a correlation between LP responses and protection from subsequent infection has not been found in naturally infected or children vaccinated with an F subunit vaccine (Fernald, 1983; Welliver, 1994), leading to speculation that this assay of cellular immune stimulation may not be measuring the protective effector mechanism (Welliver, 1994).

2.9.4 Vaccine Induced Immunoenhancement of Disease

Several commercially available vaccines containing either MLV or inactivated BRSV have been in use in cattle since the mid 1980's (Baker, 1997), but there is no published evidence that these vaccines enhance disease. Anecdotal reports exist, and vaccination with a MLV vaccine at the time of exposure has been reported to exacerbate disease (Kimman, 1989b), but most concern is indirect and as a result of vaccine trials in children in the late 1960's using alum precipitated formalin inactivated (FI) HRSV

vaccine. There was an increased frequency of severe disease among vaccinated children on subsequent infection (Chin, 1969; Fulginiti, 1969; Kapikian, 1969; Kim, 1969) and further vaccine development has been tempered by this experience. There are no HRSV vaccines currently available for use in children.

Investigation revealed the FI vaccine induced HRSV specific antibody of low neutralizing capability (Murphy, 1986) and primed for exaggerated in vitro lymphoproliferative responses compared to naturally infected children (Kim, 1976), suggesting enhanced diseases resulted from an aberrant vaccine induced immune response to infection. Children that received formalin inactivated vaccine, irrespective of subsequent natural exposure, had 5 fold greater HRSV specific lymphoproliferative responses than control children who had undergone natural RSV infection (Kim, 1976). In unvaccinated children, a higher incidence of bronchiolitis and post recovery episodes of wheezing were also found in children with high LP responses (Welliver, 1979). In subsequent investigations, using rodent models of HRSV infection, FI vaccine enhanced pathology in mice is associated with a Th2 T cell response (Graham, 1993b; Waris, 1996), is abrogated by $CD4^+$ depletion or anti IL-4 and IL-10 treatment (Connors, 1992a; 1994), and has not been reproduced by passive transfer of antibody (Connors, 1992a). Further work suggests the preferential activation of antigen specific CD4+ T-cells (as would be stimulated with an inactivated vaccine) is associated with disease, whereas involvement of CD8+ T cells in proposed regulatory and effector roles was associated with reduced illness (Srikiatkhachorn, 1997; Tang, 1997a; 1997b). In rodent models, vaccination with the F protein of RSV is associated with a Th1 cytokine profile, stimulation of CTL activity and reduced illness, whereas the G protein induces

enhanced disease associated with a Th2 T helper phenotype and absence of CD8+ CTL activity (Srikiatkhachorn, 1997).

Evidence for a similar pathogenesis in children is currently lacking, but the phenotype of the HRSV memory Th response does appear to be Th1 (Anderson, 1994). One of the most active and controversial topics of research into the pathogenesis of severe HRSV related bronchiolitis in children involves the role of IgE, a hallmark of Th2 responses (Welliver, 1980; 1981; Everard, 1995; Toms, 1996), but the pathogenesis of the enhanced disease in the rodent models does not appear to involve antibody (Connors, 1992a). Clinical trials in children using attenuated live virus vaccines, administered either parenterally or intranasally have not resulted in enhanced disease, but have also been unsuccessful in preventing disease due to low immunogenicity (Wright, 1976; Belshe, 1982) or have been excessively virulent (Kim, 1973). A preliminary trial using a recombinant F protein subunit vaccine in children resulted in short term reduction in infection and no evidence of enhanced disease (Tristram, 1994). Development of genetically engineered MLV HRSV vaccines is currently an area of active research and clinical trials are in progress (Firestone, 1996; Juhasz, 1997).

Vaccination of calves with formulations similar to the ill-fated FI preparation used in children (Mohanty, 1976; 1981) or with other inactivated vaccines inducing high lymphoproliferative responses and non-neutralizing antibodies (Taylor, 1987; 1989) have not been shown to result enhanced disease. A FI, alum precipitated BRSV vaccine has been shown to induce BRSV specific IgE in a levamisole-treated calf (Stewart, 1989a), but severe disease was not noted at challenge. Vaccination of calves

with rVV expressing the F, N or G proteins of BRSV are protective, and G protein specific enhancement of pathology is not observed as it is in rodent models of HRSV (Taylor, 1997). The experimental challenges used resulted in only mild disease in the control animals and this may have been responsible for the lack of disease enhancement. However, the use of several different commercial inactivated BRSV vaccine preparations for over a decade (Baker, 1997), with no apparent adverse effects, suggests vaccine associated immunoenhancement of disease is not easily induced in cattle.

2.9.5 Efficacy of Vaccination in Field Trials

Several field trials in Europe and North America have investigated the efficacy of MLV BRSV vaccines in young calves (Verhoeff, 1984; Thomson, 1986; Kubota, 1992; Frankena, 1994), at weaning or post weaning in beef calves (Bohlender, 1984; Van Donkersgoed, 1994) and in lactating dairy cows (Ferguson, 1997). The results of such field trials are often difficult to interpret due to the multiple etiologies of bovine respiratory disease, the sporadic nature of BRSV outbreaks, and the difficulty in establishing a diagnosis of BRSV infection (Verhoeff, 1984; Baker, 1997; Ferguson, 1997).

Four trials have been conducted in young calves that received their first vaccination when most calves had maternal antibodies. In three studies there was reduced clinical disease in vaccinated calves in groups that experienced BRSV outbreaks (Verhoeff, 1984; Thomson, 1986; Kubota, 1992), but in one of these studies significant protection was only observed in groups that were completely vaccinated and not in groups where vaccinated and control calves were co-mingled (Verhoeff, 1984), suggesting protection was incomplete and dependant on the level of challenge. In one

trial a reduction in clinical disease was observed, but a multivalent vaccine was used and this reduction was ascribed to protection from BVD virus (Frankena, 1994).

Reductions in undifferentiated respiratory disease of > 50% have been reported in large numbers of beef calves vaccinated with one or two doses of MLV BRSV prior to and at weaning (Bohlender, 1984). Other trials have shown much smaller but significant reductions in undifferentiated respiratory disease in calves vaccinated prior to weaning, but a significant effect on subsequent disease was not detected when older animals were vaccinated (Van Donkersgoed, 1994), perhaps due to prior exposure.

A significant increase in early lactation milk production and first insemination conception rates were observed in first parity cows vaccinated prior to calving with a multivalent (four-way) vaccine containing BRSV when compared to cows that received the same vaccine without BRSV (three-way) (Ferguson, 1997). Four-way vaccination was associated with increased first insemination conception rates in second parity cows, but there was no improvement in milk production. No differences were noted between three and four-way vaccinated third and higher parity cows. It was not determined if primary, or reinfections with BRSV occurred in heifers introduced into the milking herd. As respiratory disease was not described as a medical problem in this study, and primary BRSV infection can be severe in naive adult dairy cattle (Harrison, 1985; Elvander, 1996), these were most likely reinfections of subclinical severity, but with a significant effect on productivity.

2.10 Summary

Bovine and human respiratory syncytial viruses are important and ubiquitous respiratory pathogens of children and cattle. Infections in both infants and calves occur

in the face of maternal antibody, but passive antibody has a disease sparing effect. Maternal antibody suppresses antibody responses to infection, but has apparently minimal effect on protection from reinfection. Protection from systemic vaccination is reduced by maternal antibody. Virus clearance in a primary infection is dependent on cell mediated immunity and appears to involve cytotoxic T lymphocytes. Antibody appears to have a minor role in a primary infection. Reinfections are frequent and are not dependent on antigenic diversity of circulating virus strains, but rather on the short duration of mucosal immunity, which appears to involve cell mediated mechanisms. Disease in cattle upon reinfection is mild or subclinical. The correlates of immunity induced by vaccination or natural infection are not established but appear to involve anamnestic cell mediated as well as humoral responses. Serum antibody appears to be protective primarily in the lung. Vaccination of calves with both live and inactivated virus vaccines has resulted in variable degrees of protection, but these results need to be confirmed with a virulent challenge model. The majority of field trials have shown some protection from clinical disease by vaccination with modified live BRSV.

Immunopathogenic mechanisms and inflammatory reactions may contribute to bronchoconstriction and emphysema in both cattle and humans, and to pulmonary edema in calves, but the mechanisms remain unconfirmed or unidentified. Vaccination with inactivated HRSV has increased the frequency of severe disease in children but there is no published evidence disease or pathology is exacerbated by vaccination in cattle.

2.11 Formulation of Hypotheses and Objectives

This thesis deals primarily with vaccination and its effect on experimental BRSV infection in calves. When work on this thesis began, the humoral response to BRSV infection had been investigated (Kimman, 1987a; 1989e) but there was very little work describing effector cell mediated responses. Several key papers have been published on this subject in the intervening years (Taylor, 1995b; Gaddum, 1996a; Thomas, 1996), but there remained a lack of research with simultaneous investigation of serum and mucosal antibody and cell mediated effector responses. As well, investigations of the immune responses to infection and the protective or disease enhancing effects of vaccination in BRSV infection in cattle have been hampered by the lack of clinical disease induced by the models used (Belknap, 1995). The main thrust of this thesis was to address the issues of vaccine efficacy, the potential for vaccine induced enhancement of disease, the significance of qualitative differences in vaccine induced antibody responses, and the correlates of immunity to BRSV infection, using an experimental challenge model that reproduced the clinical disease and pulmonary pathologic features observed in field infections

The primary hypothesis formulated was that administration of inactivated BRSV vaccines had the potential for disease enhancement, whereas live virus immunization would be protective without disease enhancement.

Although published evidence of enhanced disease occurring in cattle that received inactivated vaccines is lacking, supported only by anecdotal reports, this aspect of the hypothesis was investigated primarily for the following reasons. Experimental investigation of the effects of a formalin inactivated (FI) BRSV vaccine similar to the

HRSV vaccine that enhanced HRSV disease in children has not been done in cattle using a model that resulted in clinical disease, and a bovine model of vaccine associated disease enhancement would be a valuable tool for human RSV vaccine development considering the similarity of human and bovine RSV-induced disease. Information regarding the immune response of children that received FI vaccine is minimal. The induction of high levels of non-neutralizing antibodies and enhanced lymphoproliferative responses were the major abnormalities described (Kim, 1976; Murphy, 1986). The mechanism of disease enhancement and how these responses may be related are only matters of speculation. Work in rodent models has indicated that FI vaccines of the type administered to children result in a Th2 T cell response, which is associated with enhanced pulmonary pathology (Graham, 1993b). It is not currently known if the results of these models are an accurate reflection of the pathogenesis of the aberrant response to RSV infection in children that received FI HRSV vaccines. There is some speculative evidence for antibody mediated or Th2-like (Kelso, 1995), mechanisms of disease enhancement operative in the pathogenesis of natural BRSV and HRSV infections (Kimman, 1989d; Everard, 1995), but antibody apparently does not have a role in disease enhancement in rodent model of HRSV (Connors, 1992a).

It has not been determined if BRSV vaccines similar to the FI HRSV vaccine administered to children, or commercial inactivated BRSV vaccines, induce Th2 T cell responses in cattle, and this was beyond the scope of this thesis. However, alum is a poor inducer of interferon gamma in ruminants (Emery, 1990), a cytokine which has been shown to induce bovine B cells to undergo an IgM to IgG2 class switch (Estes,

1996)suggesting cattle would have Th2-like T cell and antibody responses to an alum precipitated formalin inactivated BRSV vaccine.

Inactivated vaccines may also be poorly protective, as they have in other systems been shown to be poor inducers of CD8+ T cells (Morrison, 1988), crucial for viral clearance in calves (Taylor, 1995b). The protective effects of MLV vaccination were expected to be a result of enhanced memory cytotoxic activity and the induction of functional antibodies. The role of antibody in elimination of a primary BRSV infection (Taylor, 1995b) and its' association with reinfection rates have been difficult to establish (Kimman, 1989e). However, high levels of maternal antibody result in a marked reduction in disease severity (Belknap, 1991). This suggests vaccine induced prechallenge or anamnestic antibody responses should contribute to a significant reduction in virus load and pathologic lesions although elimination of BRSV infection may ultimately depend on cellular mechanisms.

The secondary hypotheses were formulated as follows:

1. Modified-live virus BRSV vaccines induce antibodies with neutralizing and fusion inhibiting properties whereas inactivated virus vaccines do not.
2. Antibody responses induced by systemic vaccination are associated with reduced disease and pathology although clearance of infection may be dependent on cell mediated mechanisms.
3. MLV vaccines prime for anamnestic cytotoxic responses whereas inactivated vaccines do not.
4. Virus clearance in a primary BRSV infection is correlated with the appearance of BRSV specific cytotoxic cells, rather than antibody responses.

Experimental Objectives

1. Compare the ability of commercial MLV and inactivated vaccines to induce functional antibodies to BRSV.
2. Develop a BRSV challenge model inducing clinical disease and pathology typical of field infections and to investigate the immune responses associated with viral clearance and /or pulmonary pathology in naïve calves.
3. Investigate the potential of a FI alum precipitated vaccine to enhance BRSV induced clinical disease and pathology, and to compare the antibody and cell mediated responses with those of calves that received MLV vaccine.
4. Investigate the correlates of immunity in calves vaccinated with MLV vaccines.

3 FUNCTIONAL ANALYSIS OF ANTIBODY RESPONSES OF FEEDLOT CATTLE TO BOVINE RESPIRATORY SYNCYTIAL VIRUS FOLLOWING VACCINATION WITH MIXED VACCINES

3.1 Abstract

The antibody response of cattle to bovine respiratory syncytial virus (BRSV) immunization was investigated using four different commercially available mixed vaccines. Forty, 5-6 month old, beef calves, randomly assigned to groups of ten, were vaccinated on day 0 and 21 with 1 of 3 inactivated vaccines, (three groups), or a modified live virus (MLV) vaccine. BRSV-specific antibody responses were measured prior to vaccination and on day 35 by using an enzyme linked immunosorbent assay (ELISA), virus neutralization assay (VN), a fusion inhibition assay (FI), and for the ability to facilitate antibody dependent, complement mediated cytotoxicity (ADCMC) of BRSV infected cells. Sera from day 35 were, in addition, analyzed by use of an IgG1, IgG2 isotype specific ELISA.

All vaccines induced significant increases in BRSV specific IgG antibody as measured by ELISA, but only one inactivated and the MLV vaccine induced significant increases in VN titers. Fusion inhibiting antibody titers were low or undetected in calves vaccinated with the inactivated vaccines. Vaccination with modified live virus induced significantly higher titers of fusion inhibiting antibodies, which are considered to be

most highly correlated with protection. The VN to ELISA and FI to ELISA ratio of the calves that received MLV vaccine were significantly greater than the calves receiving the three inactivated vaccines. Vaccination with MLV induced the highest IgG2/IgG1 ratio. This difference was small, and only significant relative to two of the inactivated vaccine groups, which were not significantly different from each other. The higher proportion of IgG2 isotype in the MLV sera was not associated with lower ADCMC; a function not attributed to this isotype. The VN and FI titers, but not the ELISA value of the sera were most predictive of ADCMC. The inactivation processes apparently alter epitopes and affect the induction of functional antibodies.

3.2 Introduction

Bovine respiratory syncytial virus (BRSV), a pneumovirus in the family *Paramyxoviridae,* is an important respiratory pathogen of cattle (Kimman, 1990) which is highly prevalent though not ubiquitous in cattle herds in western Canada (Durham, 1990). Immunization protocols for this virus, and its human counterpart, RSV, have been the subject of considerable investigation due to the potential of certain vaccines, primarily formalin inactivated preparations, to produce immunoenhancement of pathology upon subsequent challenge with the viruses (Murphy, 1986; Prince, 1986; Kimman, 1990). Investigation of the antibody responses of children and rodent models of RSV infection demonstrating this phenomena has shown, that, while high ELISA concentrations of antibodies to the F and G proteins are induced by vaccination with inactivated viruses, these have very poor neutralizing capabilities compared to those induced by vaccination with modified live virus or by natural infection (Murphy, 1986; Prince, 1986; Kimman, 1990). Documented evidence of enhanced disease in cattle

following immunization with inactivated vaccines is lacking, but the same high non-neutralizing antibody responses have been reported (Ellis, 1992b; 1995b). These results suggest that the inactivation process alters certain epitopes on the viral glycoproteins essential for the induction of neutralizing antibodies. These non-neutralizing antibodies may be poorly protective, and in addition, contribute to enhanced pathology, possibly by activation of complement without the ability to neutralize virus infectivity (Murphy, 1986; Kimman, 1989d; 1990).

Passive transfer of RSV-specific monoclonal antibodies to mice indicated that the capacity to inhibit virus mediated fusion of cells was the best correlate of a protective antibody response, with virus neutralizing and complement fixing capabilities not being as highly correlated with protection of experimentally infected mice (Taylor, 1984; 1992). Although all fusion inhibiting antibodies were neutralizing, the converse was not always true. Currently there are few data concerning the functional capacities and isotypes of BRSV-specific antibodies in cattle, especially with regard to antibodies induced by vaccination with live and inactivated antigens (Kimman, 1987b; 1989a; Ellis, 1992a; 1995a; 1995b). The purpose of this study was to determine the ability of vaccine induced antibodies to mediate fusion inhibition and complement mediated lysis of BRSV infected cells. These responses were compared to the total IgG response, as determined by ELISA, and to virus neutralizing activity. In addition, the relative proportions of the IgG1 and IgG2 isotypes were determined as a possible indicator of differences in the phenotype of the T-helper (Th) responses (Estes, 1994), as there is considerable evidence in rodent models, and some in human beings, that a Th2-type T cell response may be associated with enhanced disease, whereas a Th1-type response

has been associated with protection or natural infection (Connors, 1992b; Connors, 1994; Anderson, 1994;).

3.3 Materials and Methods

3.3.1 Experimental Design

Forty mixed breed, approximately 6 month old, weaned beef calves, weighing approximately 225 kg, were randomly assigned to four groups, each containing 10 calves. The animals were housed in a single pen in a commercial feedlot at the farm of origin. Contact with other animals was prevented. Each group of 10 calves was vaccinated with one of four commercially available mixed vaccines on days 1 and 21 of the study. Group 1 (MLV) was vaccinated with a modified live vaccine containing BRSV isolate 375 (Cattlemaster 4; SmithKline Beecham Animal Health, Exton, Pa.); group 2 (KV 1) with an inactivated preparation derived from BRSV field isolate GL266 (Virobos 4; Vetrepharm Inc, London Ont.); group 3 (KV 2) with an inactivated vaccine derived from BRSV isolate 375 (Bovilan 4K; Langford Inc, Guelph, Ont.); and group 4 (KV 3) with an inactivated vaccine utilizing a BRSV field isolate (Triangle 4; Fort Dodge Laboratories, Fort Dodge, Iowa). Participants conducting laboratory analyses were blinded as to group designation.

3.3.2 BRSV Specific IgG ELISA

BRSV specific IgG concentrations were determined using an indirect ELISA as described (Durham, 1990) with minor modifications. Briefly, partially purified antigen was prepared from lysed Vero cells infected with the BRSV RB94 isolate, and as a control, lysed uninfected Vero cells. A 1:50 dilution of serum samples from days 0 and 35 were added to duplicate wells of antigen coated plates, incubated at 37°C, and

washed. BRSV specific IgG was detected using peroxidase conjugated protein G (Zymed, San Francisco, Calif.) followed by substrate (ABTS; Kirkegaarde and Perry Laboratories, Gaithersburg, MD). Absorbance was read at 405 nm.

Optical density (OD) values were converted to ELISA units as described (Malvano, 1982) by comparing net (antigen - tissue control) sample OD values with the net OD value of a high BRSV titer standard reference serum on each plate. A standard curve, generated by linear regression of serial dilutions of the reference serum, was used to assign concentration units (Ellis, 1995b). Negative values were considered to be 0. A delta value of 20 ELISA units was considered to be evidence of seroconversion (Ellis, 1995b).

3.3.3 Virus Neutralization Assay

Serum VN titers were measured by use of a microtitration plaque reduction assay, as previously described (Ellis, 1990). Serial threefold dilution's of serum were made in medium, followed by a 1:1 dilution with 100 $TCID_{50}$ of the RB94 isolate of BRSV, yielding final dilutions of 1:6, 1:18, 1:54, 1:162. All samples were tested in duplicate and the titer was recorded as the reciprocal of the last dilution neutralizing virus infectivity in half the replicates. If both replicates yielded positive results the titer was considered to be twice the reciprocal of that dilution. Titers were recorded <6, 6, 12, 18, 36, 54, 108, 162, >162 and assigned scores of 0-8 respectively for purposes of analysis (Thrusfield, 1986).

3.3.4 Fusion Inhibition Assay

A fusion inhibition assay was performed as described (Ellis, 1995a) with minor modification. Primary fetal bovine tracheal (BT) cells were seeded onto 96 well plates

in Dulbecco's modified eagle medium (DMEM; Life Technologies, Grand Island, NY) with 5% fetal bovine serum (FBS). After 24 hours incubation, at which time they were 90% confluent, the cells were infected with 50 plaque forming units/well of RB94 BRSV and incubated 2 hours at 37°. The medium was shaken off the plates and replaced with 200*ul*/well fresh media and incubated for an additional 2 hours, after which this media was replaced with fresh media and the test sera. Threefold serial dilution's of sera were added in duplicate wells, starting at a 1:10 dilution. Three days after infection, the plates were fixed with 80% acetone, and then stained with Giemsa for 2 hours at room temperature. The stain was removed, replaced with distilled water, and the monolayers examined for the presence of syncytial cells.

The titer was recorded as the reciprocal of the last dilution to inhibit formation of syncytia in half the replicates. If both replicates yielded positive results (no syncytia), the titer was considered to be twice the reciprocal of that dilution. Titers were recorded as <10, 10, 20, 30, 60, 90, 180, 270, >270 and, for the purposes of analysis, assigned scores of 0-8 respectively (Thrusfield, 1986).

3.3.5 IgG Isotype Specific ELISA

An indirect ELISA utilizing the same antigens as the IgG ELISA was used to determine the BRSV specific IgG1 and IgG2 end point titers. End point dilution was used to establish a titer for each isotype, rather than a single dilution, in order to minimize competition between isotypes (Kimman, 1987b). Serial twofold dilution's of sera, starting at 1:50, were made in duplicate wells of antigen coated plates. Each plate included a standard high BRSV titer reference serum and a FBS negative control. Following a 1 hour incubation, the plates were washed and incubated sequentially with

murine monoclonal antibodies specific for bovine IgG1 or IgG2, (obtained from Klaus Nielsen, Agriculture Canada, Nepean, Ontario), biotinylated rabbit antimouse IgG (Zymed, San Francisco, Calif.), peroxidase conjugated avidin-biotin complex (ABC; Vector Laboratories, Burlingham, Calif.), and peroxidase substrate (ABTS).

The FBS negative control OD values were subtracted from the sample values. The net OD value for each dilution was obtained by subtracting the tissue control from the OD values in wells containing BRSV antigen. The titer was determined, as described, as the reciprocal of the highest dilution that scored > 10% of the maximum net OD value of the reference sera (Kimman, 1987b). For the purposes of analysis, the titers of 50, 100, 200, 400, 800, 1600, 3200, 6400 and 12,800 were assigned scores of 1-9 respectively (Thrusfield, 1986).

3.3.6 Antibody Dependent Complement Mediated Cytotoxicity (ADCMC)

Complement mediated lysis was determined by assessing cell viability with a colorimetric assay (Hansen, 1989), using a modification of a described procedure (Kimman, 1989a). Primary BT cells ($1x10^4$ cells / well) were seeded onto 96 well plates in DMEM with 5% FBS. Half the wells were infected with RB94 BRSV at an MOI of 1. At 48 hours after infection, when considerable cytopathic effect was visible, but no cell detachment present, the plates were washed once with warm DMEM with 1% gelatin. The serum samples, diluted 1:5 in 100*ul* DMEM with 1% gelatin, were added to 6 infected and uninfected wells and incubated at 37° C for 1 hour. 10*ul* of BRSV antibody negative bovine serum (obtained from a newborn calf prior to colostrum administration), stored at -80°C, or the same serum, heat inactivated for 45 minutes at 56° C (HIC), was added as a source of complement, and the plates incubated for a

further 45 minutes. The media was then replaced with 50*ul* DMEM with 5% FCS and 25*ul* of 5mg/ml, 3(4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide (MTT; SIGMA, St. Louis, Mo.). After an additional 2 hour incubation, 100*ul* of sodium dodecyl sulphate/dimethly formamide (SDS/DMF; SIGMA, St. Louis, Mo.) extraction buffer (Hansen, 1989), was added. Conversion of the tetrazolium salt (MTT) to colored formazan by live cells was determined by measuring absorbance at 595/655 nm.

The surviving cell number for each triplicate was determined using a standard curve generated from serially diluted infected or uninfected cells. Percent cell death was defined as the percent of the difference between the complement and HIC values for each sample. A high titer positive control serum and a FBS and media negative control were included in each assay.

3.3.7 Statistical Analysis

The significance of differences between vaccination groups for each assay, and within vaccination groups for day 0 and 35 results, were established by use of 95% confidence intervals (Martin, 1987b). Relationships between different antibody responses among all 40 calves were established by least squares linear regression using computerized software (Microsoft Excel, Microsoft Corporation, Redmond, WA)

3.4 Results

3.4.1 BRSV Specific IgG as Determined by ELISA

The indirect ELISA was used as a measure of total BRSV specific IgG. All calves, except for three receiving the MLV vaccine, showed evidence of seroconversion with an increase of >20 ELISA units (Figure 3.1). There were significant differences between all groups, with the MLV vaccine inducing the lowest response. Only two calves had a day 0 ELISA value >10, which would be consistent with residual maternal antibody.

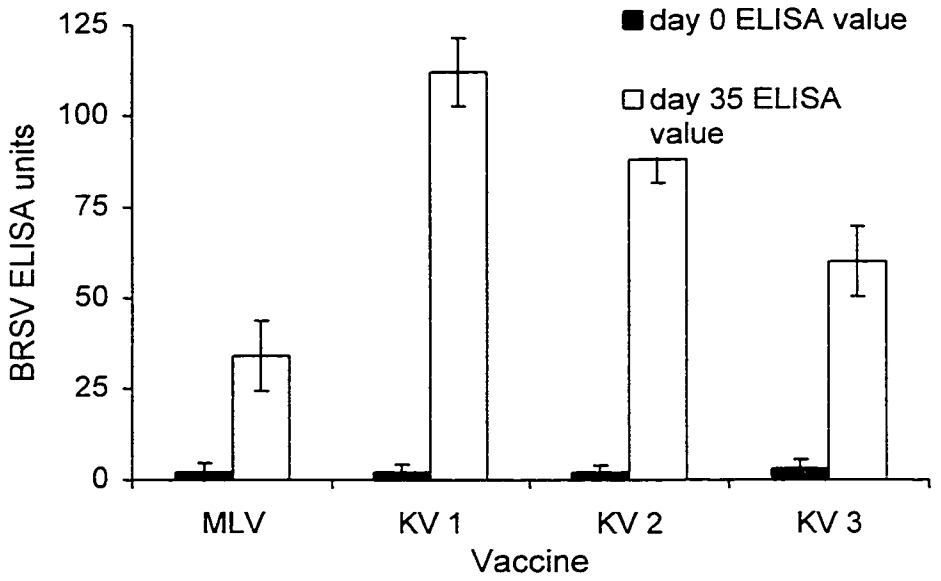

Figure 3.1 Total BRSV specific serum IgG as measured by ELISA on days 0 and 35. The assay was done as described in 3.3.2. Error bars represent 95% confidence intervals.

3.4.2 Virus Neutralizing Titers

Day 0 VN titers were similar for all groups, with a mean titer of 1:6. Only two groups, KV1 and MLV demonstrated significant increases in geometric mean titers by day 35 at the 95% level of confidence (Figure 3.2). The titer of the MLV group was

significantly higher than all other groups. The three calves which failed to demonstrate seroconversion in the ELISA assay all had evidence of seroconversion in the VN assay, with day 35 VN titers of $\geq$1:36, compared to day 0 titers of $\leq$ 1:6.

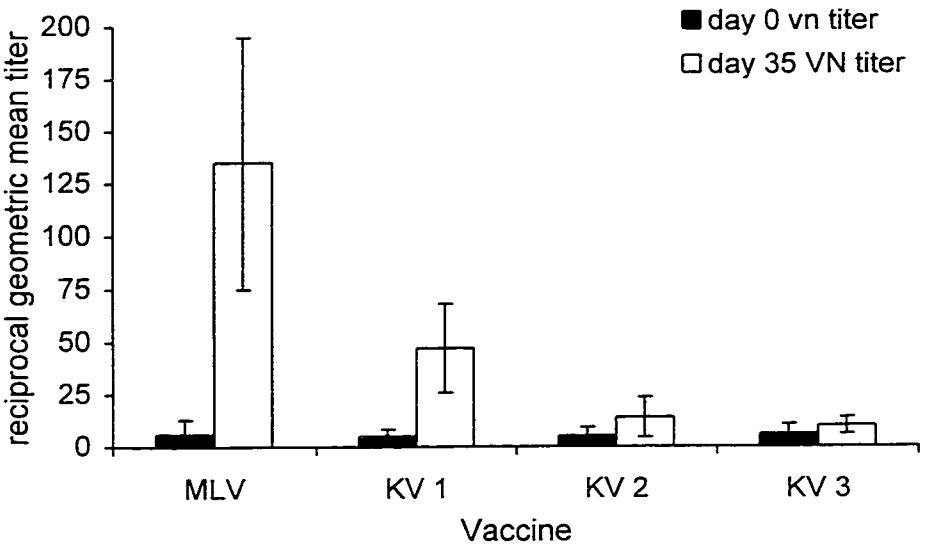

Figure 3.2 BRSV virus neutralizing antibodies on days 0 and 35.
Values are the reciprocal geometric mean titres equivalent to the mean score for each vaccine group, assigned as described in 3.3.3. Error bars represent 95% confidence intervals.

3.4.3 Fusion Inhibiting Titers

A fusion inhibition assay was used to investigate the ability of the various sera to inhibit cell to cell spread of BRSV by syncytia formation. Only 3 calves had detectable FI titers on day 0. The response to vaccination was similar to that seen in the VN assay. Only calves vaccinated with KV1 and MLV vaccines had significant increases in FI antibodies. The group administered the MLV vaccine had significantly higher titers than other groups (Figure 3.3).

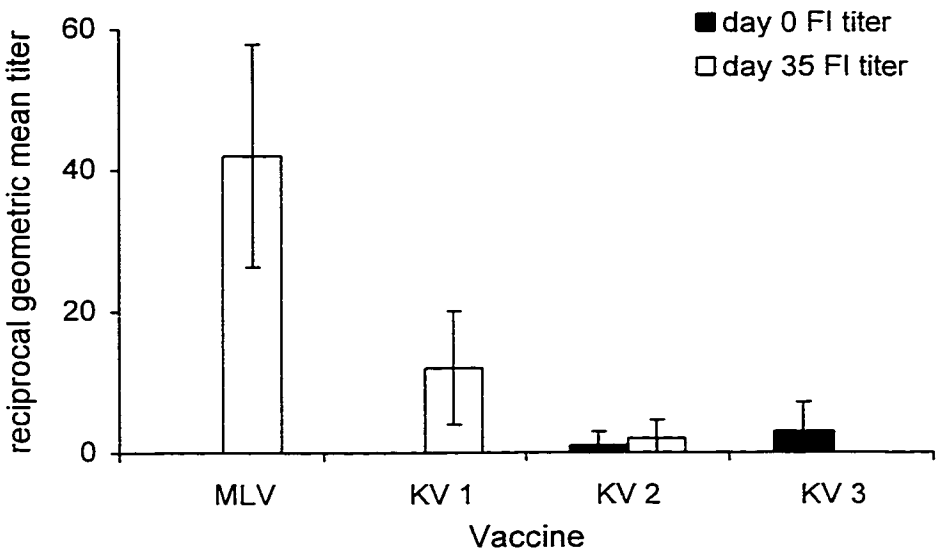

Figure 3.3 BRSV fusion inhibiting antibodies on days 0 and 35.
Values are the reciprocal geometric mean titres equivalent to the mean score of the dilutions of sera inhibiting syncytia formation of infected cells. Error bars represent 95% confidence intervals.

3.4.4 IgG1, IgG2 Isotype Specific ELISA

The MLV vaccine induced the highest ratio of IgG2/IgG1, however, the differences between groups were small, and only significant between the MLV and KV2 and KV3 calves (Figure 3.4).

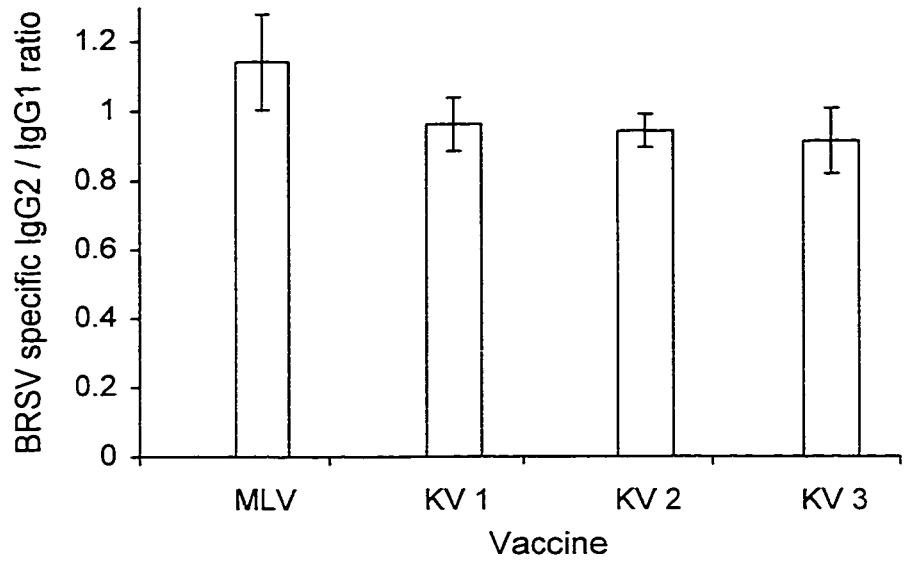

Figure 3.4 BRSV specific IgG2 to IgG1 ratios of day 35 sera.
Values determined using scores of the reciprocal endpoint serum dilutions for each isotype, as described in 3.3.5 Error bars represent 95% confidence intervals.

3.4.5 Antibody Dependent Complement Mediated Cytotoxicity

All groups demonstrated a significant post vaccination mean increase in ADCMC with one calf from each of KV2 and KV3 failing to show a post vaccination increase. The highest cytotoxicity values were obtained using sera from the MLV vaccinees, however, the mean of this group was not significantly different from either the KV1 or KV2 groups (Figure 3.5). The values obtained using sera from the KV3 group were significantly lower than from all other groups. Complement mediated lysis of infected cells in the absence of antibody (FBS or media) was < 6% in all assays. Lysis of uninfected cells was not seen with any of the sera.

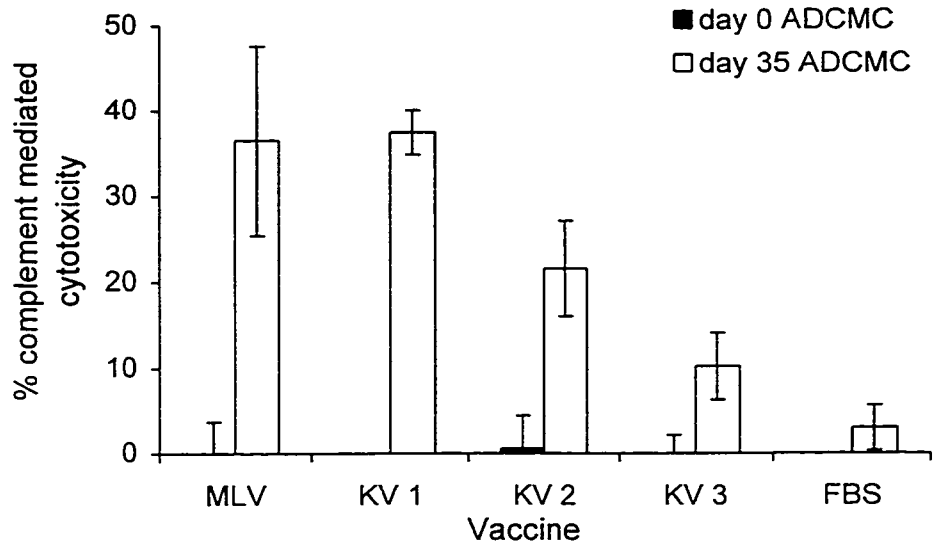

Figure 3.5 Antibody dependent complement mediated cytotoxicity (ADCMC). The ability of day 0 and 35 sera from the four vaccine groups and a fetal bovine serum negative control (FBS) to facilitate antibody dependent complement mediated cytotoxicity (ADCMC). Expressed as a percentage of BRSV infected cells killed. Error bars represent 95% confidence intervals.

3.4.6 Comparative Analysis

Virus neutralizing, and FI titer, to ELISA value ratios, were used to determined the proportion of total BRSV specific antibody having functional neutralizing and fusion inhibiting properties. In absolute terms, the sera from the calves receiving the MLV vaccine had the highest VN and FI titers. The difference was even more marked when the comparison was made as a proportion of total BRSV specific IgG (Figure 3.6). The inactivated vaccine, KV1, also induced relatively high VN titers, but this was associated with much higher ELISA values.

ELISA values were not predictive of either VN or FI titers in this study; R^2 = .014 and .067 respectively, which is in agreement with previously reported data (Ellis,

1992b). Approximately 55% of the FI titer variation could be predicted by the VN titer (R^2 = .55; P = .001).

The VN and FI titers were most predictive of ADCMC (R^2 = .57 and .50 respectively; P< .001). ELISA scores had no predictive value (R^2 = .06). Higher ADCMC values were associated with higher IgG2 (R^2 = .21; P<.005), but the predictive value was low (21%).

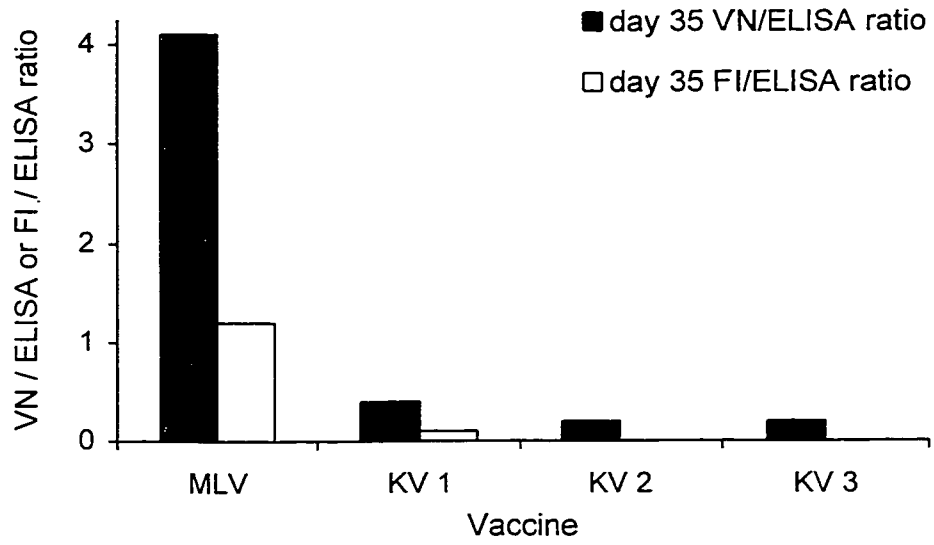

Figure 3.6 Virus neutralizing/ELISA and fusion inhibiting/ELISA ratios.
The ratios of the reciprocal geometric mean virus neutralizing or fusion inhibiting titres to the ELISA (total BRSV specific IgG) value as a means of demonstrating the relative proportion of the IgG antibody response capable of these functios in each vaccine group.

3.5 Discussion

These results confirm and extend the findings of previous studies in our laboratory. As found previously, there was a dissociation between the total BRSV-specific IgG response and virus neutralizing antibodies induced in cattle following the

administration of commercially available modified-live and inactivated vaccines (Ellis, 1992b; 1995a; 1995b). Herein we demonstrate a similar relationship between the total IgG response and the induction of antibodies capable of inhibiting fusion of virus infected cells or with the ability to fix complement on infected cells. The role of serum antibody in protection and recovery from RSV infection is controversial in calves, children, and rodent models of RSV infection (Westenbrink, 1989; Kimman, 1990). In one investigation, no differences were found in BRSV specific ELISA titers or proteins recognized by BRSV specific antibodies, between calves dying or recovering from BRSV infection (Westenbrink, 1989). Maternal antibody has not been shown to offer any protection from infection, but high titers may be associated with less severe lower respiratory tract disease (Kimman, 1988; 1990; Belknap, 1991). In children, attenuation of lower respiratory tract disease (but not infection), conferred by passive transfer of immune globulin, is dependent on a high virus neutralizing titer, not ELISA value, of the immune globulin (Groothuis, 1993; Meissner, 1993). Prophylactic administration of immune globulin selected for high virus neutralizing titer to cotton rats and mice prevented illness and markedly reduced viral replication in the lung, but was less effective in preventing replication in the upper respiratory tract. Therapeutic administration of immune globulin 5 days after infection resulted in more rapid recovery. Using an immune globulin with a low VN titer but similar ELISA value, these effects were not seen unless the dose was increased 10 times (Graham, 1993a; Siber, 1994). Work using monoclonal antibodies has established that antibodies directed against the fusion or F glycoprotein are more protective in rodent RSV challenge models than those against the G protein, while those against the N protein are not

protective (8). In experimentally infected mice, protection from disease by mAbs directed against the F protein was most highly correlated with the ability of the mAb to inhibit syncytia formation. These protective mAbs also had neutralizing activity, but this alone was not necessarily indicative of protective properties *in vivo*. The ability of the mAb to induce ADCMC of RSV infected cells was also not required for protective activity (Taylor, 1992).

This study demonstrates that the MLV BRSV vaccine induced BRSV specific antibody responses with significantly greater scores in the VN and FI assays. These are the *in vitro* antibody assays most highly correlated with disease sparing or protection in children and rodent models of RSV infection (Taylor, 1984; 1992; Graham, 1993a; Groothuis, 1993; Meissner, 1993; Siber, 1994), and therefore most likely to have the same correlations in cattle. The lower ELISA values relative to SN or FI titers seen with MLV vaccination may be due to a lower total BRSV specific IgG production, or an alteration in the epitopes required for neutralization by the preparation of the ELISA antigen, similar to that occurring in the preparation inactivated vaccines. The inactivated vaccines may have invoked a greater response to epitopes on viral proteins not generally exposed on infected cells or intact virus, or to different proteins, such as the N protein, which are poorly immunogenic, but this was not investigated. Previous work, using radioimmune precipitation or western blotting, indicates this latter possibility is less likely as serum from calves vaccinated with either MLV or inactivated virus vaccines both recognized nearly exclusively the F protein (Ellis, 1992b; 1995b). The moderate predictive value of VN titer on FI titer is expected as it has been shown, using a panel of

monoclonal antibodies, that all fusion inhibiting antibodies are neutralizing, but not all neutralizing antibodies are fusion inhibiting (Taylor, 1992).

The results of the ADCMC assay indicate that immunization with MLV vaccine induces complement-activating antibody, effective in inducing cell lysis, with a lower ELISA value than inactivated vaccines. ADCMC was most highly correlated with the other functional assays, virus neutralization and fusion inhibition, and not with ELISA value. This may be due to a similar requirement for antibodies to recognize the viral proteins on the cell membrane in their native state. This experiment, as conducted, does not support the hypothesis of complement activation by high titers of non-neutralizing antibodies on infected cell surfaces as mechanism of disease enhancement, as first proposed for children receiving inactivated vaccines (Murphy, 1986), and later suggested as a possible pathologic mechanism in field cases of BRSV in cattle (Kimman, 1989d; 1990). However, it is possible that free virus, or viral proteins released from cells, may complex with non-neutralizing antibodies and activate complement, or that these antibodies may fix complement yet not induce cell lysis (Kimman, 1990), possibilities not investigated in these experiments. Such a functional disparity, if present, could conceivably be detrimental.

The failure to demonstrate a negative correlation between IgG2 and ADCMC could be due to the inability of IgG2 to competitively block the binding of complement fixing IgG1 to infected cells (Kimman, 1989a), or the fact that sera from groups MLV and KV1 had the highest VN and FI titers as well as the highest IgG2/IgG1 ratios.

The considerable variation in antigen preparation and adjuvant among the four vaccines could conceivably result in differences in the T cell as well humoral responses

induced. This is an area of considerable interest in RSV research, as in rodent models of HRSV infection, vaccination with inactivated virus or purified viral glycoproteins not only produces antibodies with poor functional capabilities, but also induces a different T-helper (Th) (Kelso, 1995) response than live virus infection (Connors, 1992a; Graham, 1993b; Tang, 1994). There is evidence, in rodents, of a role for a Th2 type T-cell response in immunoenhancement of RSV pathology (Connors, 1992b; 1994; Tang, 1994), with protection being associated with a Th1 response (Tang, 1994). It has yet to be determined if a similar relationship exists for human beings or cattle; however, natural infection in humans does induce a Th1 memory response (Anderson, 1994) as does live virus challenge in mice (Graham, 1993b; Tang, 1994). There is considerable evidence in mice (Rizzo, 1995) and humans (Kitani, 1993; Briere, 1994; Wilkes, 1994) for cytokine mediated control of immunoglobulin isotypes, with a Th1 type response associated, in mice, with an increase in IgG2a relative to IgG1. Bovine IgM secreting B-cells can be induced to switch preferentially to IgG2 by IFNγ (Estes, 1994), indicating a similar relationship may exist in the bovine immune system and the relative proportions of IgG1 and IgG2 may be reflective of differences in the phenotype of the T-cell response. The IgG isotype ELISA results of this experiment would not suggest a difference in T-cell response induced by the different vaccines, although the MLV group did have the highest proportion of IgG2. The MLV vaccine used in this study did contain alum as an adjuvant, which in sheep is a poor inducer of IFNγ (Emery, 1990). This may have induced a different T cell response than an unadjuvanted MLV vaccine,

without affecting the production of antibodies recognizing the important neutralizing and fusion inhibiting epitopes.

To our knowledge this is the first report to directly compare the ability of various commercially available BRSV vaccines to induce fusion inhibiting and complement fixing antibodies in cattle, and to determine the predominate IgG isotype of these responses. Further studies are required to determine which assays of antibody function are most relevant to a protective response in cattle.

4 THE EFFECT OF FORMALIN-INACTIVATED VACCINE ON RESPIRATORY DISEASE ASSOCIATED WITH BOVINE RESPIRATORY SYNCYTIAL VIRUS INFECTION IN CALVES

4.1 Abstract

The effect of vaccination with a formalin-inactivated, alum-precipitated (FI), bovine respiratory syncytial virus (BRSV) vaccine on BRSV induced respiratory disease in calves was investigated. Six month old BRSV-naive calves were vaccinated with either a FI, a modified live virus (MLV), or virus antigen negative control vaccine (n = 4 per group). One month after the second vaccination, the calves were aerosol challenged with lung wash from a newborn calf infected with a field isolate of BRSV. Moderate to severe clinical disease occurred in all calves. Calves that received FI vaccine had a significantly earlier (day 2 vs. day 4-5) onset of pyrexia and dyspnea ($p<.05$). Pulmonary lesions, consisting of cranioventral atelectasis and dorsal emphysema, occurred in all groups. Two calves that received MLV, and 3 that received FI vaccine, had reduced pneumonic lung area relative to controls. Vaccination with the FI vaccine resulted in more rapid onset of clinical disease, but ultimately, reduced pulmonary pathology in most recipients.

4.2 Introduction

Human respiratory syncytial virus (HRSV) and bovine respiratory syncytial virus (BRSV) are antigenically related pneumoviruses in the family Paramyxoviridae (Smith, 1975). Both viruses are ubiquitous respiratory pathogens with similar epidemiology, and induce similar clinical disease and lesions (Kimman, 1990; Van der Poel, 1994; Baker, 1997). HRSV is the leading cause of severe lower respiratory tract disease in infants < 1 year of age (Heilman, 1990). Despite the importance of this pathogen, implementation of an efficacious vaccine has been delayed, at least in part, due to enhanced disease that occurred in paediatric subjects vaccinated in the 1960's with a formalin inactivated, alum adjuvanted vaccine (Chin, 1969; Kim, 1969). In rodent models of HRSV infection, clinical disease is minimal or absent (Graham, 1993b; Hildreth, 1993b), however, enhanced pathology occurs in animals have that received a FI vaccine and is associated with a Th2 T cell response (Waris, 1996). In a primary HRSV infection in mice, depletion of either $CD4^+$ or $CD8^+$ T cells delays viral clearance (Graham, 1991c), but eliminates illness and pathology, suggesting that the minimal pathology observed in these models occurs as a result of the inflammatory response, rather than the viral infection per se. This absence of virus induced pathology is at variance with the severe disease associated with delayed viral clearance in immunocompromised children (Fishaut, 1980; Hall, 1986) and calves (Thomas, 1984), leading to questions about the relevance of rodent models of RSV infection (Hildreth, 1993a; 1993b; Piedra, 1993). The similarity of the human and bovine diseases suggests calves would be a better model for studying the effect various vaccines may have on

response to infection. Several commercial BRSV vaccines are currently in use in cattle, and while there are anecdotal reports of vaccine-enhanced disease, this has not been documented in the literature, nor experimentally reproduced.

Similar to the rodent models, previous vaccination and challenge studies in calves have resulted in only mild or no clinical disease (Mohanty, 1976; 1981; Kimman, 1987a; 1989e; Taylor, 1989). This inability to reproduce severe disease, as often follows naturally acquired BRSV (and HRSV) infections, has limited progress in understanding the pathogenesis and immunity to BRSV in cattle, and limited the usefulness of BRSV infection in cattle as a model of HRSV infection (Belknap, 1995). The objectives of this study were to establish a challenge model that experimentally reproduced severe clinical disease following BRSV infection and to determine if clinical disease would be enhanced in calves that had received a formalin-inactivated (FI) vaccine prepared similarly to the "lot 100" vaccine used in paediatric subjects in the late 1960's. The "lot 100" vaccine preparation method was used for its historical importance as the ill-fated vaccine that was associated with enhanced disease in children, and for the ability of this and similar vaccines to preferentially induce antibody (Th2 or Th2-like) responses in calves or other animal models (Stewart, 1989b; Emery, 1990; Kakuk, 1993; Kung, 1994; Waris, 1996). For comparison, a modified live virus (MLV) vaccine was used to prime for a cell mediated response and cytotoxic T lymphocyte (CTL) activity (Nicholas, 1990), at a low dose that induced minimal pre-challenge antibody.

4.3 Materials and Methods

4.3.1 Calves

Holstein bull (n = 8) and heifer (n = 4) calves, obtained from a local dairy, were removed from their dams at birth, fed 2.5 litres of pooled colostrum and conventionally raised in isolation facilities at the University of Saskatchewan. All calves were clinically normal and shown to be free of bovine virus diarrhoea virus (BVDV) infection by virus isolation.

4.3.2 Experimental Design

Twelve Holstein calves were randomly assigned to one of the following three groups of four calves: group I; vaccinated with FI BRSV vaccine, group II; vaccinated with MLV BRSV vaccine and group III; received a tissue culture control mock vaccination for the MLV (n = 2) or the FI vaccine (n = 2). Calves were vaccinated at 4-5 months of age, revaccinated 3 wk later and challenged with BRSV via aerosol exposure 34 days after the second vaccination (day 0). Calves surviving the challenge, or not euthanized earlier for humane reasons, were euthanized 8 days after challenge (day 8).

4.3.3 Cell Culture and Vaccine Preparation

The vaccines were prepared using the BRSV RB94 isolate (SmithKline Beecham Biologicals S.A., Rixensart, Belgium) propagated in Madin-Darby bovine kidney (MDBK) cells maintained in Dulbeccos' modified Eagles medium (DMEM) with glutamax supplemented with 5% foetal bovine serum (FBS) (Gibco BRL, Grand Island, NY), 100 μg/ml sodium penicillin G, 100 μg/ml streptomycin sulphate, 50mM HEPES HEPES (N-2- hydroxyethylpiperazine-N-2-ethanesulfonic acid), and incubated

at 37° in 5% CO_2. Cell cultures were determined to be free of mycoplasma by culture on Hayflicks agar and BVD Virus by indirect immunoflourescence testing. MDBK cells were infected with BRSV at an MOI of .01 and harvested after 72 hr when the cell monolayer showed 60 - 80% cytopathic effect. The flasks were frozen at -70°C, thawed, and the lysates clarified by centrifugation at 1400xg for 10 minutes. The clarified supernatants contained $2x10^6$ plaque forming units (pfu) of BRSV per ml. Uninfected MDBK cells were grown and harvested under identical conditions for preparation of the mock vaccines. Aliquots of clarified supernatant were stored at -70°C and used as the MLV vaccine and its' mock control vaccine.

The FI BRSV and mock vaccines were prepared as described for the "lot 100" vaccine (Kim, 1969; Waris, 1996). Briefly, clarified supernatant was inactivated for 2 days at 37°C by the addition of 1:4000 formalin. The inactivated supernatant was centrifuged at 50,000xg for 1 hr at 4°C, the pellet resuspended in PBS at 1:25 of the original volume and precipitated with 10% aluminium potassium chloride. The precipitate was washed twice and resuspended in phosphate buffered saline (PBS) to 1:100 of its original volume. The protein content of the vaccine was 1.3 mg/ml and the absorption of protein was >97% (Bio Rad Protein Assay, Bio Rad, Richmond, California),

Calves were vaccinated intradermally either with 1 ml of the FI vaccine or control preparation, divided in 4 cervical sites, or for the MLV vaccine, with 100 µl diluted supernatant containing $10^{3.5}$ pfu of BRSV or an equivalent volume of uninfected supernatant.

4.3.4 Challenge Model

The "Asquith isolate" of BRSV was obtained from a calf with severe respiratory tract disease. BRSV was immunohistochemically documented in pulmonary tissue from this calf (data not presented). The isolate was passed three times in embryonic bovine tracheal (EBTR) cells, then serially passed in three colostrum-deprived new-born calves by aerosol administration of a lung wash obtained from the previous calf. All washes were done using RPMI-1640 supplemented with 1.5% FBS (Gibco BRL, Grand Island, NY). Calves were maintained on enrofloxacin (Baytril, Bayer Inc., Etobicoke, Canada) at 5 mg/kg/day from birth. A lung wash obtained from the third calf 4 days after infection was used as the challenge inoculation for this experiment. This wash contained $3x10^3$ pfu/ml BRSV and was stored at -70°C. No bacteria were cultured from the wash on blood agar. Mycoplasma were not isolated in 3 serial passages in Hayflicks broth, and it was negative for bovine herpesvirus-1 (BHV-1), parainfluenza-3 (PI_3) and BVD viruses using indirect immunoflourescence on inoculated EBTR cells and additionally by PCR testing for BVD virus (Manitoba Agriculture, Veterinary Services, Winnipeg, Manitoba). At the time of challenge, the lavage fluid was thawed and clarified by centrifugation at 300xg for 5 minutes. Each calf was inoculated on day 0 with ~10^5 pfu BRSV, using a nebulizer (Ultra-Neb 99, Devilbiss, Somerset, PA) equipped with a face mask.

4.3.5 Monitoring and Clinical Assessment

Clinical assessments were made at the same time each morning by a veterinarian blinded as to treatment group. Changes in respiratory rate (RR) were assessed using

delta values calculated using the mean RR for days -2 to 0 as the baseline for each calf. Calves were considered to have a significantly increased rate if the value was greater than the baseline plus 2x the standard deviation of the baseline mean (Wilkie, 1976). Clinical scores for cough, respiratory distress and sickness were assigned using a modification of a scoring system previously described (Babiuk, 1987 and Richard Harland, Biostar Inc. Saskatoon, Saskatchewan, personal communication). The sickness scores (SS) were assigned using combined objective and subjective observations and included the following predictive statements: SS = 1, a sick animal that, in clinical setting, would be treated and be expected to recover; SS = 2, an obviously sick animal that in a clinical setting should already have been treated, but recovery is not certain; SS = 3, an animal judged to be unlikely to recover with treatment and for which the most appropriate course of action is euthanasia; SS = 4, moribund animal. Adventitial lung sounds were recorded but not scored. Thoracic radiographs were taken of all calves between days 4-8. Arterial blood gas tensions were measured on samples collected from the coccygeal artery on day 0, 4, 5, and 6 using a blood gas analyser (Model 288, Ciba-Corning, Medfield, MA). Values were corrected for rectal temperature (Severinghaus, 1965).

4.3.6 Virus Isolation and Titration

Nasal virus shedding was assessed using nasal swabs taken on days 0 and 2-8. Swabs were placed in 1 ml of transport medium consisting of DMEM supplemented with 10% FBS (Gibco BRL, Grand Island, NY), 0.3M $MgSO_4$, 0.4M sucrose, 50mM HEPES, 200 μg/ml sodium penicillin G, 200 μg/ml streptomycin sulphate and 0.5

μg/ml amphotericin B, and frozen at -70°C. Virus shedding was quantitatively determined by a micro-isolation plaque assay with a maximum calculated sensitivity of 10 pfu per ml. Aliquots of transport medium were inoculated in serial dilutions onto EBTR cells, incubated for 56 hr and the plates fixed in 80% acetone. Viral plaques were identified by staining with an avidin-biotin immunoperoxidase technique utilising a 1:1000 dilution of a monoclonal antibody specific for the F protein of RSV (mAb19, courtesy of Dr. Geraldine Taylor, Institute for Research on Animal Diseases, Compton, Newbury, Berkshire, UK), followed by 1:1000 biotinylated rabbit antimouse IgG (Vector Laboratories, Burlingham, CA), avidin- biotin complex (Vectastain, Vector Laboratories, Burlingham, CA) and diaminobenzidene. Antibodies were diluted in Blotto Buffer (50 mM Tris HCL, pH 8.5, 10mM EDTA, .05% Tween 20, 1% Triton X-100, 2% skim milk) during incubations for 1 hr at 37° C. Nasal swabs were tested for the presence of BHV-1, PI_3 and BVD virus by indirect immunoflourescence of inoculated EBTR cells.

4.3.7 Virus Neutralising and ELISA Antibody Assays

Serum samples were obtained prior to vaccination and on days 0, 4, 6 and 8 to measure BRSV specific serum virus neutralising antibody titres and BRSV specific IgG concentrations by ELISA, as previously described (West, 1997a).

4.3.8 Cytotoxicity Assays

Chromium release cytotoxicity assays were carried out similar to previous descriptions (Gaddum, 1996a), using peripheral blood mononuclear cells (PBMC) collected prior to vaccination, and on days 4, 6 and 8 after infection and on

bronchoalveolar lavage (BAL) cells collected on day 8. Briefly, peripheral blood was collected in acid citrate dextrose, and after centrifugation at 500xg for 15 min, the buffy coat was collected and diluted in an equal volume of PBS/Alsevers. Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation at 1400xg for 25 min at 20°C over 70% Percoll (Pharmacia, Uppsala, Sweden). Cells from the interface were washed twice in PBS/Alsevers and resuspended in RPMI-1640 (Gibco BRL, Grand Island, NY) supplemented with 10% heat inactivated FBS (Gibco BRL, Grand Island, NY) 100 μg/ml sodium penicillin G, 100 μg/ml streptomycin sulphate, 50mM HEPES, $5x10^{-5}$ M 2-mercaptoethanol, and used as effectors at E:T ratios of 100:1 and 50:1. BAL cells were obtained by lavaging the right cranial lung lobe with 60 mls cold PBS. The BAL fluid was centrifuged at 200xg for 10 min, the cell pellet resuspended in RPMI-1640 (Gibco BRL, Grand Island, NY), layered over 70% Percoll (Pharmacia Uppsala, Sweden), and centrifuged at 1400xg for 25 min. Mononuclear cells recovered from the interface were washed twice in RPMI and were used as effectors at an ET ratio of 80:1.

Target cell lines were generated for each calf from 6 mm skin punch biopsies. Explant cultures were established as described (Freshney, 1986), in 25 cm^2 flasks using 1.5 ml of DMEM with 20% FBS, (Gibco BRL, Grand Island, NY), 100 μg/ml sodium penicillin G, 100 μg/ml streptomycin sulphate, 50mM HEPES HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid). The cultures were incubated at 37° in 5% CO_2 and the medium changed daily for 1 week. The explants were then removed and the cultures maintained in DMEM with 10% FBS with passage every 5 days. Cultures were used as targets after 3 - 7 passages. Cell cultures were determined to be

free of mycoplasma by culture on Hayflicks agar and BVD Virus by indirect immunoflourescence testing. Autolgous and heterologous dermal fibroblasts were used as targets. Adherent targets ($1x10^4$/well, in round bottomed 96 well plates) were infected with BRSV RB94 isolate at an MOI of 2, 18 hr prior to the assay, and were labelled with 1 μCi/well of ^{51}Cr (Amersham, Arlington Heights, IL) for 6 hr prior to the addition of effectors. Cells were incubated for 6 hr at 37° C in 5% CO_2 before supernatant was harvested (SCS, Skatron Instruments, Sterling, VA). Counts per minute (cpm) were determined using a gamma counter (Model Gamma 5500, Beckman Instrumentation, Palo Alto, CA) and percent cytotoxicity was determined by the formula; % cytotoxicity = (cpm - spontaneous release/ total release- spontaneous release) x 100.

4.3.9 Lymphocyte Proliferation (LP) Assay

BRSV specific lymphocyte proliferation (LP) was determined prior to vaccination and on day 0 and 8 as previously described (Ellis, 1992a), by measuring tritiated thymidine incorporation in antigen-stimulated PBMC. Cells were cultured in round bottom 96 well plates at a density of $3x10^6$/ml and stimulated with a 1:50 dilution (previously determined to be optimal) of BRSV-infected MDBK cell tissue culture lysate or uninfected control lysate. Cells were labelled after 6 days of culture, harvested onto glass fibre filters 18 hr later, and cpm determined using a scintillation counter (Model LS6000IC, Beckman Instrumentation, Palo Alto, CA). The data was expressed as previously described (Sharma, 1996b), using delta c.p.m. obtained by subtracting the

antigen from the control c.p.m. after first performing a square root transformation of the data.

4.3.10 Intradermal Skin Testing

On day 0, each calf was injected in two cervical sites with 100 µl of lysed, clarified, BRSV-infected (RB94 isolate) Vero cells containing $1x10^5$ pfu/ml and in two sites, in the contralateral cervical area, with lysed uninfected Vero cells (control). Punch biopsies (6mm) of the sites were collected at 24 and 48 hr and fixed in 10% neutral buffered formalin. Hemotoxylin and eosin stained sections were scored on a 0-4 scale for granulocytic and mononuclear cell infiltration. On day 7, surviving calves were reinoculated intradermally, and assessed for wheal formation at 15 minutes and biopsied at 24 hr.

4.3.11 Post Mortem Sampling

Calves were euthanized with an intravenously administered dose of barbiturate (Euthanyl Forte, MTC Pharmaceuticals, Cambridge, Canada). The respiratory tract was removed and the dorsal and ventral surfaces of the lungs were photographed as well as the cut surface of a single, standardised mid lobe cross section of both caudal lobes. Tracings were made on acetate from the projected slides of all three views, outlining both total and depressed dark red, obviously pneumonic, areas. The percentage area of pneumonic lung for each tracing was determined by computer software (Image 1, Universal Imaging Corporation, West Chester, PA). The mean of the three views was used as the percent pneumonic lung area score.

Sections of each lung lobe, taken at the edge of a pneumonic area if possible, were fixed in 10% neutral buffered formalin and processed for routine histology and immunohistochemical staining for BRSV antigen as previously described (Haines, 1989), using a polyclonal antibody to HRSV (Dako, Dimension Laboratories, Mississauga Canada).

Microbiological determinations were done using 5 gm of pneumonic lung homogenised in 5 mls of DMEM (Gibco BRL, Grand Island, NY). The homogenate was clarified by centrifugation at 1000xg for 5 min and the supernatant was inoculated onto blood agar plates, EBTR cells and into Hayflicks medium. Blood agar plates were incubated at 37°C in 5% CO_2 for up to 7 days. Colonies were identified by standard techniques. Samples were considered negative for mycoplasma after 3 passages in Hayflicks medium and subculture onto Hayflicks agar. Positive cultures were stained by indirect fluorescent antibody techniques using mAb specific for M. bovis, M. arginini, M. laidlawiii, M. bovirhinis and M. bovigenitalium. The inoculated EBTR cultures were examined daily for CPE and repassaged on day 5. Cultures showing CPE on the first passage, and all cultures 5 days after the second passage, were stained for BRSV, BHV-1, PI_3 virus and BVD virus by indirect immunoflourescence.

4.3.12 Data Analysis

The scored clinical parameters, arterial oxygen and tension data and lymphoproliferative responses were evaluated using a Kruskal-Wallis one way analysis of variance to establish differences between groups and differences over time. When the P value was significant ($P<0.05$), differences between groups were evaluated by the

Mann-Whitney U test and differences among groups by the Wilcoxon signed rank test using exact two tailed probabilities to establish significance ($P<0.05$). When no differences between treatment group could be established, differences over time were evaluated using pooled data from all groups. A reduction in the sample size of all groups on day 5 precluded meaningful statistical analysis by group past this point. Analyses were done using computerised software (Statistix, Analytical Software, Tallahassee, FL).

4.4 Results

4.4.1 Clinical Data

All calves in all groups had increased respiratory rate (RR) delta values over time ($P\leq0.008$) (Figure 4.1). Using pooled data from all groups of calves, there were significant increases in RR on days 4-8 ($P<0.001$). Significant differences among groups occurred only on days 2 and 3. Calves in group I (FI) had a significantly higher delta RR than either group II (MLV) or III (control) ($P<0.03$). This was reflected in moderate to marked respiratory distress in all calves in group I from approximately 48-72 hr after infection. Dyspnea with an expiratory grunt was observed in 6 calves on days 3-7 (group I; n = 1, group II; n =3, group III; n = 2).

Increased rectal temperatures of 40 - 41 °C were recorded in all calves, with peak fevers occurring between days 2-6 (Figure 4.2). Significant differences among groups occurred only on days 2 and 3 when group I had significantly higher temperatures than group II ($P<0.03$), but not group III ($P= 0.057$). After day 4, group I had the lowest rectal temperatures, however this difference was not significant

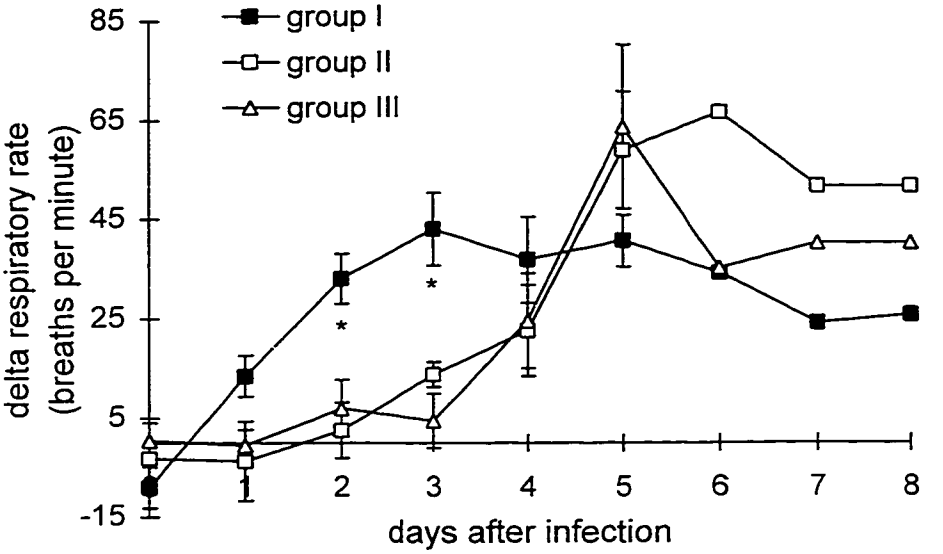

Figure 4.1 Delta respiratory rates (breaths per minute increase over pre-infection baseline; mean ± SE).
Calves were vaccinated with FI BRSV (group I), MLV BRSV (group II) or sham vaccinated (group III) and challenged with BRSV on day 0. *Significant difference betweeen group I and groups II and III (P<0.03; Mann-Whitney U test).

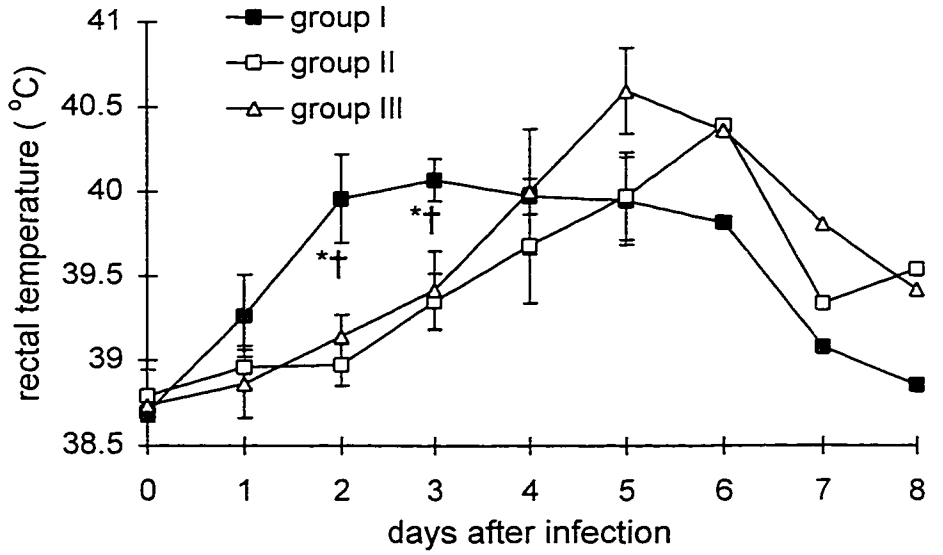

Figure 4.2 Rectal temperatures (°C; mean ± SE).
Calves were vaccinated with FI BRSV (group I), MLV BRSV (group II) or sham vaccinated (group III) and challenged with BRSV on day 0. * Significant differencebetweeen group I and groups II and III (P<0.03); Mann-Whitney U test). † Difference between groups I and III not significant (P=0.057; Mann-Whitney U test).

Frequent spontaneous coughing was observed in most calves on days 5 - 8 as were harsh, increased breath sounds (data not shown). Adventitial lung sounds (wheezes and rales) were noted in 9 calves, usually beginning on day 4, and persisting in 4 calves to day 8. There was no group difference, nor was there a positive correlation between severity of disease, or bronchiolitis observed at necropsy, and the presence or persistence of adventitial lung sounds (data not shown).

The highest sickness scores (SS) (representing the veterinary clinician's overall assessment of the severity of illness) for most calves were recorded on days 5-7 (Figure 4.3). The SS of the calves in group I reflect the trend of earlier onset, with subsequent earlier attenuation, of most clinical signs compared to the other groups. No significant group differences were found on paired analysis, however, aside from the one calf which was euthanized on day 5 (SS = 3), sickness scores for group I never exceeded 1. All other calves received SS of ≥ 2, with 5 calves assigned a SS of 3. On day 5, one calf died (group III) and 3 others (one from each group), in severe respiratory distress, were euthanized for humane reasons.

There was a significant decrease in PaO_2 on days 4 and 6 ($P<0.003$; $P<0.02$, pooled data) (Table 1). Although the lowest values recorded were in groups II and III, there were no significant group differences in PaO_2 at any time.

Thoracic radiographs of all calves showed a diffuse pattern of patchy consolidation, involving up to two thirds of the anterioventral lung. Bronchograms, lucent areas of cavitation and oedematous intrapulmonary pleural planes were variably present. In two calves (groups I and III), there was mild pneumothorax. (Figure 4.4).

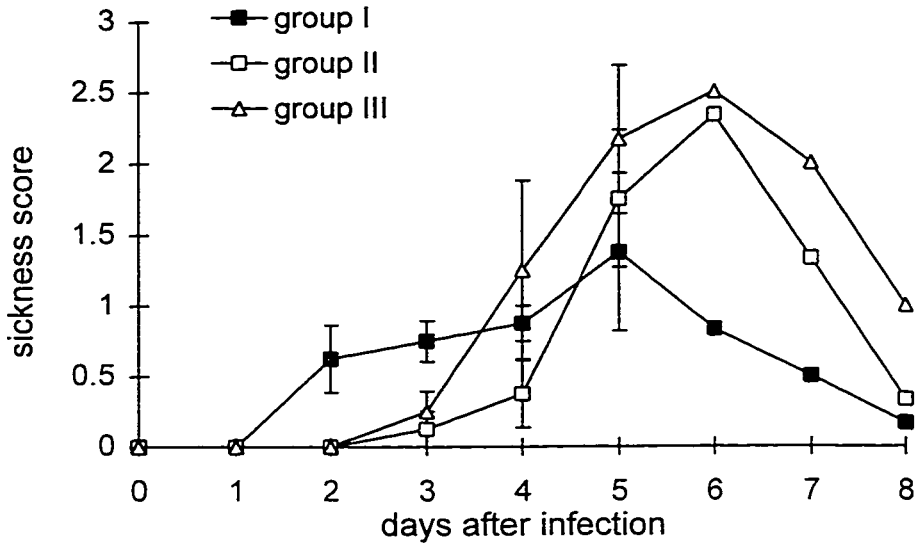

Figure 4.3 Sickness scores (0-4; mean ± SE).
Calves were vaccinated with FI BRSV (group I), MLV BRSV (group II) or sham vaccinated (group III) and challenged with BRSV on day 0.

Table 4.1 Arterial oxygen tension (PaO_2, mm Hg) and lung lesion scores (mean % pneumonic lung area from dorsal, ventral and cross sectional views).
Calves were vaccinated with FI BRSV (Group I), MLV BRSV (Group II), or control vaccine (Group III). Calves were aerosol challenged with BRSV on day 0.

	PaO_2 mm Hg				lesional lung score
	day 0	day 4*	day 5	day 6†	
Group I	[a]94.9	63.6	nd		41
	98.7	100.4	nd	66.9	7.7
	89.7	86.4	nd	66.2	10.3
	107.7	93.7	nd	77.9	4.6
Group II	[a]98.2	53	54		40.8
	106.8	73.3	nd	61.4	16.2
	93.7	76.7	57	55.5	28.2
	96.1	95	nd	75.7	5.1
Group III	[b]90.1	49.1			28.5
	[a]89.1	69.6	48.0		33.4
	104.6	72	nd	64.9	23.8
	93.9	58	nd	nd	26

[a]calves euthanized or [b]dying on day 5. nd = not done. *† Significant decreases in PaO2, pooled data (*P<0.003; †P<0.02).

Figure 4.4 Thoracic radiograph of a calf (group III) 7 days after BRSV challenge. There is a diffuse anterioventral pattern of patchy consolidation (large black arrow). The decending aorta (arrowhead) and the dorsal edge of the lung (small black arrows) are clearly defined, indicative of pneumomediatinum and pneumothorax respectively. There is also subcutaneous emphysema (white arrow).

4.4.2 Virus Shedding

Nasal virus shedding was detected in all calves for a minimum of 4 consecutive days, beginning in all but one calf (group I) on day 2. BRSV was isolated from 2 calves

on day 7 and none of the 8 calves on day 8. There were no significant differences in either the titre or duration of nasal virus shedding between vaccine groups. When calves were stratified into two groups based on sickness score (n = 6 per group), higher titres of nasal virus shedding on days 2 and 3 were associated with a subsequent SS ≥ 2.5 (day 2, P<0.005; day 3 P=0.054) (Figure 4.5). These six calves also had the highest percent lesional lung scores. BRSV was isolated from the lungs of calves necropsied on day 5, but not day 8. BHV-1, PI_3, and BVD viruses were not isolated from the nasal swabs or lungs.

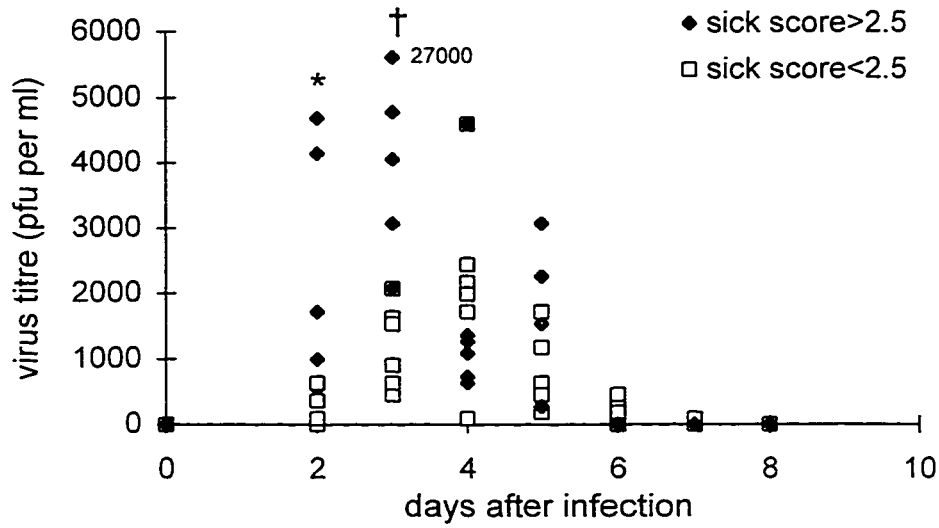

Figure 4.5 Nasal BRSV shedding (pfu/ml) with calves stratified according to peak sickness score (SS).
* Significant difference (P<0.005) between SS>2.5 (n=6) and SS<2.5 (n=6).
†Difference not significant (P=0.054, Mann-Whitney U test.

4.4.3 Antibody Responses

All calves were seronegative by VN assay for BRSV prior to vaccination. At challenge, all calves in group I had high titres of BRSV specific IgG as determined by ELISA (Figure 4.6), and 3 had low VN titres (Table 4.2). ELISA titres on day 0 for

calves in groups II and III remained unchanged from pre-vaccination values although one calf in group I did have pre-challenge VN titre of 1:18. In group I (n = 3), anamnestic responses were evident in both ELISA and VN assays on day 6. All surviving calves in group II (n = 3) seroconverted, as measured by both VN and ELISA assays, typical of a primed response. Only one of the remaining 2 calves in group III seroconverted as measured by VN assay, on day 8.

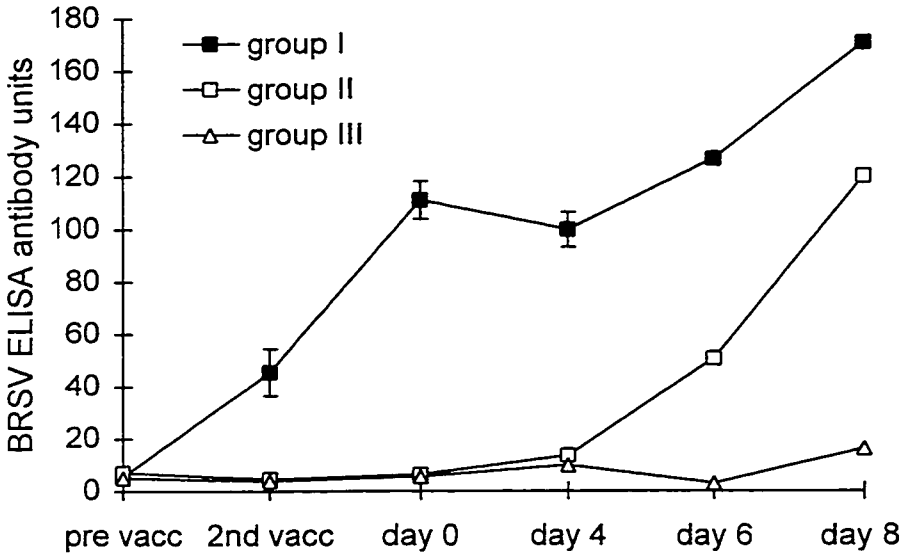

Figure 4.6 BRSV specific IgG as determined by ELISA (ELISA units, mean ± SE) Calves were vaccinated with FI BRSV (Group I), MLV BRSV (Group II), or control vaccine (Group III) on days –55 and –35 and aerosol challenged with BRSV on day 0.

4.4.4 Intradermal Skin Testing

Histologically, the response to intradermally inoculated BRSV on day 0 in 3 of the calves in group I was characterised by a marked infiltration of neutrophils with few eosinophils and moderate oedema at both 24 and 48 hr. There was mild influx of monocyte/macrophages in 2 calves at 48 hr. The fourth calf in group I and calves in

group II had undetectable or very mild inflammatory responses that were not different from the calves in group III that received the control vaccine. An immediate (within 30 minutes) wheal formation was noted in 3 of the calves in group I on day 0. When the intradermal inoculation was repeated in all 8 surviving calves on day 7, wheals ranging from 1.5-2.5 cm diameter were observed within 10 minutes in 2/3 calves in group II. A milder reaction (≤ 1 cm) was observed to the control antigen in these calves. Histologically, the 24 hr intradermal response of all calves was similar to the response observed after the day 0 inoculation.

Table 4.2 Geometric scores of BRSV neutralizing antibody titres.

	day of experiment					
	-55	-34	0	4	6	8
Group I	0	0	2	2		
	0	0	2	2	8	11
	0	0	0	1	2	6
	0	2	3	2	5	8
Group II	0	1	3	2		
	0	0	0	0	2	6
	0	0	0	0	6	12
	0	0	0	2	6	7
Group III	0	0	0	0		
	0	0	0	0		
	0	0	0	0	0	0
	0	0	0	0	0	3

Calves were vaccinated with FI BRSV (Group I), MLV BRSV (Group II) or control vaccine (Group III) on days -55 and -34 and challenged with BRSV on day 0. Scores were assigned as described in 3.3.3.

4.4.5 Lymphocyte Proliferative Responses

The calves in group I had uniformly high vaccine-induced LP responses to BRSV antigen that were significantly different from pre-vaccination values ($P<0.03$) and from the other groups ($P<0.015$) (Figure 4.7). On day 8 all surviving group II and III calves had low to moderate LP responses.

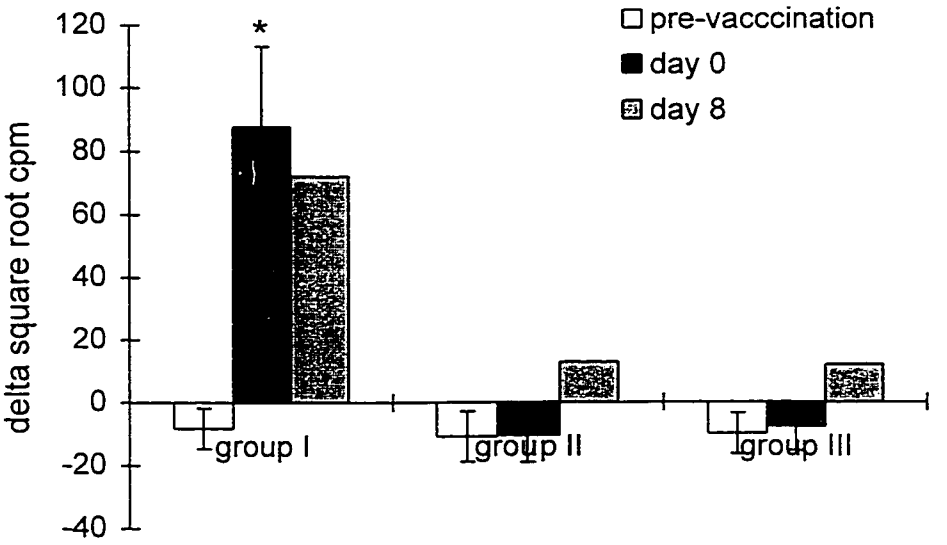

Figure 4.7 Lymphoproliferative responses (LP) to BRSV (mean ± SE). Calves were vaccinated with FI BRSV (Group I), MLV BRSV (Group II), or control vaccine (Group III). * Significant LP response over prevaccination values ($P<0.03$) and significantly different from other groups ($P<0.015$, Mann-Whitney U test).

4.4.6 Cell Mediated Cytotoxic Responses

Cytotoxic responses could not be demonstrated in PBMC throughout the experiment (data not shown). BAL cells collected on day 8 from two calves in each of groups II and III mediated moderate cytotoxic activity (22-68%) restricted to BRSV

infected autologous targets (Table 4.3). Marginal levels of cytotoxic activity (8-14%) were mediated by BAL cells from two calves in group I and one calf in group II.

Table 4.3 Percent Cytotoxicity mediated by BAL cells collected on day 8 after BRSV challenge against autologous and heterologous dermal fibroblast targets.

	Autologous		Heterologous	
	BRSV+	BRSV-	BRSV+	BRSV-
Group I	8[a]	0.3	2	-2
	14	0.3	7	1
	4	5	-5	-1
Group II	9.2	0.4	-0.9	-3
	68	-1	-1.9	2.3
	43.1	1.5	0	2.9
Group III	22	0.5	3.7	10
	38	0	3	-1.6

Calves were vaccinated with FI BRSV (Group I), MLV BRSV (Group II) or control vaccine (Group III) [a]Percent specific release at E:T ratio 80:1.

4.4.7 Pulmonary Pathology, Histopathology and Immunohistochemistry

All calves necropsied on day 5 had similar lesions. The lungs were oedematous and the caudal lobes were voluminous with rounded margins and multiple 2-5 cm bullae (Figure 4.8). Large (15-20 cm), dissecting, haemorrhagic, bullae were present in two calves (groups I and III), one of which also had extensive pneumomediastinum (group I). In all calves, there was cranioventral consolidation and atelectasis, with extensive subpleural emphysema and diffuse, moderate interlobular oedema and emphysema.

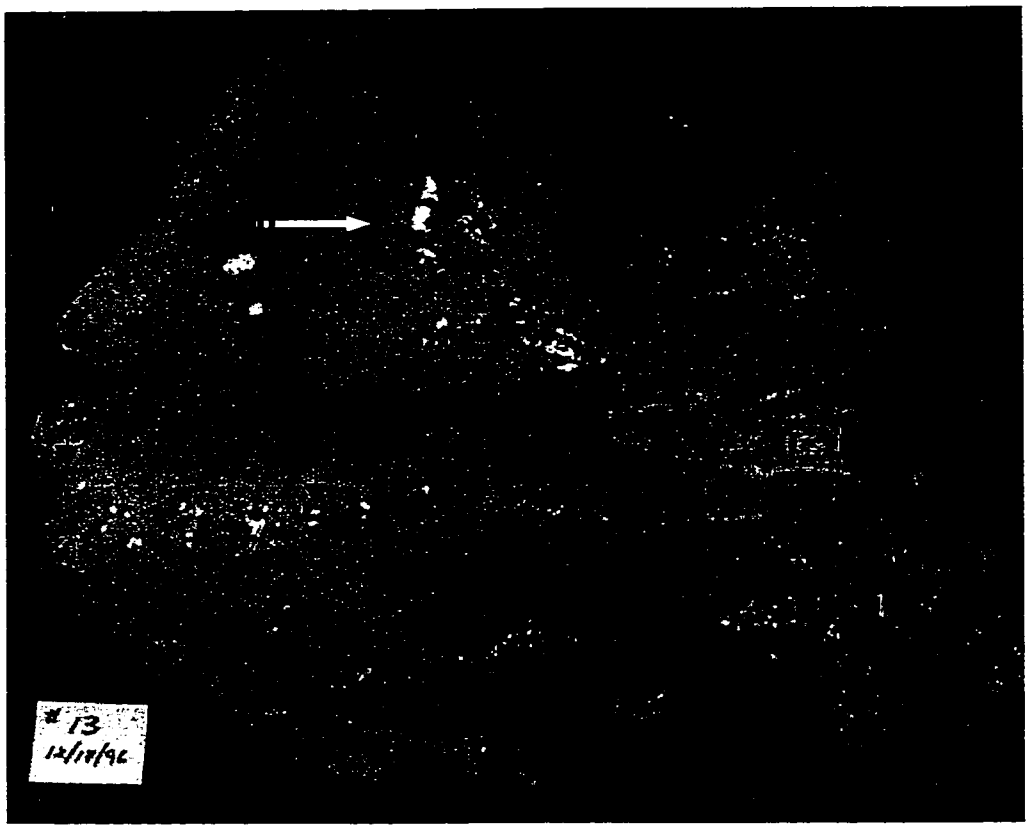

Figure 4.8 Lungs from a calf that received a sham vaccine (group III) 5 days after aerosol inoculation with BRSV.
There are dark, depressed atelectatic cranioventral lesions (large black arrow). The dorsal lobes are voluminous with large hemorrhagic (large white arrow) and several smaller bullae (small black arrows).

The gross lesions in the calves necropsied on day 8 were variable but could be described in two groups. In the two calves from group III, and one of the calves from group II, the distribution and extent of the cranioventral consolidation was the same as that seen in calves euthanized on day 5, but less confluent. The caudal lobes were moderately hyperinflated, with 2-15 cm bullae present in two calves (groups II and III), one of which had also had marked pneumomediastinum, peri-renal retroperitoneal emphysema and subcutaneous emphysema (group III). The other two calves in group II and the 3

calves in group I had multifocal to locally extensive cranioventral consolidation. Hyperinflation of the dorsal lobes was mild or absent and two calves (groups I and II) had a 3 cm emphysematous bulla. The pneumonic lung area scores (Table 4.1) (dark red depressed areas) varied considerably within groups except for the control calves (group III), which clustered around a mean of 28% (range 23.8-33.4%). Three calves in group I had considerably less pneumonic lung area than the controls (4.6-10.5%), while the fourth calf, euthanized on day 5, had the most extensive consolidation recorded (41%). The calves in group II were distributed evenly throughout the range of values recorded (5.3- 40.8%). Calves that were euthanized on day 5 had the highest percentage pneumonic lung area in all groups.

The calves necropsied on day 5 all had histologic lesions of a severe subacute bronchointerstitial pneumonia characterised by extensive necrosis of bronchial and bronchiolar epithelium. Interstitial and interlobular emphysema was extensive and severe. Within areas of consolidation and atelectasis, the bronchiolar lumens were occluded with exfoliated and necrotic epithelial cells and neutrophils and macrophages. BRSV antigen was readily demonstrated in these calves by immunohistochemistry (IHC) (Figure 4.9). By day 8, bronchiolar lumens in affected lobules were occluded by eosinophillic inflammatory debris in which rare foci of BRSV antigen were detected by IHC, but there was extensive regeneration of airway epithelium, with focal type II pneumocyte proliferation and nascent bronchiolitis obliterans. Histologic evidence of bacterial involvement was absent except for sections from one middle lobe of one calf (group II). Two histologic features were unique to the group I calves; there was a more marked infiltration of plasma cells and lymphocytes in the bronchial submucosa in two

of the calves, and a mild to moderate peribronchial eosinophil infiltrate was present in all group I calves.

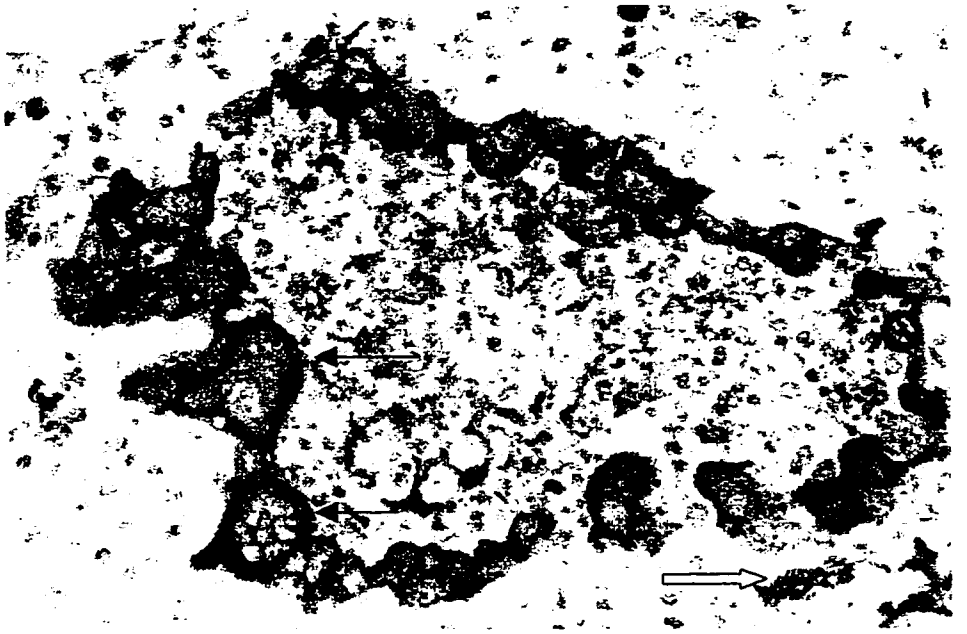

Figure 4.9 Photomicrograph of a cranial lung lobe of a calf that received a control vaccine (group III) and died 5 days after aerosol infection with BRSV. Positive immunohistochemical staining for BRSV antigen is seen in syncytial cells in the bronchiole (small arrows) and multifocally in the alveolar epithelium (white arrow) The bronchiolar lumen is occluded by necrotic epithelium and neutrophils and the lobule is atelectatic.

An untyped Mycoplasma sp. was isolated from the lung of one calf from each of groups II and III. Pasteurella multocida was isolated from one lobe of one group II calf euthanized on day 8. Bacterial pathogens were not isolated from other lobes of this calf or from other calves.

4.5 Discussion

To our knowledge, this is the first report of experimental reproduction of severe clinical disease similar to that encountered in outbreaks of naturally acquired BRSV infection. Moderate to severe clinical disease was observed in all 12 calves, resulting in the death or euthanasia of four calves on day 5. This is the same mortality rate (25%) observed in the outbreak of respiratory disease from which the Asquith challenge virus originated (unpublished data), and is consistent with reports of other BRSV outbreaks (Pirie, 1981; Ellis, 1996c). There are three previous reports of experimental BRSV infections that produced significant clinical disease, all using combined intranasal, intratracheal inoculation of a low passage field isolate for 4 consecutive days at up to a 10 fold higher infectious particle daily inoculation than used in this study (Bryson, 1983; Belknap, 1991; Ciszewski, 1991). The challenge virus in the present study was passaged in neonatal calves, rather than cell culture, which may account for its' apparent increased virulence. It is thought there is a rapid selective loss of virulence on cell culture passage (Kimman, 1990; Belknap, 1995; van der Poel, 1996), however the factors responsible for passage-associated attenuation are unknown. Synergism with recognised pathogens was ruled out.

Calves vaccinated with the formalin inactivated, alum precipitated vaccine had an earlier onset of pyrexia and respiratory distress, and faster resolution of clinical respiratory disease, in surviving calves, than did control calves or those that received MLV vaccine. Similarly, although details of clinical findings are scant, a high percentage of children that received the lot 100 vaccine required hospitalisation when subsequently infected with HRSV (Kim, 1969), however, in contrast to the calves in

this study, they experienced an apparent overall enhancement of disease rather than an earlier onset and resolution. This may suggest a different pathogenic mechanism, but could also be due to differences in the severity of the challenge, as all of the unvaccinated calves experienced severe disease, whereas most unvaccinated and infected children developed only mild symptoms. The FI vaccine in this experiment appears to have induced disease enhancing as well as some partially protective immune responses. The early acute clinical disease was not a non-specific reaction to the cellular components in the challenge inoculation, as has been demonstrated in rodent RSV models of vaccine enhanced disease (Piedra, 1993), as the control calves vaccinated with uninfected tissue culture components would then have responded similarly, but did not in our study. The immediate skin reactions noted in 3/4 calves given FI vaccine suggests that a pulmonary type I hypersensitivity reaction may have been partially responsible for the early enhanced clinical disease. The lack of immediate clinical signs at aerosol challenge, as observed in Micropolyspora faeni-induced bovine pulmonary hypersensitivity (Wilkie, 1976), does not preclude involvement of a type I hypersensitivity, as there may have been insufficient antigen in the original inoculation and viral multiplication may have been required to elicit a detectable reaction. A pulmonary Arthus reaction has also been proposed to explain the enhanced disease observed in the recipients of the lot 100 vaccine (Murphy, 1986). The high titres of non-neutralising antibodies induced by the lot 100 vaccine were suspected of mediating this phenomenon in the immunised children (Murphy, 1986). Likewise, in this study, acute respiratory distress was highly correlated with the presence of non-neutralising antibodies at the time of virus exposure. The marked vasocentric neutrophil infiltration

24 hr following intradermal inoculation in 3/4 calves that received FI vaccine was typical of an Arthus reaction.

The FI vaccine induced a strong BRSV-specific lymphocyte proliferative response. This is in contrast to the low or absent responses of the calves given the MLV vaccine, or the control unvaccinated calves, either before or after challenge. These results are consistent with previous reports in calves following infection or similar types of vaccination (Taylor, 1987; Ellis, 1992a), and with the finding that children given the lot 100 vaccine, irrespective of subsequent natural exposure, had 5 fold greater HRSV-specific LP responses than children who had not received the lot 100 vaccine and had undergone natural HRSV infection (Kim, 1976). It has been proposed that this exaggerated response was involved in the enhanced disease in the vaccinated children (Kim, 1976). This is supported by a study in naturally infected children, where a higher incidence of bronchiolitis and post-recovery episodes of wheezing were found in children with high LP responses (Welliver, 1979). High LP responses in the calves given FI vaccine may suggest the induction of a high number of BRSV specific T-cells, or perhaps a Th1 to Th2 shift. This response correlated with the uniformly high prechallenge antibody responses, and, apparently, earlier onset of acute respiratory disease following exposure to BRSV. The two most severely affected calves given FI vaccine also had a more marked mononuclear peribronchiolar infiltration.

The gross lesions seen in the lungs of calves in this experiment are consistent with previous observations made following natural BRSV infection (Pirie, 1981; Van Den Ingh, 1982; Kimman, 1989c; Bryson, 1993; Ellis, 1996c). The cranioventral distribution of red, collapsed lobules is also consistent with previous results of other

experimental challenges (Bryson, 1983; Belknap, 1991; Belknap, 1995; Otto, 1996), but extensive dorsocaudal pulmonary oedema, emphysema and bullus formation, with subcutaneous emphysema, have only been previously described following natural infection. Several immunopathologic mechanisms, including types 1 and 3 hypersensitivities, have been proposed to explain this severe form of BRSV induced disease (Kimman, 1989c; Kimman, 1989d; Stewart, 1989c; Kimman, 1990; Baker, 1991). In this experiment, the sickness scores and respiratory rates of the most severely affected calves peaked on days 4-6, which in the calves that were vaccinated, coincided with the rise in antibody titres. However, 2/4 control calves died on day 5 and only one of the remaining 2 had detectable serum neutralising antibody by day 8. Therefore, it is unlikely that an antibody induced hypersensitivity (i.e. type 1, or 3), contributed to the observed pathology. Moreover, the absence of a detectable DTH reaction, excessive pulmonary infiltration or significant in vitro lymphocyte proliferative response post challenge, speaks against the involvement of a type 4 hypersensitivity. The development of severe pulmonary emphysema was independent of treatment group or any of the immune response parameters examined in this experiment. The early acute respiratory disease seen in the calves that received FI vaccine appeared to be a separate disease process.

Various proinflammatory mediators (histamine, leukotriene C-4, eosinophil cationic protein, IL-6, IL-8, IL-11, RANTES and TNF-alpha) capable of altering local inflammation or airway physiology have been described in the nasal secretions of RSV infected children (Welliver, 1981; Volovitz, 1988; Colocho Zelaya, 1994; Noah, 1995; Einarsson, 1996; Becker, 1997), and have been shown to be induced by HRSV

infection of epithelial cells (Noah, 1993; Arnold, 1994; Einarsson, 1996; Becker, 1997; Saito, 1997). Similar findings have not been described for BRSV infection, but experimental BRSV infection has been shown to result in increased airway resistance and airway sensitivity to histamine (LeBlanc, 1991). A virally induced, chemokine mediated airway hyperresponsiveness, leading to severe bronchoconstriction and secondary widespread emphysema, may be an important pathologic mechanism contributing to the lesions in the dorsal lung lobes.

The higher early nasal virus shedding seen in the calves that had the highest sickness scores suggests that the ability to control initial viral replication is associated with disease sparing (Stewart, 1990). We were unable to demonstrate a statistically significant influence of either vaccination strategy on nasal virus shedding, but the lower pneumonic lung scores seen in 2/4 calves that received MLV and 3/4 calves given FI vaccine, may indicate varying degrees of partial pulmonary protection, as expected with a systemic vaccination (Kimman, 1989e).

The immune mechanisms that are responsible for controlling RSV replication in calves and children are not fully understood. In this experiment, low BRSV VN antibody titres prior to challenge appeared to have no beneficial effect, as two of the four calves with pre-challenge VN titres were euthanized on day 5. The rapid rise in VN titres in all vaccinated calves between days 4-6 coincided with a drop in nasal virus recovery, however the same pattern was seen in the two surviving control calves, only one of which seroconverted by day 8, indicating that recovery from a severe challenge can occur independently of a serum antibody response, but does not preclude a possible

benefit from neutralising antibody. Depletion of $CD8^+$ T-cells in BRSV infected calves results in enhanced pulmonary pathology and persistent infection in the face of a normal antibody response (Taylor, 1995b). However, passive serum antibody has been shown in calves (Kimman, 1988; 1989e; Belknap, 1991) and children (Groothuis, 1993) to provide amelioration of BRSV and RSV induced LRT disease, but have no or marginal effect on infection or URT shedding. Together, these findings suggest serum neutralising antibody may attenuate pathology, but virus clearance is dependent on cell mediated mechanisms, most likely $CD8^+$ CTL activity, and our results are consistent with these findings. The uniformly high titres of non-neutralising antibodies that were induced by the FI vaccine in all group I calves are similar to previous reports in cattle using various commercially available inactivated vaccines, rodent models of RSV following FI vaccination, and the children receiving the original lot 100 vaccine (Murphy, 1986; 1989; West, 1997a). These antibodies are considered to be non-protective (Taylor, 1992; Siber, 1994), and in this experiment had no consistent disease-sparing effect, however there is no evidence that these antibodies contributed to increased pulmonary pathology even though they were uniformly associated with the observed early acute respiratory disease.

We were unable to demonstrate BRSV specific CTL activity in PBMC of calves recovering from BRSV infection. The requirement for $CD8^+$ T-cells for virus clearance in calves (Taylor, 1995b) and the influx of these cells into the lungs following infection (Thomas, 1996), suggests this is a critical immune effector mechanism in calves. Ex-vivo $CD8^+$ T cell mediated CTL activity has been previously reported using PBMC

obtained from calves and lambs experimentally infected with BRSV (Sharma, 1995; 1996b; Gaddum, 1996a) with similar CTL activity demonstrated in lung lavage cells. Our inability to repeat these results may be related to the severity of our challenge compared to previous work. CTL activity has been demonstrated in peripheral blood of infants with mild RSV infections but was absent in those with severe infections (Isaacs, 1987). In the present experiment, the demonstration of cellular cytotoxicity, restricted to autologous BRSV infected targets, in BAL cells indicates such activity was induced, but possibly due to rapid trafficking to the lung in the face of severe infection, was not detectable in the peripheral circulation. The low levels of cytotoxicity in the calves vaccinated with the FI vaccine may be due to the decreased severity of the lung lesions in these calves, or may suggest an alternative means of virus clearance. It was anticipated that the MLV vaccine would prime for a more effective CTL response than the FI virus. In mice, priming with live RSV or F protein vaccinia recombinants induces CTL activity, whereas inactivated virus or FG glycoprotein vaccines in an alum adjuvant do not (Nicholas, 1990). The MLV vaccine used in this experiment was a very low dose that induced a detectable antibody response in only one of four calves with no detectable proliferative response or DTH. The only evidence for priming in these calves was the rapid secondary antibody response relative to the control calves. The small dose in this vaccine may have primed for a CTL response, but the resulting CTL precursor frequency may have been too low to be protective. It remains to be seen whether a larger parenteral dose of MLV vaccine that induced detectable post-vaccinal cellular and antibody responses, would be more protective than the dose used in this experiment.

All of the calves in group I had high LP responses, inconsistently associated with a reduction in disease. Likewise, in children, no correlation was found between LP responses induced by vaccination or natural infection and protection from subsequent infection (Fernald, 1983; Welliver, 1994), leading to speculation that this assay of cellular immune stimulation may not be measuring the protective effector mechanism (Welliver, 1994). The phenotype of the proliferating cell population in PBMC from cattle that were administered vaccine containing either MLV or inactivated BRSV was $CD4^-$ (Ellis, 1992a). The established importance of $CD8^+$ rather than $CD4^+$ T cells for viral clearance in cattle (Taylor, 1995b), may partially explain this lack of correlation, provided the same conditions apply in humans.

In conclusion, we have demonstrated that experimental infection of calves using lung lavage fluid from a BRSV-infected calf can induce clinical disease and pathology typical of severe natural infections. The calves that received the FI vaccine had an earlier onset of clinical disease, compared to the control calves or those vaccinated with MLV, that was possibly immune mediated. Protection from infection was not induced by either a low dose MLV, or high dose FI alum precipitated BRSV vaccine. Moderate but inconsistent protection from pulmonary pathology was observed in the calves that received the FI vaccine, and to a lesser extent, the MLV vaccine. Low titres of neutralising antibody were not protective at challenge, nor was serum antibody necessary for recovery. The protective immune mechanism was not identified. The development of the most severe pulmonary lesions, consisting of dorsal emphysema was independent of treatment group and the immune response parameters examined.

5 THE EFFECT OF MODIFIED-LIVE VIRUS VACCINE ON RESPIRATORY DISEASE ASSOCIATED WITH BOVINE RESPIRATORY SYNCYTIAL VIRUS INFECTION IN CALVES

5.1 Abstract

The effect of vaccination with mixed modified-live (MLV) vaccines containing bovine respiratory syncytial virus (BRSV) on BRSV induced respiratory disease in calves was investigated. Two to four week old colostrum fed, BRSV-seronegative-calves were vaccinated with either, 2 doses of MLV delivered either intramuscularly (IM) or intradermally (ID), with a single dose IM of MLV or MLV with an adjuvant (four groups, n = 6 per group), or remained as unvaccinated controls (n = 9). Calves were challenged 3 weeks after the second or only vaccination with lung wash obtained from a new-born calf infected with a field isolate of BRSV. Moderate to severe disease occurred in all control calves. Mild to moderate disease was observed in some calves in all vaccine groups, with the onset of symptoms in some calves occurring 1-2 days earlier than in unvaccinated calves. A significant overall reduction in clinical disease was observed in groups that received two vaccinations. Virus shedding and pulmonary pathology were significantly reduced in all vaccinated groups. The group that received a single vaccination with unadjuvanted MLV had reduced protection, correlated with reduced vaccine-induced BRSV specific cell-mediated responses. Virus clearance in the

control calves appeared dependent on cell mediated cytotoxicity. Protection afforded by vaccination was associated with accelerated antibody and cytotoxic responses but other mechanisms may also be involved.

5.2 Introduction

Bovine respiratory syncytial virus is a ubiquitous and important respiratory pathogen of cattle that can cause severe disease in naive animals of all ages, but primarily affects young calves in recurrent seasonal outbreaks (Kimman, 1990; Van der Poel, 1994; Baker, 1997). Clinical disease is characterised by pyrexia, coughing, and tachypnea, often progressing rapidly to dyspnea and occasionally to death (Bryson, 1983; Baker, 1997). BRSV is considered to be one of the predisposing viral etiologies leading to secondary Pasteurella sp. infections in bovine respiratory disease complex (BRD), but often secondary infections are absent in acute deaths attributable to BRSV (Kimman, 1989c; Bryson, 1993).

Several commercial BRSV vaccines are currently in use in cattle (Baker, 1997), but there is little published data regarding the efficacy of BRSV vaccination. Field studies have shown a variable effect on clinical disease, treatment rate or productivity of young calves, weaned animals, and cows attributable to BRSV vaccination, perhaps related to variations in virus prevalence or prior exposure (Verhoeff, 1984; Thomson, 1986; Frankena, 1994; Van Donkersgoed, 1994; Ferguson, 1997). In previous experimental infection models, the inability to induce significant clinical disease has limited the extrapolation of the effects of vaccination described in these experiments to the field situation (Mohanty, 1981; Kimman, 1987a; 1989e; Taylor, 1987; 1989; 1997). In addition, there are anecdotal reports of enhanced disease occurring in

vaccinated cattle that have not been documented in the literature or adequately tested, and warrant experimental investigation.

Recent experimental evidence suggests that virus clearance in a primary BRSV challenge is critically dependent on cellular immunity mediated by CD8+ T-cells and apparently independent of the antibody response (Taylor, 1995b). The immune mechanisms responsible for the disease reduction in previously exposed or vaccinated animals are not fully understood (Kimman, 1989e; 1990; Taylor, 1995b; Baker, 1997). High titres of passive antibody have been shown to have a disease sparing effect (Belknap, 1991), and reinfection of calves with or without prechallenge antibody titers is associated with anamnestic antibody responses and reduced virus shedding compared to the primary infection (Kimman, 1987a; 1989e), suggesting that vaccine induced prechallenge or anamnestic antibody, in addition to accelerated cell mediated responses, may also contribute to protection from severe disease in vaccinated animals. The objectives of this study were to investigate the potential of a mixed vaccine containing modified live virus BRSV to protect calves from clinical disease and pulmonary pathology when exposed to a virulent experimental BRSV challenge. The vaccine was used according to label instructions (two doses delivered intramuscularly), as a single dose or was delivered intradermally at one quarter of the dose. For comparative purposes a mixed MLV vaccine containing an adjuvant was administered according to label instructions as a single intramuscular dose. Vaccine induced cell mediated and antibody responses, and the kinetics of these responses post challenge, were determined and correlated with the kinetics of virus clearance in attempting to determine which immune responses are associated with the protection afforded by vaccination.

5.3 Materials and methods

5.3.1 Experimental Design

Thirty-three neonatal calves were obtained from a local dairy. They were removed from their dams at birth, fed 2 liters of a pool of BRSV antibody-negative colostrum and transferred to isolation facilities at the WCVM within 12 hours of birth. Calves were randomly assigned to one of 4 test (vaccinated) groups (n = 6) or 1 control group (n = 9) with the following designations: Group 1 calves received no treatment. Groups 2-3 were vaccinated with multivalent product containing modified-live BRSV, BHV-1, PI3, BVD (Bovishield 4, Smithkline Beecham, West Chester, PA). Group 2 calves were vaccinated according to label diredtions, twice intramuscularly at 3 week intervals with 2 ml (MLV 2x IM); Group 3 calves received two doses of 0.5 ml (0.25 ml administered in 2 cervical sites) intradermally at three week intervals (MLV 2x ID); Group 4 received a single 2 ml as a single intramuscular vaccination (MLV 1x); Group 5 received a single 2 ml intramuscular vaccination of a multivalent vaccine containing modified-live BRSV, BHV-1 PI3, BVD with a saponin adjuvant (Pyramid 4, Fort Dodge, Fort Dodge Iowa) (MLV Adj 1x). Calves received their first (or only) vaccination at 2-4 weeks of age and were challenged three weeks after the last vaccination. The challenge model utilized lung wash obtained from a newborn calf infected with BRSV as previously described. The lung wash was confirmed negative for bacterial contamination, Mycoplasma spp. and bovine herpes-1 (BHV-1), parainfluenza-3 (PI_3), BVD viruses using standard diagnostic methods. Aerosolized lung wash containing ~$10^{4.9\text{-}5.2}$ pfu BRSV was delivered using an ultrasonic nebulizer (Ultra-Neb 99, Devilbiss, Somerset, PA) and face mask on day 0. Thoughout this study the days refered to as days 0 though

8 refer to days after challenge. Calves were euthanized by barbiturate overdose (Euthanyl Forte, MTC Pharmaceuticals, Cambridge, Canada) 8 days after challenge (day 8) if not euthanized earlier for humane reasons.

5.3.2 Clinical Assessment

Clinical assessments were made at the same time each morning by a veterinarian blinded as to treatment group. Scores for heart rate, respiratory rate, respiratory effort, rectal temperature, cough, nasal discharge and depression were assigned as described in appendix 1 and the sum of these scores was used as the cumulative clinical score (CCS). The sickness scores (SS) were assigned using combined objective and subjective observations, after all other scores were assigned, using a modification of a scoring system previously described (Babiuk, 1987, and Richard Harland, Biostar Inc. Saskatoon, Saskatchewan). These scores were a reflection of the severity of clinical disease as it would be assessed in the field, and included the following descriptive and predictive statements: SS = 0.5, an animal for which there is a suspicion of disease, but would not be treated or easily noticed, SS = 1, a sick animal that, in clinical setting, would probably be treated and be expected to recover; SS = 2, an obviously sick animal that in a clinical setting should already have been treated, but recovery is not certain; SS = 3, an animal with severe disease, unlikely to recover even with treatment, and for which the most appropriate course of action is euthanasia; SS = 4, a non-responsive moribund animal. Intermediate scores were assigned at the discretion of the veterinarian. Adventitial lung sounds were recorded but not scored. Arterial blood gas tensions were measured on samples collected from the caudal thoracic aorta (Will,

1972) on day 6, using a blood gas analyser (Model 288, Ciba-Corning, Medfield, MA). Values were corrected for rectal temperature.

5.3.3 Bronchoalveolar Lavage

Bronchoalveolar lavage (BAL) was done on 6 group 1 calves and 3 calves from each of groups 2-5 on days 0, 5, 6, and 8. In addition 3 group 1 and 3 group 2 calves were lavaged on days 0, 5, and 8. The BAL was performed on standing calves in a manner similar to previous descriptions (Fogarty, 1983; Kimman, 1986). After nasal instillation of topical 2% lidocaine, a sterile, lubricated, 65 cm x 0.25" inside diameter (0.375" outside diameter) PVC naso-tracheal tube was passed through the larynx. A sterile, 120 cm, 16 French PVC tube was passed through the naso-tracheal tube until it gently lodged in a small airway, and was then withdrawn slightly. Lavage was performed with three aliquots of 50 ml of 0.01M PBS, with 10-25 mls recovered on each wash. Aliquots of the first wash were always used for virus isolation and antibody determination, the remaining washes were pooled for use in cellular cytotoxicity assays. BAL supernatants used for antibody assays were centrifuged at 500g to remove debris and 1 mM phenyl-methyl-sulphonyflouride (PMSF, Sigma, St Louis, MO) was added to inhibit proteolysis prior to storage at -70°C (Elson, 1984).

5.3.4 Collection of Specimens

Nasal swabs were obtained on day 0, and 4-8 for mucosal antibody detection. Swabs were saturated with nasal secretions, placed in 1ml of ELISA working buffer (0.01M phosphate buffer, pH 7.2, with 0.75M NaCl, 0.05% Tween 20) with 0.2% gelatin and 1 mM PMSF and stored at -70°C.

Nasal swabs were taken on days 0-8 and deep bronchial swabs were taken at necropsy on day 8 for virus isolation. Swabs were placed in 1 ml of transport medium consisting of DMEM supplemented with 20% FBS (Gibco BRL, Grand Island, NY), 0.5M, 50mM HEPES (N-2- hydroxyethylpiperazine-N-2-ethanesulfonic acid), 200 μg/ml sodium penicillin G, 200 μg/ml streptomycin sulphate and 0.5 μg/ml amphotericin B, and frozen at -70°C. Equal volumes of BAL fluid were added to a 1.0M $MgSO_4$ transport medium and stored at -70°C (Fernie, 1980).

Serum samples were obtained for antibody assays prior to vaccination and on days 0, and 4-8. Blood was collected in acid citrate dextrose and heparin tubes for blastogenesis and IFNγ assays respectively, prior to vaccination and on days 0 and 8. Lavage specimens and deep bronchial swabs taken at necropsy on all calves were inoculated onto cysteine heart agar, blood agar and Hayflicks broth. Identification of bacterial and mycoplasmal pathogens was done using standard diagnostic techniques.

5.3.5 Virus Isolation and Titration

Virus shedding was quantitatively determined by a micro-isolation plaque assay, with a maximum calculated sensitivity of 10 pfu per ml, as previously described (West, 1998), but with minor modifications. Aliquots of nasal swab transport medium and BAL fluid were inoculated in serial dilutions into 96 well plates and $1x10^4$ EBTR cells/well were added. The plates were incubated for 3 hrs at 37°, the medium pipetted off and replaced then incubated for a further 48 hr before the plates were fixed with 80% acetone. Viral plaques were identified by staining with an avidin-biotin immunoperoxidase technique utilising a 1:3000 dilution of a rabbit poloyclonal antisera

to RSV (Dako, Dimension Laboratories, Mississauga Canada), followed by 1:1000 biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingham, CA), avidin- biotin complex (Vectastain, Vector Laboratories, Burlingham, CA) and diaminobenzidene. Antibodies were diluted in Blotto Buffer (50 mM Tris HCL, pH 8.5, 10mM EDTA, .05% Tween 20, 1% Triton X-100, 2% skim milk) during incubations for 1 hr at 37° C.

5.3.6 Virus Neutralising and ELISA Antibody Assays

5.3.6.1 BRSV Specific Assays

BRSV specific neutralising serum antibody titres and BRSV specific IgG ELISA assays were performed as previously described (West, 1997a). BRSV specific IgM and IgA were determined using a modification of the described ELISA assay. Briefly, the plates were coated 1 hr at 37°C with BRSV antigen diluted in carbonate buffer (pH 9.5), washed and blocked with 0.2% gelatin in carbonate buffer. Samples were added and incubated at 37°C for 1 hr. The plates were washed, followed by addition of either a 1:1000 dilution of a mAb to bovine IgM (Sigma, St. Louis, MO), or a 1:300 dilution of mAb against bovine IgA (Serotec Inc, Raleigh, NC). The assays were completed using a 1:1000 dilution of biotinylated rabbit antimouse (Zymed, San Francisco, CA) incubated for 1 hr 37°C, followed by a 30-minute incubation with peroxidase labelled avidin-biotin complex (Vectastain ABC, Vector Laboratories, Burlingham, CA). ABTS was used as the enzyme substrate. Sample and antibody dilutions were made in ELISA working buffer with 0.2% gelatin. Serum samples were diluted 1:50. Nasal swabs and BAL specimens were used at a 1:2 dilution. The same serum standards were used for the IgG and IgM assay. A pool of nasal secretions from 5

naturally exposed cattle, collected 8 days after experimental reinfection with BRSV, was used for the standards in the IgA assay. ELISA units were assigned as described (West, 1997a).

5.3.6.2 ELISA for the Detection of Antibodies to Challenge Inoculum

The isotype specific assays described above were used to detect serum IgG and IgM reactive with components of the challenge lung in prevaccination and day 0 samples. ELISA plates were coated with antigen prepared by pelleting the lung wash at 100,000g for 2 hours and resuspending to a working dilution of 1:4 of the original volume in carbonate coating buffer. Convalescent serum from an unvaccinated field case of BRSV was used as a positive standard. High OD values, relative to a fetal bovine serum negative control, were obtained with this serum and test samples values were expressed as a percent OD of this standard.

5.3.7 Cellular Assays of Immune Responses

5.3.7.1 Ex-vivo BAL Cell Cytotoxicity Assays

BAL cells collected on days 5, 6 and 8 were used as effectors at an E:T ratio of 100:1 mononuclear BAL cells to target cells in a chromium release cytotoxicity assay in a manner similar to previous descriptions (Gaddum, 1996a) and as described in 4.3.8. In this experiment BAL cells were not Ficol separated. Clumps of cells were allowed to sediment out and the remaining suspended cells pipetted off, centrifuged at 200g and resuspended in RPMI with 10% FBS. Three weeks prior to the experiment, explant cultures of dermal cells were established from each animal using 6 mm skin biopsies as described in 4.3.8. Targets consisted of control and BRSV infected autologous and heterologous dermal fibroblasts.

5.3.7.2 Lymphocyte Proliferation (LP) Assay

BRSV specific lymphocyte proliferation (LP) was determined prior to vaccination and on day 0 and 8 similar to previous descriptions, by measuring tritiated thymidine incorporation in antigen-stimulated PBMC (Ellis, 1992a). Cells were cultured in round bottom 96 well plates at a density of $3x10^6$/ml and stimulated with a 1:100 dilution (previously determined to be optimal) of BRSV-infected Vero cell tissue culture lysate or uninfected control lysate. Cells were labelled after 6 days of culture, harvested onto glass fibre filters 18 hr later, and cpm determined using a scintillation counter (Model LS6000IC, Beckman Instrumentation, Palo Alto, CA). The data was expressed as a stimulation index derived from the ratio of antigen cpm/control cpm.

5.3.7.3 BRSV Specific Whole Blood IFNγ Assay

Interferon γ was determined before vaccination and on days 0 and 8, on plasma collected from heparinized peripheral blood cultures stimulated with 25 μl of heat inactivated BRSV-infected Vero cell tissue culture lysate, or uninfected control lysate, for 18 hours in 24 well plates, as previously described (Rothel, 1992). Plasma was stored at -70°C. IFNγ was quantitatively determined using recombinant bovine IFNγ (Ciba–Geigy, East Hanover, NJ) standards in an antigen capture ELISA as previously described (Ellis, 1994). Briefly, ELISA plates were coated overnight at 4 °C with a 1:4000 dilution (in carbonate buffer) of a monoclonal antibody specific for bovine interferon gamma (mAb 2-2-1, provided by The Veterinary Infectious Disease Organisation, VIDO, Saskatoon, Canada). Plasma samples were added and serially diluted (1:2) in the plate using PBS with .05% Tween 20 (PBST). Plates were incubated

for 2 hours at room temperature, washed, and incubated for one hour at room temperature with a rabbit antisera raised against bovine interferon gamma, diluted 1:400 in PBST with 0.1% gelatin (PBST-g). This was followed by a one hour incubation with biotinylated goat anti-rabbit IgG (Zymed, San Francisco, CA) diluted 1:7500 in PBST-g, and a 1/2 hr incubation with peroxidase labelled avidin-biotin complex (Vectastain ABC, Vector Laboratories, Burlingham, CA). ABTS was used as the enzyme substrate. Four calves had small amounts (< 100 pico gm/ml) of IFNγ in the control antigen stimulated cultures, and these values were subtracted from the antigen stimulated values to obtain BRSV specific IFNγ.

5.3.8 Post Mortem Analysis

Prior to euthanasia, calves were weighed. The lungs were removed, weighed, and lung volume determined by water displacement. The dorsal and ventral surfaces of the lungs were photographed. Tracings were made on acetate from the projected slides of both views, outlining both total and depressed dark red, obviously pneumonic, areas. The percentage area of lesional lung for each tracing was determined by computer software (Image 1, Universal Imaging Corporation, West Chester, PA). The mean of the two views was considered the percent lesional lung area.

Sections of each lung lobe, taken at the edge of a pneumonic area if possible, were fixed in 10% neutral buffered formalin and processed for routine histology.

5.3.9 Data Analysis

The serum, nasal and BAL antibody concentrations, clinical scores, and nasal virus titres collected over time were evaluated using a univariate two way ANOVA for

repeated measures, using treatment as a grouping factor. The overall effect of group, time and group by time interaction were considered significant if $p < 0.05$. Post hoc comparisons over time within groups were done using a one way ANOVA for repeated measures, and if the overall F was significant multiple pairwise comparisons were done using the Tukeys HSD test. Comparisons among groups were done using Tukeys HSD test for unequal N (Spjotvol and Stoline test) with significance set at $\alpha = 0.05$. Prior to analysis, the ELISA antibody values and nasal virus titres were square root transformed. The serum neutralising antibody titres were transformed by assigning geometric scores of 1 - 10 corresponding to serial dilution titres of < 1:4 to > 1:324 (West, 1997a). Differences in the sickness scores over time for each group were established using the Friedman ANOVA, and differences between the control and vaccine groups using the Kruskal-Wallis one way analysis of variance and pairwise Mann-Whitney U tests, employing Bonferroni's correction to maintain an overall $\alpha = 0.05$.

The BAL antibody, cellular cytotoxicity and virus isolation data were analysed after pooling results from all vaccine treatment groups. The day 5, 6 and 8 BAL cytotoxicity and day 5 and 6 BAL virus titres were compared among groups using the Mann-Whitney U test and two tailed exact p values. Day 6 arterial oxygen tensions (PaO_2) and post mortem percent lesional lung areas were evaluated using a one way ANOVA. When the p value was significant ($p < 0.05$), the vaccine treatment groups were compared to the control group using Bonferroni multiple pairwise t-tests with an overall $\alpha = 0.05$.

The lymphoproliferative and interferon gamma responses and sickness scores were evaluated using a Kruskal-Wallis one way analysis of variance. When the p value was significant ($p<0.05$), differences between control and vaccine treatment groups were established using multiple pairwise Mann-Whitney U tests, controlling for type one error using Bonferroni's correction to maintain an overall $\alpha = 0.05$. Regessional analyses and Pearson correlations were done for various parameters, as indicated in the text. The Spearman rank correlation was used for variables not meeting the assumptions of normality. Analyses were done using computerised software (Statistica, Statsoft, Tulsa, OK).

5.4 Results

5.4.1 Clinical Data

5.4.1.1 Clinical observations

Moderate to severe clinical respiratory disease occurred in all unvaccinated calves, with one calf, in severe respiratory distress, euthanized on day 5. Clinical disease was observed in some calves of all vaccine treatment groups. The individual clinical parameters all demonstrated a similar pattern to the combined cumulative sickness score (Figure 5.1). In the repeated measures ANOVA of the cumulative clinical scores (CCS) there was a significant effect of group ($p < .02$), time ($p < .001$) and group by time interaction ($p < .001$). The overall group effects were only significant between the control group and groups 2 and 3 (two doses of MLV IM or ID respectively) In the post hoc groupwise comparisons over time, the CCS of group 1 were significantly different from groups 2 and 3 on day 5, groups 2, 3, and 5 (one dose adjuvantd MLV) on day 6

and all vaccine treatment groups on day 7. There were no significant differences in CCS among vaccine treatment groups at any time point; however, there were differences in the time effect between groups (Table 5.1). Group 2 was the only group that did not have a significant increase in CCS over time ($p = 0.19$). The most significant clinical disease in group 5 occurred on days 2-4, whereas the pattern of clinical disease in group 4 paralleled that of the control calves. There was an overall trend for the vaccinated calves to have an earlier onset but faster resolution of clinical disease. This trend was more obvious in the rectal temperatures (Figure 5.2) than the CCS, with the temperatures of the calves in group 5 significantly higher than the control (group 1) calves on day 2 ($p < 0.05$ HSD). Only group 2 was significantly different from the control group in the overall repeated measures ANOVA, largely due to this early clinical response.

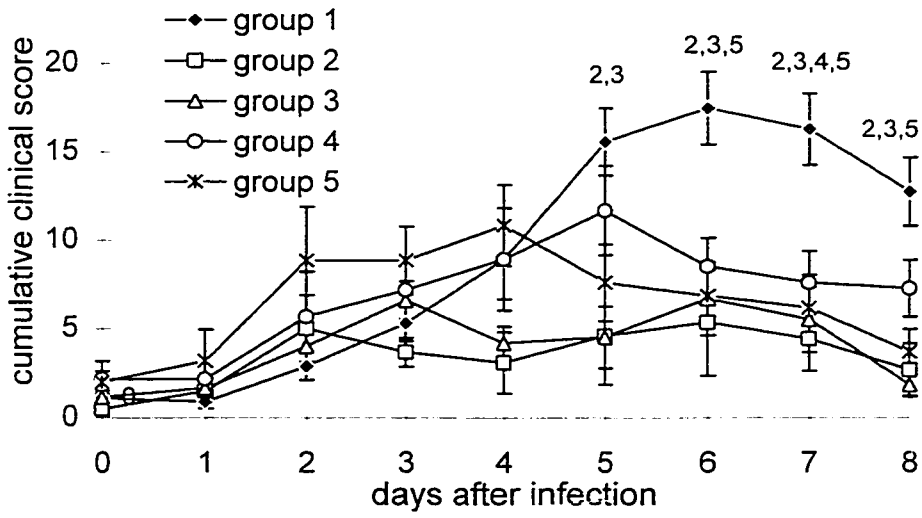

Figure 5.1 Cumulative clinical score (CCS, mean ± SE).
CCS assigned using the method described in Appendix 2 for calves that were vaccinated as described in 5.3.1 (group 1 = controls, group 2 = MLV 2x IM, group 3 = MLV 2x ID, group 4 = MLV 1x, group 5 = MLV Adj 1x), and challenged with BRSV on day 0. Data labels indicate vaccine treatment groups that were significantly different from group 1 on that day ($p < 0.05$, HSD). There were no differences between groups 2-5.

Table 5.1 Days of significant increase in Cumulative Clinical Score (CCS), by treatment group.

	Treatment Group				
day[a]	1	2	3	4	5
1	-	-	-	-	-
2	-	-	-	-	<0.01
3	-	-	<0.03	-	<0.01
4	<0.001[b]	-	-	<0.07	<0.001
5	<0.001	-	-	<0.001	-
6	<0.001	-	<0.02	<0.015	-
7	<0.001	-	-	-	-
8	<0.001	-	-	-	-

Days that the CCS for each treatment group was significantly greater ($p < 0.05$ HSD) than pre-infection (day 0) values. [a]Refers to days after infection (day 0). [b]p value, Tukey's HSD.

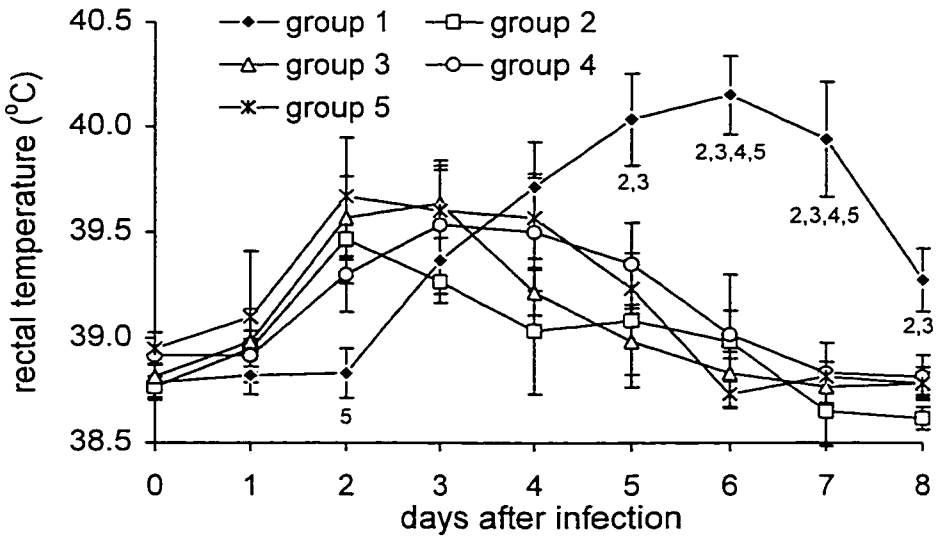

Figure 5.2 Rectal temperature (°C, mean ± SE).
Calves were vaccinated as described in 5.3.1 (group 1 = controls, group 2 = MLV 2x IM, group 3 = MLV 2x ID, group 4 = MLV 1x, group 5 = MLV Adj 1x), and challenged with BRSV on day 0. Data labels indicate vaccine treatment groups that were significantly different from group 1 on that day ($p < 0.05$, HSD). There were no differences between groups 2-5.

The sickness scores (SS) (Figure 5.3) follow a pattern similar to the CCS. These scores more accurately demonstrate the differences between the mildly and severely affected animals. The peak SS was more predictive of both day 6 PaO_2 and % lesional lung area than peak CCS (R^2 = .50 and .51 vs. .35 and .37 respectively $p < 0.001$). Significant differences among groups occurred on days 5-7. On days 5-7, groups 2, 3, and 5 and on day 6 group 4, were significantly different than group 1 (Mann-Whitney U with Bonferroni's correction). Significant differences over time were observed in groups 1, 4 and 5 ($p > 0.00001$, $p < 0.002$ and $p = .048$, respectively, Fiedman ANOVA), but not groups 2 and 3 ($p = 0.53$ and 0.14 respectively.

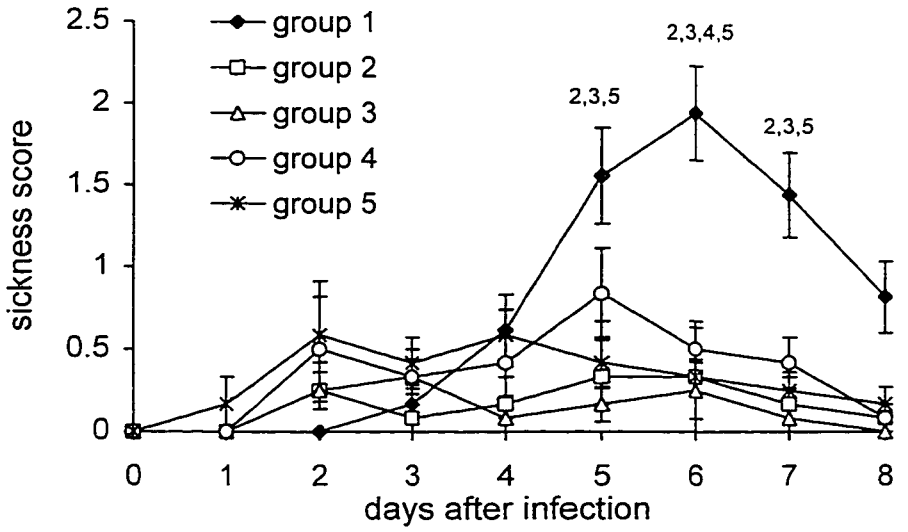

Figure 5.3 Sickness Score (SS, mean ± SE).

Calves were vaccinated as described in 5.3.1 (group 1 = controls, group 2 = MLV 2x IM, group 3 = MLV 2x ID, group 4 = MLV 1x, group 5 = MLV Adj 1x), and challenged with BRSV on day 0. Data labels indicate vaccine treatment groups that were significantly different from group 1 on that day ($p < 0.05$, Mann-Whitney U with Bonferroni's correction). There were no differences between groups 2-5.

In a field situation, all of the unvaccinated calves would have been considered sick (peak SS ≥1) with 7/9 considered to be seriously ill (SS ≥ 2), whereas in 15/24 vaccinated calves the disease was subclinical (SS≤ 0.5) and only 4/24 would have been considered seriously ill (SS = 2). The mean peak SS of all vaccine treatment groups, except group 4, in which clinical disease was observed in 5/6 calves, were significantly lower than the control group ($p < 0.05$). There were no differences between the vaccine treatment groups 2-5 ($p = 0.23$, Kruskal-Wallis).

5.4.1.2 Arterial PO_2

Vaccination was associated with a significant reduction in hypoxia relative to the control calves in treatment groups 2, 3 and 5 ($p<0.035$) but not in group 4 ($p = 0.064$) (Figure 5.4). The PaO_2 values of group 1 ranged from 33 -70 mmHg and vaccinated calves from 60 - 95, with 8 calves > 85 mmHg, or essentially normal.

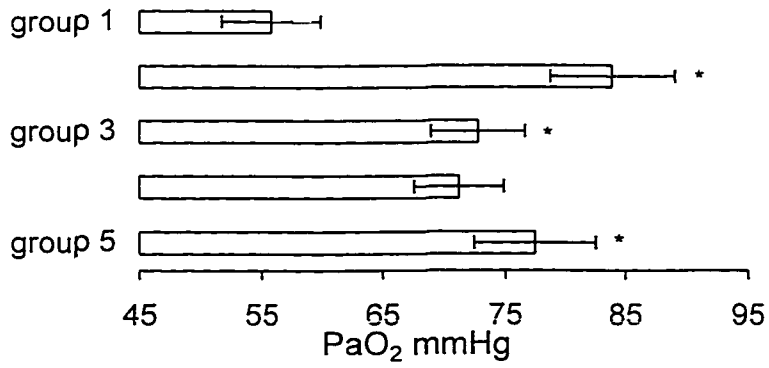

Figure 5.4 Arterial PO_2 mmHg on samples (mean ± SE) taken on day 6 after infection. Calves were vaccinated as described in 5.3.1 (group 1 = controls, group 2 = MLV 2x IM, group 3 = MLV 2x ID, group 4 = MLV 1x, group 5 = MLV Adj 1x). *Significantly different from group1 ($p < 0.05$, Bonferroni's).

5.4.2 Virus Shedding

Nasal virus shedding, usually beginning on day 2, was detected in all but two calves (groups 3 and 4). In the ANOVA for repeated measures, group, time and time by group interaction were all significant ($p < 0.001$). No significant differences were found between vaccine groups ($p > 0.87$) and all vaccine groups were different to group 1, so the pattern of virus shedding was further analysed using pooled data from groups 2-5. In the unvaccinated calves, the peak of virus shedding occurred on days 4-5, followed by a precipitous drop on day 6 and the elimination of virus shedding on day 7 (Figure 5.5). In the vaccinated calves, shedding peaked on days 3-4 at a significantly lower titres, with the first significant drop in titre beginning one day earlier than the unvaccinated calves (day 5) followed by clearance in all but 2 calves on day 6 (Figure 5.5).

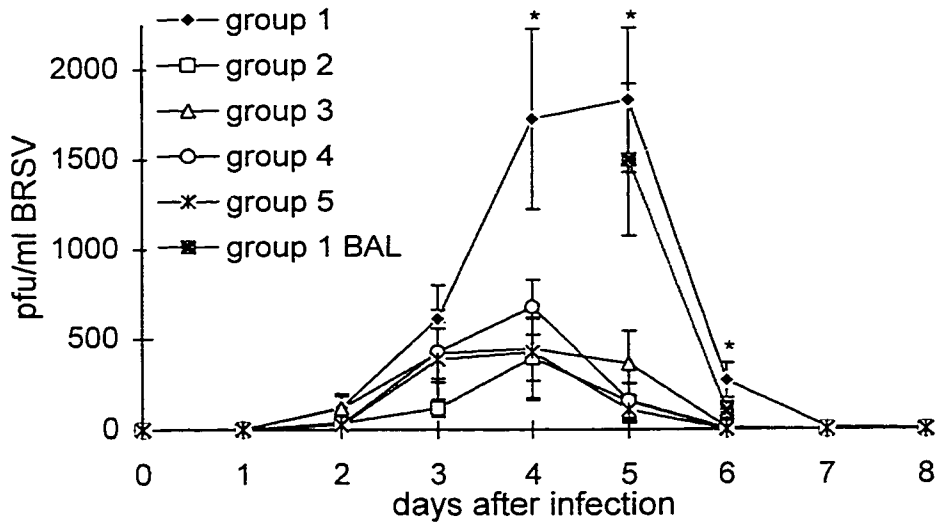

Figure 5.5 Nasal and pulmonary virus shedding (pfu, mean ± SE).
Shedding determined as described in 5.3.5, for calves that were vaccinated as described in 5.3.1 (group 1 = controls, group 2 = MLV 2x IM, group 3 = MLV 2x ID, group 4 = MLV 1x, group 5 = MLV Adj 1x). * Group 1 nasal shedding significantly different from vaccinated groups (using pooled data from groups 2-5) ($p < 0.05$, HSD).

The pattern and titer of virus recovery from lung lavage fluid in control calves was similar to that observed in the nose (Figure 5.5). Virus was recovered at low titres (< 400 pfu/ ml) in only 3/15 vaccinated calves sampled on day 5.

The nasal virus titre on days 4-5 was significantly correlated to both the % lesional lung and subsequent SS on days 4-8 (R = 0.63-0.83 and 0.61-0.82). Similar correlations were found when both groups were analysed separately.

5.4.3 Antibody Responses

Four calves, two in the control group and 1 in groups 3 and 5, had minimal serum virus neutralising antibody titers to BRSV of 1:4 prior to vaccination. All other animals were seronegative. In the ANOVA for repeated measures, the effect of group, time and group by time interaction were significant ($p < 0.001$) for all antibody parameters examined, except IgM in serum, BAL fluid and nasal secretions, where the effect of group was not significant ($p \geq 0.28$). In post hoc analysis of time effect by group, a significant prechallenge, vaccine-induced, BRSV specific serum IgG and VN antibody response was evident only in groups 2 and 3 (Figure 5.6, 5.7). This response was significantly different, in groupwise comparisons, from all other groups on days 0 and 4 with the exception of group 4 on day 4 in the VN assay. Group 5 was the only vaccine treatment group that did not have a significant increase in BRSV specific serum IgM on day 0 compared to prevaccination values, but there were no significant differences between vaccine groups at challenge (Figure 5.7). Samples were not taken prior to vaccination for assessment of mucosal antibody response, so the prechallenge effect of vaccination could not be examined directly, however, there were no differences

in the day 0 ELISA values between the control or vaccinated calves for either nasal or BAL fluid antibody ($p \geq 0.96$ and $p \geq 0.62$, respectively; t-test) (Figure 5.8).

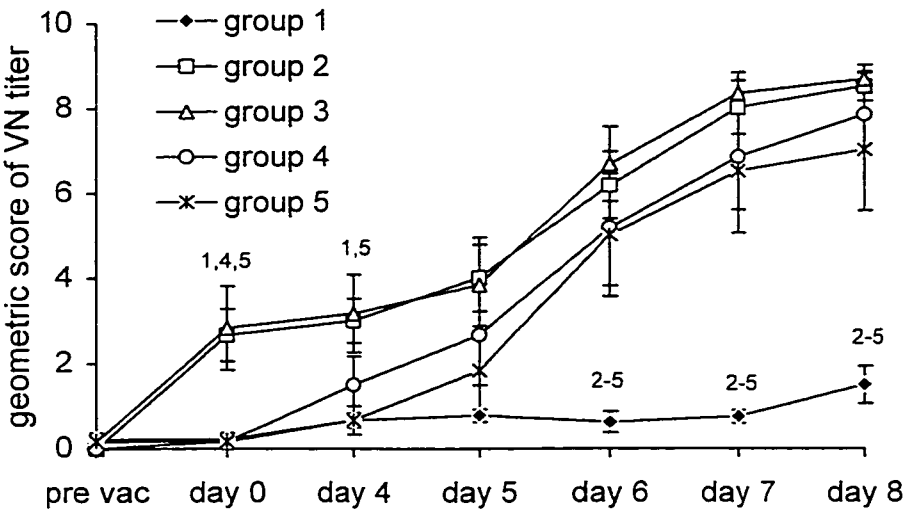

Figure 5.6 Geometric score of the reciprocal dilution's of virus neutralizing serum antibody titres (mean ± SE).
Calves were vaccinated as described in 5.3.1 (group 1 = controls, group 2 = MLV 2x IM, group 3 = MLV 2x ID, group 4 = MLV 1x, group 5 = MLV Adj 1x). Data labels indicate groups significantly different from the groups adjacent to the label.

The times of the first significant post challenge responses are summarised by group in Table 5.2. A significant change over time was not observed for any group in any assay between challenge and day 4. Anamnestic responses in groups 2 and 3 occurred at approximately the same time as groups 4 and 5 seroconverted (day 5-6), with a nearly simultaneous rise in all isotypes at both mucosal sites and in serum. Significant group differences among the vaccine treatment groups after day 4 were observed only in the serum IgG response, with group 4 being lower than the two groups receiving two doses of vaccine (2 and 3). There was a trend for group 5 to have a

slightly earlier response in several assays and to have higher IgM and IgA responses on days 5 or 6. These differences were not significant when compared to the other vaccine treatment groups, but were the only significant differences among the vaccine groups relative to the control group on those days (Figures 5.7, 5.8).

Table 5.2 Day of first significant post challenge response in mucosal and serum antibody

	Treatment Group					
	1	**2**	**3**	**4**	**5**	**2-5**
Serum VN	8[a]	(+) 6	(+) 6	(-) 6	(-) 6	5[†]
Serum IgM	5	(+) nc	(+) nc	(+) 6	(-) 5	5[†]
Serum IgG	>8	(+) 6	(+) 6	(-) 6	(-) 5	5[†]
Nasal IgM	7	(-) 6	(-) 6	(-) 6	(-) 6	5[†]
Nasal IgA	7	(-) 6	(-) 6	(-) 6	(-) 5	5[†]
Nasal IgG	>8	(-) 6	(-) 6	(-) 6	(-) 6	6[†]
BAL IgM	≥6					(-) 5*
BAL IgA	8					(-) 5*
BAL IgG	>8					(-) 5*

[a]First day post challenge that a significant ($p < 0.05$, HSD) antibody response was detected. (+/-) refers to the presence or absence of a prechallenge vaccine induced antibody response. nc = no change post challenge. [†] n = 24. * n = 18.

In group 1, post challenge antibody responses, except serum IgM, were detectable 1 to 3 days later than in the vaccinated calves. IgM was the predominant antibody response noted in the unvaccinated calves. BRSV specific IgM was first detected in the serum, BAL fluid and nasal secretions of some calves on days 5, 6 and 7 respectively, and by day 8, exceeded the serum and BAL fluid response observed in groups 2-5. A modest IgA response was observed in the nasal secretions and BAL fluid on days 7 and 8 respectively. A significant increase in BRSV serum neutralising

antibody titre did not occur until day 8, although 4 calves developed a titre of 1:4 on day 4, with no further increase till day 8. An IgG response was not detected by day 8.

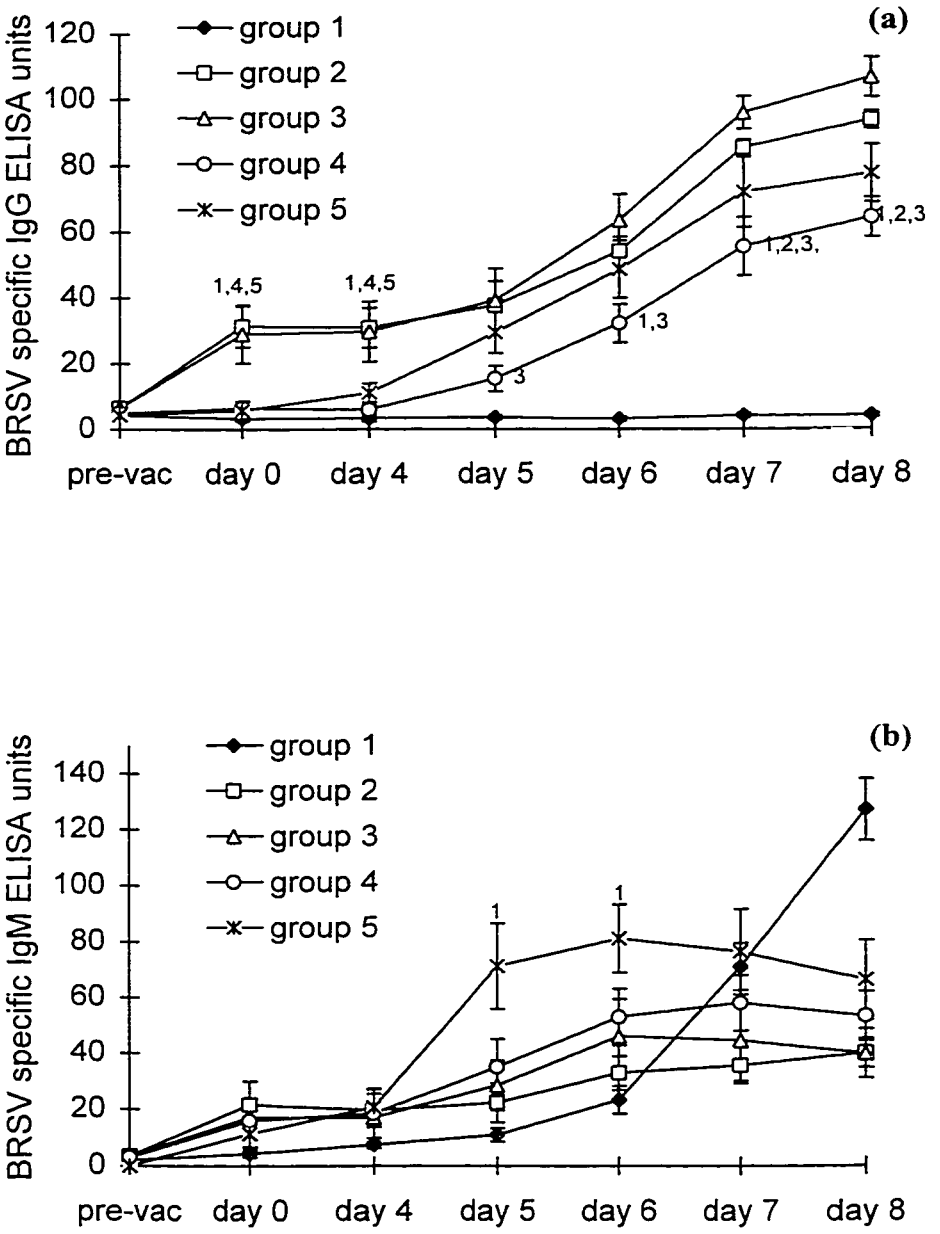

Figure 5.7 Serum BRSV specific IgG (a) and IgM (b) (ELISA units, mean ± SE). Calves were vaccinated as described in 5.3.1 (group 1 = controls, group 2 = MLV 2x IM, group 3 = MLV 2x ID, group 4 = MLV 1x, group 5 = MLV Adj 1x). Data labels indicate the group(s) significantly ($p < 0.05$, HSD) different from the group(s) adjacent to the label.

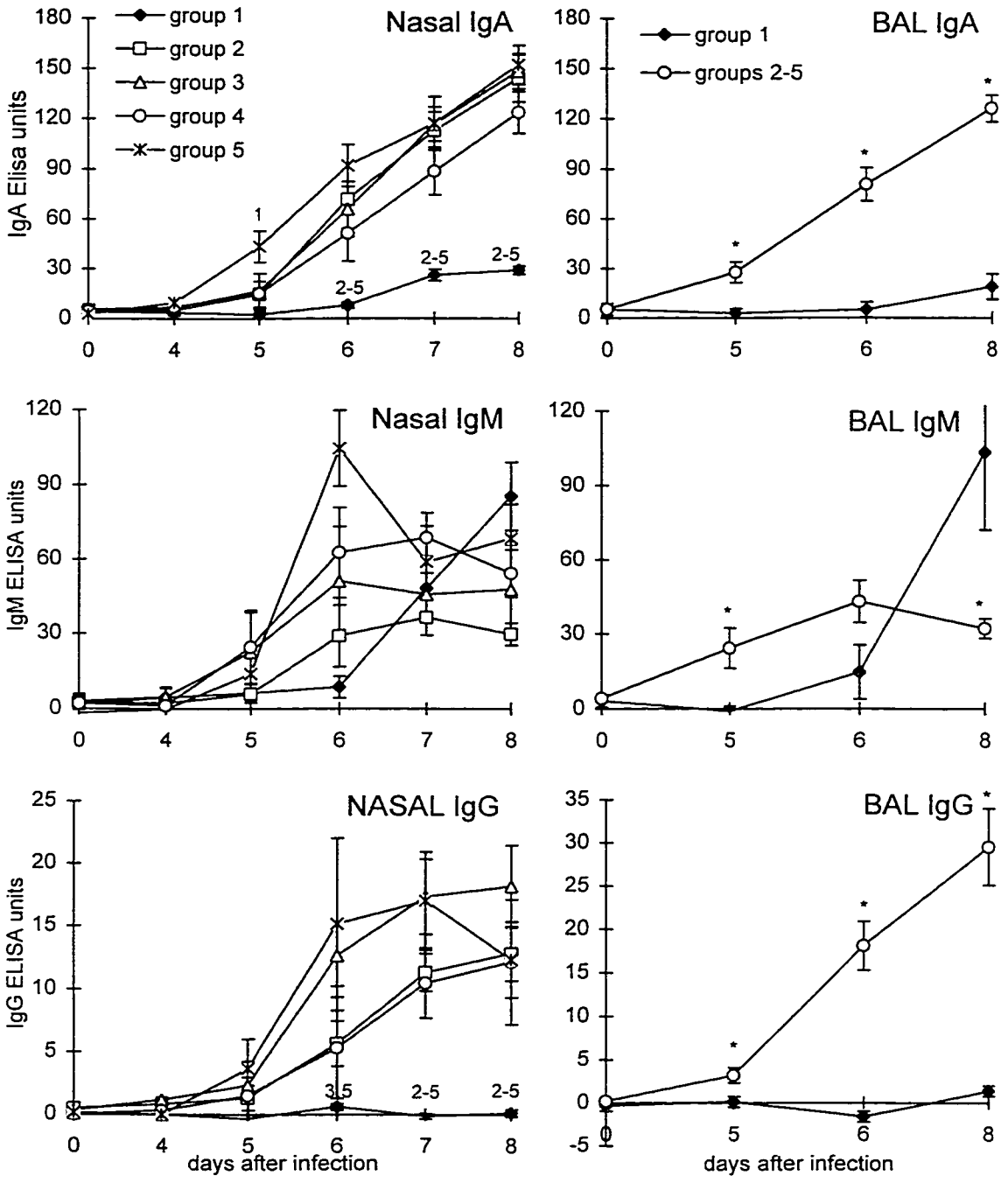

Figure 5.8 BRSV specific IgA, IgM and IgG (ELISA units, mean ± SE), in nasal secretions and BAL fluid.

Calves were vaccinated as described in 5.3.1 (group 1 = controls, group 2 = MLV 2x IM, group 3 = MLV 2x ID, group 4 = MLV 1x, group 5 = MLV Adj 1x). The BAL data for groups 2-5 represents pooled data from 15 calves. Labels indicate significant group differences. Data labels indicate the group(s) significantly different ($p < 0.05$, HSD) from the group(s) adjacent to the label. *Significant differences in BAL antibody between control and vaccinated calves.

There were no significant post vaccination increases in either IgG or IgM ($p > 0.42$), specific for components of the challenge lung wash in any group, although group 2 did have significantly higher post vaccination IgG than the control group ($p = 0.037$ t-test). Small postvaccination increases in IgG were noted in some calves only in groups 2 and 3, but the post vaccination lung wash specific IgG was correlated to the day 0 BRSV specific serum IgG ($R = 0.57$, $p < 0.05$). These increases are most probably due to the detection of BRSV antigen in the lung wash. There was no correlation with day 2-4 SS ($R < 0.05$).

5.4.4 Cellular Immune Responses

5.4.4.1 Lymphocyte Proliferation

The day 0 stimulation indices of groups 2, 3, and 5, but not 4, were significantly greater than group 1 ($p < 0.03$; Mann Whitney U with Bonferroni's correction) (Figure 5.9). On day 8 after challenge, there were no group differences ($p = 0.25$, Kruskal-Wallis), with a general decrease in SI except in the control calves and group 4.

5.4.4.2 Whole Blood IFNγ Assay

BRSV specific IFNγ was not detected in stimulated peripheral blood of any calves prior to vaccination (data not shown), or any of the control calves prior to challenge. IFNγ was detectable on day 0 in most vaccinated calves from groups 2, 3, and 5 but only 2/6 calves from group 4 (Figure 5.10). By day 8 after challenge, IFNγ was detectable in 3/8 control calves and all of the vaccinated calves. This response in

the vaccinated calves was significantly higher than on day 0, and all groups were significantly different from the control calves ($p < 0.003$).

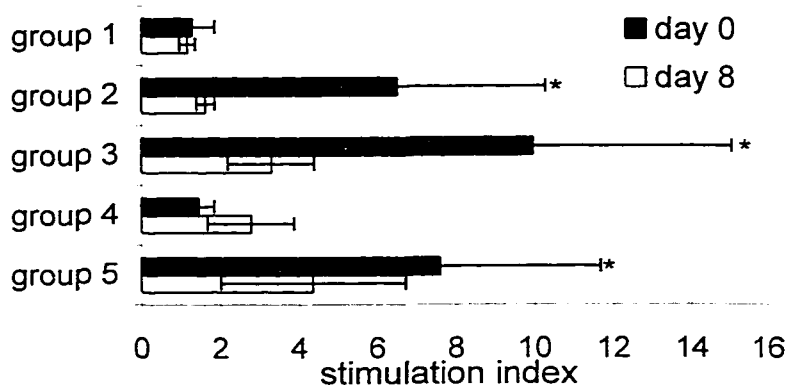

Figure 5.9 BRSV blastogenesis stimulation index (mean ± SE), on days 0 and 8. Calves were vaccinated as described in 5.3.1 (group 1 = controls, group 2 = MLV 2x IM, group 3 = MLV 2x ID, group 4 = MLV 1x, group 5 = MLV Adj 1x. *Significantly different from group1 ($p < 0.05$, Mann-Whitney U with Bonferroni's correction).

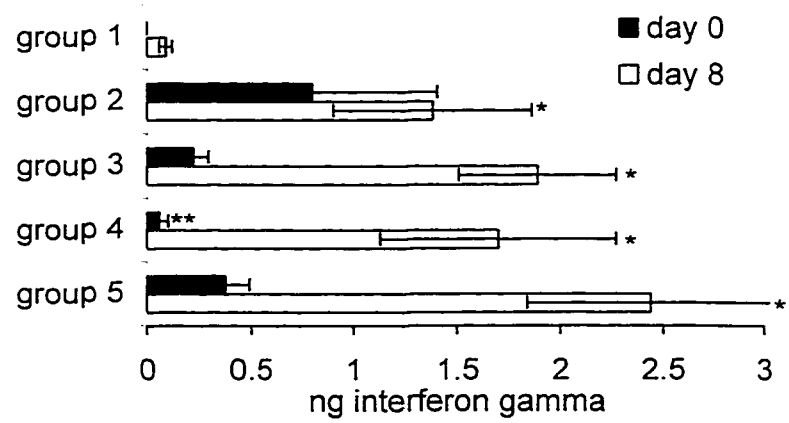

Figure 5.10 BRSV specific IFN γ (ng) detected in whole blood cultures.
IFN γ (mean ± SE) detected in plasma after 18 hr incubation as described in 5.3.7.3, for calves vaccinated as described in 5.3.1 (group 1 = controls, group 2 = MLV 2x IM, group 3 = MLV 2x ID, group 4 = MLV 1x, group 5 = MLV Adj 1x.) *Significantly different from group 1 ($p < 0.05$, Mann-Whitney U with Bonferroni's correction). ** Only 2/6 calves responding.

5.4.4.3 Cytotoxic Responses

On day 5, antigen specific lysis from 4- 32 % was detected in BAL cells from 6/15 vaccinated calves and 0/9 control calves. By day 6 a cytotoxic response was detected in nearly all calves (4/5 control, 9/12 vaccinated). This response was maintained in the 7/8 control calves and 8/15 vaccinated calves on day 8. There was a trend for the response in the vaccinated calves to be detected, and peak earlier, than in the control calves, (Figure 5.11) but significant differences were not detected on days 5, 6 or 8 ($p = .19$, 0.23 and 0.11, respectively, Mann Whitney U).

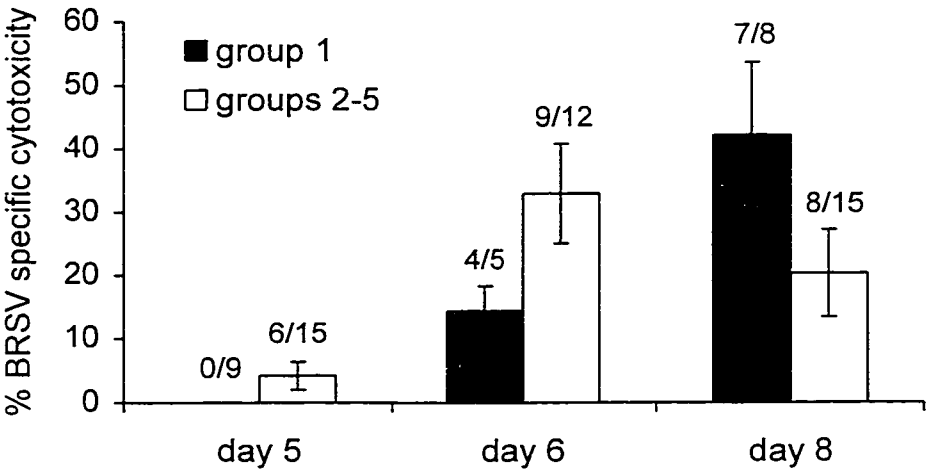

Figure 5.11 BRSV specific cytotoxicity of autologous targets mediated by BAL cells. BRSV specific cytotoxicity (mean ± SE), mediated by BAL cells in a 6 hr ^{51}Cr release assay for control (group 1) and vaccinated calves (groups 2-5). Labels indicate the number of calves with a positive response (> 5% specific lysis) relative to the number of animals assessed on each day.

Cytotoxicity mediated by BAL cells were in most cases restricted to autologous BRSV infected cells. Lysis of uninfected cells was < 5% and these values were subtracted to obtain antigen specific lysis. Lysis of BRSV infected heterologous cells

(dermal cells from other calves in the same experiment) was observed in 4 calves. This was most likely due to MHC compatibility, as all calves came from the same farm, and reciprocal lysis was observed in two cases, but MHC unrestricted cytotoxic mechanisms cannot be ruled out.

5.4.5 Pulmonary Pathology and Histopathology

In all but 2 calves (groups 2 and 5), there were variable amounts of lobular consolidation and atelectasis of the ventral lung, involving up to 63% of the lung area (Figure 5.12). Mild to moderate hyperinflation of the caudal lobes was noted in several calves, but most obvious in 6/9 unvaccinated calves, two of which had moderate caudal lobe interlobular emphysema with bulla formation. Moderate to severe interlobular oedema was observed in 5 calves in group 1 and 1 calf in group 2. A significant reduction in the area of consolidated and atelectatic lung, relative to the unvaccinated calves, was noted in all vaccinated groups ($p \leq 0.03$) (Figure 5.12), but was less obvious in group 4. The group 1 calves also had the highest lung weight to body weight and lung volume to body weight ratios, suggesting more severe edema and emphysema, but these differences were not statistically significant ($p = 0.076$ and $p = 0.19$, respectively ANOVA).

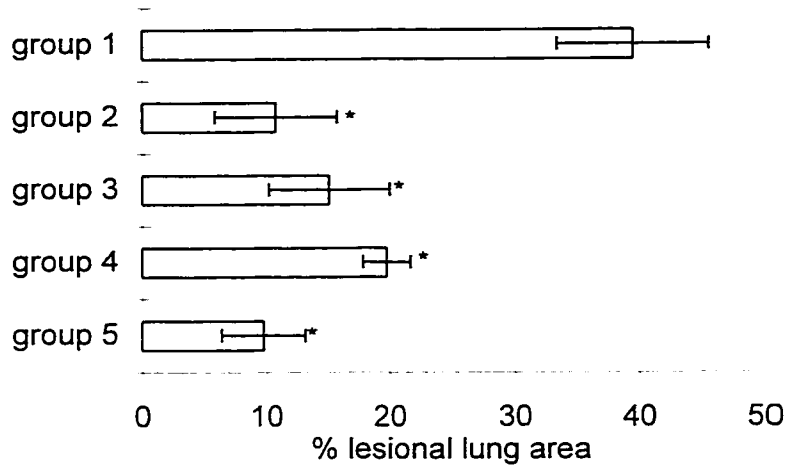

Figure 5.12 Pulmonary atelectasis and consolidation expressed as a % of lung surface area (mean ± SE).
Calves were vaccinated as described in 5.3.1 (group 1 = controls, group 2 = MLV 2x IM, group 3 = MLV 2x ID, group 4 = MLV 1x, group 5 = MLV Adj 1x.) *Significantly different from group1 ($p < 0.03$, Bonferroni).

Histologically, the appearance of the pulmonary lesions of the calf necropsied on day 5 was that of a severe subacute bronchointerstitial pneumonia characterised by extensive necrosis of bronchiolar epithelium and formation of numerous syncytial cells. By day 8, eosinophilic debris and neutrophils often occluded bronchiolar lumens in affected lobules, but there was extensive regeneration of airway epithelium, with little evidence of active infection or syncytial cell formation. The calves in group 4 tended to have the most marked airway and interstitial neutrophil infiltration in affected lobules. Nascent bronchiolitis obliterans was present in several of the more affected calves in group 1. Histologic evidence of bacterial involvement was absent, although Pasteurella multocida was isolated from the lung of one calf (group 4) and Acholeplasma laidlawii, a mycoplasmal organism generally considered to be non-pathogenic (Woldehiwet, 1990; Jungi, 1996), was isolated from 14 calves (all groups). There was no correlation

between a positive culture and the severity of lung lesions or peak sickness score (R = 0.13 and 0.18, Spearman R).

5.5 Discussion

To our knowledge, this is the first time the efficacy of commercial modified live BRSV vaccines have been investigated using an experimental challenge model that induces severe clinical disease similar to that encountered in outbreaks of naturally acquired BRSV infection. Moderate to severe respiratory disease, characterised clinically by pyrexia, coughing, hyperpnea and dyspnea, accompanied by moderate to severe arterial hypoxemia, occurred in all unvaccinated calves similar to descriptions of severe field infections (Pirie, 1981; Lekeux, 1985). There was a reduction in clinical disease, relative to the controls, in all vaccine treatment groups, however only groups 2 and 3, that received two vaccinations prior to challenge, had a significant overall reduction in the cumulative sickness score (CCS) relative to the control group, and only group 2 did not have a significant change in CCS over time. The efficacy of the vaccine in preventing clinically significant disease is more accurately demonstrated using the sickness score (SS). Using this parameter there was no significant change over time in either group that received 2 doses of vaccine.

Although there were no significant differences among the vaccine treatment groups, the CCS and SS of the groups that received a single vaccine tended to be higher, and in group 4, there was no significant difference from the control group in peak sickness score. In field trials, a single dose of MLV BRSV vaccine without adjuvant was associated with significant reductions in undifferentiated respiratory disease, but protection was slightly reduced compared to two doses and our results are in agreement

with these findings (Bohlender, 1984). In group 5, that received a single dose of MLV with an adjuvant, clinical disease peaked between days 2-4, several days before the unvaccinated calves, a pattern that was evident to a lesser extent in all vaccinated groups. The lung lesion scores and blood gas values for group 5 indicates protection was comparable or superior to groups 2 and 3, and the elevated clinical scores resulting from the early disease were not associated with increased pulmonary pathology. In group 4, the pattern of disease was more closely correlated over time to that observed in the unvaccinated calves, peaking between days 4-6, and was associated with increased pulmonary pathology, indicating a reduction in protection. If the peak SS or the peak CCS are correlated to the lung lesion scores and PaO_2, using only values obtained from days 3-8, rather than days 2-8, there is an increase in the regression coefficient (R^2 = 0.64 vs. 0.50 , $p < 0.001$). The CCS from days 2 and 3 have no predictive value for % lesional lung ($R^2 < 0.05$), whereas those from days 5-8 do ($R^2 = 0.45$-0.6, $p < 0.0001$).

Early clinical disease, characterised by pyrexia and hyperpnea, was observed in some calves in all vaccine treatment groups, but was most obvious in group 5. Similar disease was observed in calves vaccinated with a formalin inactivated (FI) BRSV vaccine following challenge with lung wash (West, 1997b), but has not been described in vaccinated calves challenged with BRSV grown in tissue culture. The calves vaccinated with the FI BRSV preparation all had very high BRSV specific non-neutralising serum IgG titres and lymphoproliferative responses, accompanied in some calves by an immediate reaction to intradermal BRSV, suggesting several possible immune mediated mechanisms for disease enhancement (West, 1997b). Calves that received a sham FI vaccine prepared from uninfected cell cultures did not have the same

response, suggesting that it was not due to sensitisation to cellular components of the challenge inoculation. In this experiment, the unvaccinated calves did not receive a similar control preparation and this possibility cannot be ruled out. BRSV specific antibody could not be detected in group 5 until day 5 after challenge, and there is a no correlation with day 0 and 4 antibody and day 2-3 clinical parameters, precluding the involvement of BRSV specific antibody. There is a positive correlation (R = 0.63) between day 5 IgM and day 6 IgG and day 2-3 respiratory rate and temperature, implying the ability to make a strong antibody response, rather than the presence of antibody, predisposes to this phenomena. The absence of a serologic response to the lung wash indicates that it was not a vaccine induced type 3 hypersensitivity reaction to non-viral components of the challenge inoculation. In most of the vaccinated calves there was a detectable post vaccination response to BRSV evident in the blastogenesis and interferon gamma assays. There were weak but significant correlations (R = 0.3-0.6), among vaccinated calves, between the day 0 blastogenesis delta cpm, day 0 and 8 IFNγ, and the clinical parameters respiratory rate, temperature, SS and CCS. These correlations were positive from days 1-3 and negative on days 4-7, indicating an association with both early disease, and later, with protection. It is also possible that there was similar vaccine induced response to non-viral components of the challenge inoculum. This could not be directly tested retrospectively, but there was a similar, though usually lower, positive correlation between the clinical parameters on day 2 and cpm for the control antigen used in the blastogenesis assay, but there was no difference in the control antigen cpm in vaccinated and unvaccinated calves (p = 0.67). In the IFNγ

assay, which used the same antigen, a minimal response to control antigen was detectable only in 4 calves, only one of which had an elevated temperature on day 2, however, the response to the Vero cell control antigen used in both these assays is of questionable relevance. The lack of association of early clinical disease with the severity of lung lesions further suggests this response is immune mediated, and not due to the viral infection *per sae,* but the mechanism, although it appears to associated with in vitro assays of cellular immunity, is undetermined.

The gross lesions seen in the lungs of calves in this experiment are consistent with previous observations made following natural BRSV infection (Pirie, 1981; Van Den Ingh, 1982; Kimman, 1989c; Bryson, 1993; Ellis, 1996c). Cranioventral atelectasis and consolidation was present in all groups, but significant edema and emphysema, primarily involving the caudal lobes, was found in 5 severely affected unvaccinated calves and one vaccinated calf. These lesions were present in the one calf necropsied on day 5 post infection, similar to previous findings (West, 1997b), suggesting this pathologic process is concurrent with the onset of severe clinical disease. A type 3 hypersensitivity has been proposed to explain these lesions associated with severe BRSV infections (Kimman, 1989c; 1989d; 1990; Baker, 1991), supported by evidence of BRSV specific IgG and IgM in field cases dying of acute BRSV infection. BRSV specific IgM was not detected in the nasal mucosa or lungs of most unvaccinated calves until days 7 and 8, considerably later than the onset of severe disease, but as very low levels of IgM were detected in the serum of some calves as early as day 5, the potential contribution of an Arthus reaction to pulmonary pathology cannot be ruled out. Higher titres of IgM were present in all vaccinated calves early in

the infection, in which edema and emphysema were largely absent, but virus antigen may have been insufficient for the development of these lesions. The delayed appearance of IgM at mucosal sites may be real, or due to differences in assay sensitivity, or due to consumption at these sites by high viral load. Anecdotally, one control calf which had a delayed serum IgM response (day 7-8) also had the lowest lung weight to body weight ratio (indicating the absence of pulmonary edema) in the entire study in spite of having a lung lesion score of $> 60\%$.

The significant correlation of the titre of nasal virus shedding with the severity of pulmonary lesions and clinical disease is in agreement with previous observations (Stewart, 1990; West, 1997b). The protective effect of vaccination is evident primarily in the suppression of peak virus shedding, relative to unvaccinated animals, on days 4 and 5, rather than protection from infection. The clearance of nasal virus occurred rapidly in both vaccinated and unvaccinated calves, beginning on days 5 and 6 respectively. Simultaneous clearance was observed in the lungs and nose of the unvaccinated calves and coincided with the appearance of BRSV dependent cytotoxic cells in the lung. The only detectable antibody response in these calves at the time of virus clearance was very low amounts of serum IgM. The serum virus neutralising titre of all but three of these calves remained unchanged through to day 8, in spite of a marked rise in IgM, implying that these antibodies were primarily non-neutralising, and unlikely to have been an important mechanism of virus clearance (Siber, 1992; 1994; Taylor, 1992;). Depletion of $CD8^+$ T-cells in BRSV infected, unvaccinated calves, results in enhanced pulmonary pathology and persistent infection in the face of a normal

antibody response (Taylor, 1995b). Our observations are in agreement with the relative unimportance of antibody in BRSV clearance in a primary infection and support the proposed role of CD8+ cytotoxic T-cells in protection (Gaddum, 1996a).

Nasal virus shedding occurred in most vaccinated calves through to day 5, demonstrating the previously described ineffectiveness of a parenterally administered MLV or inactivated vaccine in preventing BRSV infection (Mohanty, 1981; Kimman, 1989e; Taylor, 1989). Both nasal and pulmonary virus replication was reduced relative to unvaccinated calves, in agreement with some previous observations (Mohanty, 1981; Taylor, 1989), but at variance with other reports describing the effect of live virus vaccination in calves and in primate and cotton rat models of human RSV. In these latter reports, mucosal, rather than parenteral priming was required to limit replication in the nose, but pulmonary protection was provided by both routes (Kimman, 1989e; Murphy, 1989; Crowe, 1993).

The immune mechanisms, which are responsible for suppression of nasal BRSV replication in the vaccinated calves, are not clear. Only groups 1 and 2 had significant post vaccination BRSV specific serum IgG and VN antibody between days 0 and 4. These antibodies did not prevent infection, as there were no differences among vaccinated groups or between control and vaccinated groups before day 4. A weak but significant correlation was found between day 4 serum IgG and day 4 virus titre ($R = -0.52$, $p < 0.05$). Mucosal antibodies were not induced by vaccination in any calves, appearing only on day 5, simultaneous with the first significant increase in serum antibodies. This coincided with the first fall in virus shedding. Day 5 serum IgG, VN titre and nasal IgA antibody had a weak combined predictive value for day 5 virus titre

($R^2 = 0.32$, $p < 0.003$). Of these responses, mucosal IgA is probably most important, as at this time, the serum antibody in groups 4 and 5 was still less than the post vaccination response of groups 2 and 3, but virus clearance occurred simultaneously in all groups. Similarly weak associations between virus shedding and antibody have been observed previously in calves and humans even when both priming and reinfection were via the mucosal route. Virus could not be recovered from calves reinfected with BRSV even though the mucosal antibody response was not detected before day 6 after infection. (Kimman, 1987a; 1989e), but calves primed intramuscularly, nasal virus shedding was not reduced even though the mucosal antibody responses were similar. Resistance to reinfection with RSV in human volunteers also does not correlate with nasal IgA (Hall, 1991). The ability to mount a mucosal IgA response in mucosally primed animals, more than the response itself, has been correlated with reduced shedding, suggesting unidentified mucosal cell mediated mechanisms were involved in protection (Kimman, 1987a; 1989e; Oien, 1994; Johnson, 1996). In this experiment, systemic vaccination with MLV reduced nasal virus shedding and primed for a rapid mucosal antibody response, but a strong direct association was not apparent.

On day 5, cytotoxic BAL cells were detected in the lungs of a few vaccinated calves. On day 6, these responses were evident in most calves, most exceptions being those calves which had cleared nasal virus on day 5. By day 8 there was an attenuation of the cytolytic activity, whereas in the unvaccinated calves it appeared to peak at this time, suggesting an overall acceleration of the response in the vaccinated calves, even though this was not obviously apparent on day 5. If these results are extrapolated to the

nasal mucosa, nasal virus clearance in the vaccinated calves was coincident with the appearance of both mucosal antibody and cytotoxic cells.

Virus clearance in the vaccinated calves appeared to be, as, or perhaps more rapid, in the lung than in the nose. Most calves cleared the infection prior to the first sampling on day 5, precluding direct correlations between virus shedding and immune responses, but cytotoxic responses were present in 6/12 isolation negative calves on day 5. The percent lesional lung area was best predicted by a parsimonious model including day 6 serum IgG, day 5 serum IgM and day 0 stimulation index ($R^2 = 0.55$, $p < 0.0001$). Nasal IgA was also significantly associated with reduced lung lesions, but not until day 8 ($R^2 = 0.47$, $p < 0.001$), and did not increase the predictive value of the model, implying the ability to mount a pulmonary mucosal response but not necessarily antibody, may be associated with reduced pulmonary pathology. Passive serum antibody has been shown in calves (Kimman, 1988; Belknap, 1991) and children (Groothuis, 1993; Meissner, 1993) to provide amelioration of BRSV and RSV induced lower respiratory tract disease, but have no or marginal effect on infection or upper respiratory tract shedding. In this experiment there appears to have been a greater contribution of serum antibody to virus clearance in the lung than in the nose. Other cellular effector mechanisms not investigated in this experiment, such as adherent cell mediated, MHC unrestricted cytotoxicity, (Campos, 1985) may also be involved.

Although there were no statistically significant differences among the vaccine treatment groups, with respect to the various clinical parameters, the group 4 calves, that received a single dose of MLV vaccine without adjuvant, were least protected from clinical disease or pulmonary pathology, and did not have a significant difference in day

6 PaO_2 from the control calves. The post vaccination lymphoproliferative responses of this group were not different from the control calves, and only 2/6 calves had detectable post vaccination BRSV specific IFNγ. Variable lymphoproliferative responses have been previously reported in calves following natural infection or vaccination with MLV vaccines (Taylor 1987, Ellis 1992). In children, lymphoproliferative responses induced by either vaccination or natural exposure to RSV (Fernald, 1983; Welliver, 1994) are not correlated with protection from illness or infection. In one report, strong lymphoproliferative responses induced by an inactivated vaccine have been shown in calves to be correlated to both IgG antibody and protection (Taylor, 1987). Similarly, in this experiment, evidence of vaccine induced T cell priming appeared to be a correlate of protection. The group 4 calves also had a delayed serum IgG response compared to group 5, even though 5/6 calves had vaccine induced IgM, further suggesting a reduction in primed T-cell help. This delayed antibody response may contribute to the reduced protection, but other cellular effector mechanisms may also be involved. Post challenge BRSV specific IFNγ responses in peripheral blood leukocytes (PBL) have not been previously reported in cattle vaccinated with MLV vaccines. PBL from calves vaccinated with FI, alum adjuvanted BRSV, have been reported to produce half the IFNγ of unvaccinated calves 10 days after infection with no difference at 5 days post infection. The enhanced IFNγ production 8 days post infection in the calves vaccinated with MLV in this experiment would suggest these different vaccines induce phenotypically disparate immune responses. The memory cytokine response of human PBMC stimulated with RSV is primarily TH -1, with consistent production of IFNγ, and

our results suggest this cytokine is similarly induced by BRSV infection or MLV vaccination in cattle. In this experiment, IFNγ appears to be a useful correlate of MLV vaccine induced protection.

In conclusion, the results of this experiment indicate that a significant reduction in clinical disease and pulmonary pathology can be afforded by vaccination with commercial MLV BRSV vaccines. A MLV vaccine administered twice intramuscularly, or intradermally at one quarter of the dose, and a single dose of an adjuvanted MLV vaccine provided equal protection from pulmonary pathology. A single unadjuvanted intramuscular dose was less effective, and was associated with reduced in vitro lymphoproliferative responses, decreased IFNγ production and slightly delayed serum IgG. The onset of disease in some calves in all of the vaccine groups was observed 2 days earlier than in the control calves that was not dependant on antibody, but appeared to be weakly correlated to IFNg and lymphoproliferative responses to BRSV, but a similar reaction to cellular components of the challenge could not be ruled out. This early disease was not associated with increased pulmonary pathology.

Virus clearance in the nose and lung of unvaccinated calves began on day 6, before the detection of mucosal antibody, and appeared dependent on the appearance of BRSV specific cytotoxic cells. Most vaccinated calves shed virus, but peak virus titre was suppressed compared to unvaccinated controls. In vaccinated calves, clearance of nasal infection began on day 5, coincident with the simultaneous appearance of mucosal antibody and cytotoxic cells in the lung. There was a weak correlation between serum and mucosal antibody responses and virus shedding in the nose ($R^2 = 0.32$). Pulmonary

lesions were best predicted by serum IgG, IgM and lymphoproliferative responses (R^2 = 0.55) but the weak predictive value of this model suggests other immune responses may be involved.

6 SUMMARIZING DISCUSSION AND CONCLUSIONS

6.1 Introduction

This work has expanded the current knowledge of infection and immunity to BRSV and the effects of the qualitative and quantitative differences in the immune responses induced by inactivated and live virus vaccines. Several areas of this investigation have repeated earlier works, and have confirmed and expanded those results by simultaneous examination of both cell mediated and humoral immune responses. Perhaps the greatest contribution has been the ability to examine the correlates of immunity in a model that induced severe disease typical of field infections. This model has also reproduced the typical pathology associated with BRSV infection and will enable further investigations into the pathogenesis of BRSV induced pulmonary lesions.

6.2 Summarizing Discussion

6.2.1 Induction of Functional Antibodies by Vaccination with MLV and Inactivated Vaccines

The protection associated with passive antibody has been shown to be dependent on the virus neutralizing titer (Siber, 1992; Groothuis, 1993), and more specifically, with the ability of the antibodies to inhibit syncytia formation by RSV infected cells (Taylor, 1992; Thomas, 1998). The third chapter of this thesis expanded on previous work illustrating commercial inactivated BRSV vaccines induce antibodies with poor virus neutralizing properties (Ellis, 1995b), by showing that these antibodies also have no or low, fusion inhibiting capability, relative to those induced by MLV vaccination.

The inactivation processes appear to alter the conformational epitopes required for the induction of neutralizing and fusion inhibiting antibodies. This could be tested by absorption of a neutralizing antisera with these preparations and assaying for a reduction in neutralizing titer. The ability to facilitate complement mediated lysis of BRSV infected cells was correlated with virus neutralizing properties, but recent evidence, using passive transfer of monoclonal antibodies, would suggest this property is not a significant correlate of protection in calves (Thomas, 1998). Similar proportions of IgG1 and IgG2 were induced by both MLV and inactivated vaccines and this is in agreement with another recent report that has also indicated inactivated vaccines, live virus vaccines and natural infection all induce similar ratios of IgG1/IgG2 (Schrijver, 1996b). IgG isotype ratios in this experiment could not be used to infer differences in T-cell responses between the vaccines. While there is some evidence that IFNγ can preferentially induce class switching of bovine B-cells from IgM to IgG2 (Estes, 1994), and IL4 promotes IgG1 and IgE (Estes, 1995; 1996), a recent investigation indicates most Th clones isolated from cattle immune to various infections are Th0, secrete both IL-4 and IFNγ, and provide help for both IgG1 and IgG2 simultaneously (Brown, 1997).

The weak association of serum antibody with protection in subsequent chapters of this thesis raises concerns regarding the relevance of the ability of MLV vaccines to preferentially induce neutralizing and fusion inhibiting, versus non-neutralizing serum antibodies. In our experiments, protection from pulmonary pathology appeared similar with both FI and MLV vaccines. One possible explanation for both these findings, which appear to contradict the protective effects of fusion inhibiting passive antibody

(Thomas, 1998), is the relatively low titers of prechallenge BRSV specific antibody obtained with the MLV vaccines used, and the requirement for passive antibody to be at a high titer to be effective (Kimman, 1988; Groothuis, 1993; Siber, 1994). Although the ELISA values for BRSV specific IgG were quite different between the calves that received MLV and FI vaccine the pre and post challenge VN responses were very similar. It must be kept in mind that protection ascribed to passive antibody in all experiments was assesed in the face of competent cell mediated immunity. The suppression of an induced antibody response by maternal antibody may be accompanied by a cell mediated response more polarized towards Th1. If in fact there is a detrimental effect of an early induced antibody response, or a Th response skewed more towards Th2, the effect of maternal antibody and induced antibody would not be expected to be the same.

6.2.2 The Effect of Formalin Inactivated Vaccine on Response to BRSV Challenge

The objective of the fourth chapter was primarily to investigate the potential of BRSV vaccination to enhance disease in cattle. Vaccine induced immunoenhancement of BRSV associated respiratory disease has not been described in cattle as it has in children (Kim, 1969), but the similarities in the diseases associated with RSV infection in both species suggest it is a potential problem worthy of investigation. A FI alum-precipitated vaccine induced high titers of non-neutralizing BRSV specific serum antibodies and exaggerated lymphoproliferative responses in calves, as observed in children that received a similar vaccine (Kim, 1976; Murphy, 1986). There was an early onset of acute clinical respiratory tract disease in calves that received FI vaccine, but the mechanism of this is unresolved. An overall enhancement of disease was not noted, but

rather in 3/4 calves that received FI vaccine, there appeared to be a significant reduction in pulmonary pathology, suggesting this may be a different phenomena than that which occurred in the children, where both the frequency of severe disease and duration of illness were increased in vaccinees (Kim, 1969). Pulmonary lesions with severe caudal lobe emphysema and edema, typical of severe natural infection were reproduced by the experimental challenge. The severity and character of the pulmonary lesions appeared to be independent of treatment (FI vaccine, MLV vaccine, and sham vaccine) and the immune response parameters investigated (BRSV specific IgG, virus neutralizing serum antibody, lymphoproliferative response or DTH). Previous speculations of immune mediated mechanisms in the pathogenesis of BRSV induced disease and pathology (Kimman, 1989c; 1989d; 1990; Stewart, 1989c; Baker, 1991) could not be substantiated. The pulmonary lesions appeared more likely to result from the direct or indirect effects of viral infection, than from the immune responses investigated. An influence of treatment on virus shedding was not detected, perhaps due to the small sample sizes.

Clearance of virus in the control calves was not dependent on serum neutralizing antibody, but cytotoxic cells were detected in the lungs of control calves and calves that received MLV vaccination. The clearance of virus could not be temporally associated with these cells as this was not investigated until day 8, 2 days after virus clearance. Significant cytotoxic activity was not detected in BAL cells in calves that received MLV vaccine which may be due to the decreased severity of the lung lesions in these calves, rather than suggest an alternative means of virus clearance. A more complete assessment of the ability of MLV and inactivated vaccines to prime for cytotoxic

responses was precluded, as we were unable to detect ex-vivo cytotoxic activity in PBMC at any time point. A marginal protective effect without any evidence of early acute disease was observed in calves that received the MLV vaccine, but the dose of MLV vaccine used in this experiment was approximately 2 logs lower than most commercial vaccines.

6.2.3 The Effect of MLV Vaccine on Response to BRSV Challenge

The objectives of the fifth chapter were to investigate the ability of commercial MLV vaccines to protect against BRSV challenge and attempt to determine which immune responses correlated with reduced disease expression. The results of this experiment indicate that a significant reduction in clinical disease and pulmonary pathology can be afforded by vaccination with commercial MLV BRSV vaccines. A MLV vaccine administered twice intramuscularly, or intradermally at one quarter of the dose, and a single dose of an adjuvanted MLV vaccine provided equal protection from pulmonary pathology. A single unadjuvanted intramuscular dose was less effective, and was associated with reduced in vitro lymphoproliferative responses, decreased BRSV specific IFNγ production and slightly delayed post challenge serum IgG responses.

Virus clearance in the nose and lung of unvaccinated calves began on day 6, and appeared dependent on the appearance of BRSV specific cytotoxic cells in the lung, and began before the detection of mucosal antibody. Small amounts of serum IgM were detected at this time, but appeared to be non-neutralizing, and therefore are of questionable significance. This is in agreement with the results of our previous

experiment, and supports the conclusions of others regarding the importance of cellular responses rather than antibody for clearance of viral infection (Taylor, 1995b).

Most vaccinated calves shed virus, as expected with a systemic vaccine (Kimman, 1989e), but in this experiment a suppression of peak virus titre compared to unvaccinated controls was evident. The presence of vaccine induced serum antibody at challenge was not required for subsequent reductions in virus shedding or pulmonary pathology. In calves vaccinated with MLV, clearance of nasal infection began on day 5, coincident with the simultaneous appearance of mucosal antibody, secondary serum antibody responses, and cytotoxic cells in the lung. Day 5 serum IgG, VN titre and nasal IgA antibody had a weak combined predictive value for day 5 virus titre ($R^2 = 0.32$, $p < 0.003$). Reduction in pulmonary lesions was best predicted by day 6 serum IgG, day 5 serum IgM and prechallenge lymphoproliferative responses ($R^2 = 0.55$) but the weak predictive value of this model suggests other immune responses may be involved.

In some calves in all of the vaccine groups, clinical respiratory disease was observed 2 days earlier than in the control calves, similar to the calves that received FI vaccine in the previous experiment. This early clinical response was not associated with increased pulmonary pathology, and was the most clinically apparent disease noted in many vaccinated calves. Antibody specific for BRSV could not be detected in half of the affected calves at this time nor did calves have a detectable antibody response to the challenge inoculum. The response was weakly correlated to blastogenesis and IFNγ responses to BRSV. The calves vaccinated with the FI vaccine had high titres of BRSV specific IgG, but also strong lymphoproliferative responses. As the response in calves

that received both MLV and FI vaccine was similar, it is likely that in both cases this early acute disease was associated with cellular rather than antibody mediated mechanisms. A similar reaction to cellular components of the challenge could not be ruled out, as the control calves in this experiment were not vaccinated with a sham MLV vaccine, as were the calves in the FI vaccine experiment. Even if this is a specific reaction to BRSV, it may not be relevant in a natural infection unless it is shown to be dependent on virus replication and not just viral antigen in the challenge inoculum.

6.3 Conclusions

1. MLV BRSV vaccines preferrentially induce antibodies with fusion inhibiting properties.
2. Clinical disease and pulmonary pathology induced by an experimental BRSV challenge that results in disease typical of severe field infections is reduced by systemic vaccination with the commercial MLV or the experimental FI BRSV vaccines used in these studies.
3. Both the MLV and the inactivated vaccines are associated with a similar earlier clinical response to the BRSV challenge used in these studies, but this was not associated with enhanced pulmonary pathology
4. Virus clearance in unvaccinated calves is associated with the appearance of BRSV specific cytotoxic cells in the lung and appears to be independent of antibody.
5. Prechallenge vaccine induced serum antibody is not required for protection associated with vaccination.

6. Anamnestic serum and mucosal antibody responses associated with systemically administered MLV vaccine have a low but significant negative predictive value for virus shedding and pulmonary pathology.
7. Vaccination with MLV is associated with only a marginal acceleration of the BRSV specific cytotoxic response.
8. Pulmonary emphysema and edema could not be associated with antibody or cell mediated (lymphoproliferative and DTH) immune responses to BRSV.

6.4 Prospects for Further Research

In our studies, using a BRSV challenge model that resulted in severe respiratory disease typical of naturally acquired BRSV infections, we have shown that the overall effect of both MLV and FI vaccines appears to be protective rather than disease enhancing. However, both types of vaccination induced an early acute disease and further work is required to determine if this is an artifact of the challenge model or vaccine induced and thus likely to occur subsequent to natural infections.

Further work is needed to determine the relative importance of vaccine induced antibody or cell mediated responses in protection, perhaps through T-cell subset depletion of calves at priming or before challenge. Identification of bovine allele-specific CTL epitopes is being undertaken (Gaddum, 1996c) and vaccination with viral proteins or peptides that contain only CTL or antibody epitopes for defined bovine MHC haplotypes may soon be possible.

There is some indication from this work that cellular cytotoxic responses are more rapid in calves vaccinated with MLV vaccine, as would be expected with a

replicating viral vaccine. This enhancement was not as marked as others have described in ovine models of BRSV infection subsequent to mucosal priming or vaccination with inactivated vaccines with a Quil-A adjuvant (Sharma, 1992; 1996b). This may be due to species differences, but suggests there is significant room for improvement in augmenting this response, either by route of administration, dose or appropriate adjuvant. Inactivated vaccines would be expected to be inferior in this regard (Nicholas, 1990), but at this time there is no indication that this is so in calves, or that they are unprotective. The ability of appropriate adjuvants to induce MHC I presentation of inert antigens (Sharma, 1996b) and the effect this has on protection in calves needs to be investigated.

Protection in most BRSV outbreaks would require vaccination of very young calves in the face of maternal antibody. Previous work suggests in these circumstances, only the mucosal route of immunization reduces shedding in a subsequent challenge (Kimman, 1987a; 1989e). Our experiments indicate significant protection from disease can be obtained by systemic vaccination even though shedding occurs. Protection from clinical disease in calves vaccinated systemically in the face of maternal antibody needs to be determined in a similar challenge model that results in respiratory disease with pulmonary lesions.

The pathogenesis of BRSV associated pulmonary pathology remains obscure, but the ability to reproduce these lesions experimentally should allow further work to be done in this area. The possible role of virus induced imflammatory mediators in the deveolopment of these lesions has been mentioned in the text. Several cytokines, particularly those in the IL-6 family, are induced during HRSV infection by activation

of the cellular transcription factor, NF-κB (Bitko, 1997). Similar studies have not been done for BRSV. Several inhibitors of NF-κB activation have been described, one of which is aspirin (Bitko, 1997; Bergmann, 1998). Using these inhibitors, one could determine the contribution of these mediators in the disease process and also investigate a potential therapeutic option.

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APPENDIX I

7 APPENDICES

7.1 APPENDIX 1: Cumulative Clinical Score (CCS) Grading System

Respiratory Rate

Rate	Grade
< 30	0
31-40	1
41-50	2
51-60	3
61-75	4
>75	5

Heart Rate

Rate	Grade
< 71	0
71-85	1
86-100	2
101-115	3
115-130	4
>130	5

Rectal temperature

°C	Grade
<39	0
39-39.5	1
39.5-39.9	2
40-40.4	3
40.5-40.9	4
>41	5

Breathing Effort or Respiratory Distress

0 Eupnea
1 Mild abdominal breathing
2 Obvious abdominal breathing
3 Obvious abdominal and intercostal breathing with audible expiration
4 Expiratory grunt with intermittant mouth breathing
5 Expiratory grunt, open mouth breathing, frothy saliva

Nasal Discharge

0 normal
1 increased serous discharge
2 serous with mucopurulent strands
3 moderate mucopurulent
4 severe mucopurulent or purulent

Cough Score

0 none, either spontaneous or induced
1 mild induced with tracheal squeeze
2 occaisional spontaneous cough or easily induced
3 frequent spontaneous cough, especially with exercise
4 frequent spontaneous cough at rest or prolonged episode when induced

Demeanor

0 bright, alert
1 mildly depressed
2 moderately depressed
3 severely depressed
4 moribund

The sum of the scores for the various clinical parameters was expressed as the Cumulative Clinical Score (CCS).

APPENDIX II

7.2 APPENDIX 2: A Comparison Of Diagnostic Methods For The Detection Of Bovine Respiratory Syncytial Virus In Experimental Clinical Specimens

7.2.1 Summary

Virus shedding was monitored in nasal secretions of 12 calves experimentally infected with bovine respiratory syncytial virus (BRSV) using an antigen capture enzyme-linked immunosorbent assay (ELISA) detecting the nucleoprotein (N protein) antigen of BRSV, a polymerase chain reaction (PCR) amplifying the fusion protein of BRSV, and by a microisolation assay combined with immunoperoxidase staining for the F protein of BRSV. Under the conditions of this study, similar limits of detection and quantitative results were obtained from all three assays. BRSV was detected in nasal secretions of all calves for a minimum of 4 days. Virus shedding began on day 2 after infection, peaked on days 3-5, and was cleared in most calves by day 8. The PCR, and to a lesser extent the ELISA, may detect virus shedding for a longer period after infection than virus isolation, possibly due to neutralization of the virus by rising mucosal antibody. Simulated environmental conditions likely to be experienced during transport of clinical field specimens markedly reduced the sensitivity of virus isolation but had a minimal effect on the results of the NP ELISA. Actual field transport conditions (overnight on ice) had minimal apparent effect on the results of the PCR assay. The less stringent specimen handling requirements, combined with low limits of detection, of both the nucleoprotein ELISA and PCR, indicate either of these assays are more suitable for diagnostic applications than virus isolation.

7.2.2 Introduction

Bovine respiratory syncytial viruses (BRSV) are prevalent pathogens in the cattle populations of North America and Europe (Wellemans, 1975; Baker, 1985b; Kimman, 1986). The acute respiratory disease resultant from infection by these viruses can affect cattle of all ages, and is characterized by increased respiratory rate, nasal discharge, fever, and cough, that occur coincident with viral shedding (Baker, 1985b). There are numerous types of assays used to successfully detect BRSV antibodies in convalescent bovine sera (Gillete, 1983; Baker, 1985b; Westenbrink, 1987; Rhodes, 1989; Dubovi, 1993), however this diagnostic methodology results in a retrospective diagnosis and may yield equivocal results in young calves due to the presence of maternal antibodies (Kimman, 1989e; Belknap, 1991). Traditionally, virus isolation to identify acute infections has been consistently difficult, owing in large part to the lability of the virus external to the bovine respiratory tract (Baker, 1985b; Dubovi, 1993). Several alternative diagnostic methods to detect BRSV antigens in clinical specimens have been developed, including immunofluorescent staining of exfoliate respiratory epithelial cells (Kimman, 1986), immunohistochemical staining of lung sections (Haines, 1989) and commercial antigen-capture enzyme immunoassays (ELISA) developed for the detection of nucleocapsid and fusion proteins of human respiratory syncytial virus (HRSV) (Osorio, 1989; Oberst, 1993a). Application of diagnostic tests for HRSV to the detection of BRSV is based on the extensive antigenic cross-reactivity among HRSV and BRSV isolates (Stott, 1984a; Beeler, 1989; Lerch, 1989; Baker, 1992; Taylor, 1992; Oberst, 1993a); however, large scale application of these assays in veterinary diagnostic laboratories is currently prohibited by the relatively

high cost of the HRSV ELISA kits. A polymerase chain reaction (PCR) has also been described (Oberst, 1993a; 1993b; Vilcek, 1994), and while this technique is not commonly available in veterinary diagnostic laboratories at present, its use is likely to increase.

The purpose of this study was to develop an antigen capture ELISA specific for the nucleoprotein of BRSV and to compare this assay with a virus microisolation technique, and a PCR specific for the fusion protein of BRSV, for the ability to detect nasal virus shedding in nasal swab specimens from calves experimentally infected with BRSV.

7.2.3 Materials and Methods

7.2.3.1 Viruses and Propagation

The Asquith isolate of BRSV, obtained from an affected calf on a dairy farm with an outbreak of severe respiratory tract disease, was used as the challenge virus. The following six isolates of BRSV were used as positive controls for virus isolation or the nucleoprotein ELISA: 1) the SmithKline Beecham Animal Health vaccine strain (SmithKline Beecham Animal Health, Lincoln, Nebraska, USA) which is a derivative of the 375 isolate, hereafter designated "SBAH-375" (Lehmkuhl, 1979); 2) the SmithKline Beecham Animal Heath vaccine strain (SmithKline Beecham Biologicals S.A., Rixensart, Belgium) which is a derivative of RB94, hereafter designated "SBAH-RB94" (Wellemans, 1975); 3) the Minnesota isolate, hereafter designated "MN" (Baker, 1986a); 4) the American Type Culture Collection strain VR-794 (A 51908) hereafter designated "Mohanty" (Mohanty, 1975); 5) a field isolate, B3130, from the Western

College of Veterinary Medicine, hereafter designated, "WCVM", and 6) a low passage field isolate from SmithKline Beecham Animal Health, hereafter designated "SBAH-165". BRSV isolates were grown and titered in embryonic bovine tracheal (EBTR) cells or Madin-Darby bovine kidney cells in Dulbecco's modified eagle medium (DMEM; Life Technologies, Grand Island, New York, USA), supplemented with 5% heat inactivated fetal bovine serum (FBS; Life Technologies, Grand Island, New York, USA), 100 units/mL penicillin, and 100 mg/mL streptomycin.

7.2.3.2 Experimental BRSV Infections and Collection of Clinical Specimens

The calves were raised and experimentally infected as described in 4.3.1, 4.3.2 and 4.3.4. Nasal secretions were collected using rayon tipped swabs (Culturette, Becton Dickinson, Cockeysville, Maryland, USA) on days 0 and 2-8. Swabs used for virus isolation and the NP ELISA were placed in 1 mL of transport medium (Fernie, 1980) consisting of DMEM (Life Technologies, Grand Island, New York, USA) supplemented with 10% FBS (Life Technologies, Grand Island, New York, USA), 0.3M $MgSO_4$, 0.4M sucrose, 50mM HEPES (N-2- hydroxyethylpiperazine-N-2-ethanesulfonic acid), 200 μg/mL sodium penicillin G, 200 μg/mL streptomycin sulfate and 0.5 μg/mL amphotericin B, and frozen at -70°C within two hours of collection. As magnesium interfered with the PCR, a second swab for this assay was placed in transport medium without $MgSO_4$ or sucrose, and frozen at -70°C.

7.2.3.3 Antigen Capture Indirect Elisa

An antigen capture ELISA to detect the nucleoprotein (N protein) of BRSV (NP ELISA) was performed using a rabbit polyclonal antiserum (IgG fraction) to HRSV

(Dako Corporation, Carpinteria, California, USA) as the capture antibody. The antibody was diluted 1:1000 in carbonate coating buffer (pH 9.6) and 100 μL/well were added to Immulon-4 (Dynatech Laboratories, Chantilly, Virginia, USA) 96-well flat-bottomed microtiter plates and incubated overnight at 4°C. An equal volume of 4 % normal rabbit serum (NRS, v/v in coating buffer) was then added and incubated 30 min at 37°C followed by five washes in wash solution (distilled water with 0.05 % Tween 20). Tubes containing the nasal swabs were thawed, mixed vigorously using a vortex mixer, and 90 μL transport medium were added to the wells followed by addition of NP-40 detergent (10 μL of 10 % v/v in phosphate buffered saline). Plates were incubated for 45 min at 37°C, followed by eight washes in wash solution. A monoclonal antibody, with previously documented specificity for nucleoprotein of BRSV (Stott, 1984a) (#6; Institute for Research on Animal Diseases, Compton, Newbury, Berkshire, UK), was diluted 1: 300 in working buffer (0.01M phosphate buffer pH 7.2, 0.05 % Tween 20, 4.5 % NaCl, 2 % NRS), and 100 ml was added to microtiter wells. The plates were incubated for 30 min at 37°C and washed eight times. This was followed by biotinylated rat anti-mouse antibodies (Zymed Laboratories, San Francisco, California), diluted 1:200 in working buffer and, subsequently, avidin-biotin complex reagent (Vectastain-ABC, Vector Laboratories, Burlingame, California, USA), diluted to 1 drop of each reagent A and B per 20 mL of PBS with 2% NRS. The plates were incubated for 30 min at 37°C and washed eight times between steps. One hundred μL of peroxidase substrate (ABTS; Kirkegaarde and Perry Laboratories, Gaithersburg, Maryland, USA) were added and the reaction stopped after 10 min by the addition of 100 μL of 1% sodium

dodecyl sulphate (SDS). Optical densities (OD) were read at a wavelength of 405 nm using a 96 - well microplate reader (Model #3550; Bio-Rad Laboratories, Richmond, California, USA). Each ELISA plate contained 8 wells of positive ($5x10^3$ PFU SBAH-RB94, a 1/200 dilution of standard stock virus in virus transport medium) and negative (nasal secretions collected before infection) controls. A sample was considered positive if the OD was greater than the mean OD of the negative controls plus 2 times the standard deviation of the mean of the negative controls. BRSV ELISA units were assigned using a standard curve generated by linear regression of serial dilutions of stock virus (Malvano, 1982). The standard curve encompassed the range of OD's obtained for the positive samples and the standardized dilution of the positive control was near the center of the curve. To standardize results between plates, units were further transformed to a ratio of sample units/ positive control units.

To determine if the N protein capture ELISA could detect different isolates of BRSV, supernatants from cell cultures infected with SBAH-375, SBAH-RB94, MN, WCVM, and Mohanty isolates of BRSV were diluted 1/500 in PBS and performed in an assay conducted on the same plate as a titration of SBAH-165. The stability of the viral antigens was investigated by repeating the assay on 12 specimens after 2 additional freeze-thaw cycles, followed by storage at 4°C for 24 h.

7.2.3.4 Polymerase Chain Reaction

The PCR assays were done by Andre Hamel at Manitoba Agriculture, Veterinary Services Branch, Winnipeg, Manitoba. The swabs were stored at -70°C, then shipped overnight on ice packs, thawed, and a one-tube reverse transcription PCR assay

performed using 100 μL of transport medium as previously described (Hamel, 1995), using primers specific for the F protein of BRSV according to previously described sequences (Oberst, 1993b). Subjective visual assessments of relative band density were made using a 1 to 4+ scale to obtain a crude quantitative assessment of shedding in positive samples for comparison with the other assays.

7.2.3.5 Virus Isolation

Virus shedding was quantitatively determined on 100 μL of transport medium by a microisolation plaque assay with a maximum sensitivity of 10 PFU per mL. The swabs were thawed, vortexed and aliquots of the transport medium were serially diluted in DMEM (Life Technologies, Grand Island, New York, USA) supplemented with 5% FBS (Life Technologies, Grand Island, New York, USA), 50mM HEPES, 200 μg/mL sodium penicillin G, 200 μg/mL streptomycin sulfate and 0.5 μg/mL amphotericin B in 96-well tissue culture plates. Specimens were refrozen at -70°C until used in the NP ELISA. Positive (SBAH-RB94 and Asquith inoculated) and negative (day 0 nasal secretion samples) were included in each assay. EBTR cells were added to the wells, and the medium was changed after 2 h incubation at 37°C. The plates were incubated for a further 54 h at 37°C in 5% CO_2 and then fixed with 80% acetone in PBS for 15 min. Viral plaques were identified by staining with an avidin-biotin immunoperoxidase technique utilizing a 1:1000 dilution of a monoclonal antibody specific for the F protein of HRSV (Taylor, 1992) (#19; Institute for Research on Animal Diseases, Compton, Newbury, Berkshire, UK) as the detection antibody, followed by 1:1000 biotinylated rabbit anti-mouse (Vector Laboratories, Burlingham, California, USA), avidin- biotin

complex (Vectastain-ABC, Vector Laboratories, Burlingham, California, USA) and diaminobenzidene. We have subsequently used a 1: 3000 dilution of a rabbit polyclonal antibody to HRSV (Dako Corporation, Carpinteria, California, USA) as the detection antibody followed by a 1: 1000 dilution of biotinylated goat anti-rabbit (Zymed Laboratories, San Francisco, California), with similar results. Antibodies were diluted in Blotto Buffer (50 mM Tris HCL, pH 8.5, 10 mM EDTA, .05% Tween 20, 1% Triton X-100, 2% skim milk.) with incubations at 37°C for 1 h. Plates were washed 4x in PBS with 0.05% Tween 20 between steps. To assess virus stability, the assay was repeated on twelve nasal swabs subjected to two more freeze-thaw cycles and again after a further 24 h at 4°C.

7.2.3.6 Statistical Analysis

The degree of linear association between quantitative results of the different assays were established by Pearson correlation's, the significance of differences between repeated assays of the same samples were determined by paired t tests, and inter-assay agreement between positive and negative samples was expressed by the kappa statistic using computerized software (Statistix; Analytical Software, Tallahassee, Florida, USA and SAS Institute Inc, Cary, North Carolina, USA).

7.2.4 Results

7.2.4.1 Comparative Analysis of Assay Results

There was agreement between all three assays in 67/83 samples, with negative results in all day 0 samples. Discrepancies between assays are summarized in Table A1. Differences in the ability to detect virus shedding between the three assays occurred at

the beginning and end of detectable shedding. Nasal virus shedding was detected by virus isolation in all calves for a minimum of 4 consecutive days, starting in all but one of the calves on day 2. Virus isolation and the NP ELISA appeared equally sensitive in detecting early shedding with a single, but different, calf negative by each assay. The calf negative by isolation was positive by both NP ELISA and PCR. Four calves were negative by PCR on day 2 that were positive by virus isolation, a trend that was reversed on days 6-8, with 9 specimens on these days positive by PCR that were negative by isolation. The NP ELISA also yielded positive results in four of the same 9 cases, but all calves were negative on day 8 by both the NP ELISA and isolation. The PCR assay extended the duration of detection of BRSV to day 8 for 3 calves. The percent agreement for day 2-8 specimens between the various assays was highest for virus isolation and the NP ELISA at 91.5% (kappa 0.839), followed by PCR and the NP ELISA at 86% (kappa 0.720). The lowest agreement was between isolation and PCR at 77.5% (kappa 0.570).

Table A.1 Testing of 83 nasal secretion samples from experimentally infected calves for bovine respiratory syncytial virus by nucleoprotein ELISA (ELISA), polymerase chain reaction (PCR), and microisolation plaque assay (VI)

	ELISA +	ELISA -
VI+/PCR+	47	0
VI+/PCR-	5	1
VI-/PCR+	5	5
VI-/PCR-	0	20
Total	57	26

7.2.4.2 Quantitative Assessment of Virus Shedding

A similar pattern of virus shedding was obtained from the results of the NP ELISA and microisolation assay, with peak virus excretion occurring on days 3-5 after infection (Figure A1). There was a correlation coefficient of 0.72 ($p<0.001$) between PFU and NP ELISA units for the day 2-8 nasal swabs. The subjective visual quantitative estimation of shedding by PCR appeared more closely correlated to the ELISA units ($R = 0.63$), than the PFU as determined by microisolation ($R = 0.42$).

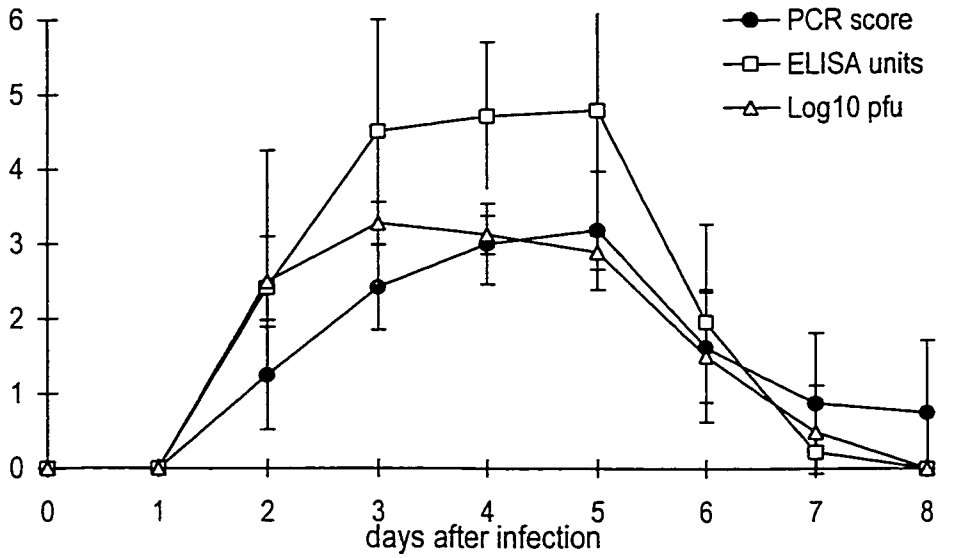

Figure A.1 Quantitative shedding of BRSV in the nasal secretions of 12 experimentally infected calves as determined by nucleoprotein ELISA and microisolation plaque assay, (mean ± SE). PFU = plaque forming units per ml. The PCR score represents a subjective assessment of band density on a 1-4 scale, not a true quantitative assessment.

7.2.4.3 The Effect of Storage Conditions on Virus Isolation and the nucleoprotein ELISA

After two additional freeze thaw cycles, there was a mean reduction in PFU of 79.5% ($p<0.05$) when compared to the results of the original microisolation assay. Storage at 4°C for a further 24 hr resulted in a mean reduction of >99%, with 5/12 samples becoming negative. The same sample treatment resulted in small (25%) but significant ($p<0.05$) drop in ELISA units in the NP ELISA. All samples remained positive (data not shown).

7.2.4.4 Ability of the nucleoprotein Elisa to Detect Different BRSV Isolates

The mean ODs obtained with the standardized dilution of cell culture supernatants of the 6 BRSV isolates were each greater than 1.0, indicating strong positive reactions.

7.2.5 Discussion

Field cases of acute respiratory disease caused by BRSV are difficult to diagnose antemortem due to the lack of pathognomonic signs and the difficulty of isolating the agent from clinical samples (Baker, 1985b; Dubovi, 1993). Under experimental conditions, virus isolation has been used to quantitate virus shedding with levels and duration of shedding similar to that reported in this experiment (Kimman, 1989e; Taylor, 1995b). The very high isolation rate obtained from nasal swabs in these experimental situations is most likely due to the immediate inoculation of the samples. In this experiment rapid -70°C cryo-preservation of the samples and possibly the high magnesium sulfate concentration in the transport medium contributed to preserved virus

viability. Stabilization of human RSV, with no loss of infectivity after multiple freeze-thaw cycles or several days at 4°C, has been reported using a similar medium containing 1M $MgSO_4$ and 1.2M sucrose (Fernie, 1980). The toxicity of this medium in the microisolation assay required the concentrations used in this experiment to be reduced to 0.3M and 0.4M respectively. The markedly reduced rate of virus recovery from specimens exposed to conditions likely to be encountered in transport, indicate, that at this reduced concentration, this medium is unlikely to increase the success rate of virus isolation in field specimens to an acceptable level. A higher concentration of $MgSO_4$ may result in better preservation of infective virus, which could be inoculated into larger tissue culture vessels, but this would increase both the time and the expense of this assay.

The use of a monoclonal antibody specific for the nucleoprotein of BRSV in an antigen capture ELISA proved to be a rapid, inexpensive and sensitive technique that could be used to screen large numbers of clinical specimens for BRSV during an acute infection. Virus shedding was detected by ELISA in five specimens which were negative on isolation. Four of these were on days 6 and 7 suggesting that neutralization of the virus by rising mucosal antibody may have resulted in apparent lack of shedding as detected in the microisolation assay, or that viral antigen is detectable for a longer period of time than viable virions. The NP ELISA appeared equally sensitive in detecting several different isolates of BRSV that were grown under standard laboratory conditions. The use of a monoclonal antibody that is specific for an invariant structural protein of the virus diminished the possibility that the assay might not detect disparate field isolates of BRSV, as could occur with the use of monoclonal antibodies specific

for envelope glycoproteins of respiratory syncytial viruses. These proteins contain epitopes that may vary among different isolates of respiratory syncytial viruses (Lerch, 1989; Baker, 1992). The NP ELISA lends itself to easy quantitation of virus shedding with significant correlation to virus isolation. The NP ELISA could be used in place of standard titration methods to quantitate the amount of BRSV for use in laboratory procedures. The small drop in sensitivity after multiple freeze-thaw cycles and storage at 4°C, indicates it is a significantly more robust assay than isolation for the detection of BRSV in field specimens. This is in agreement with previous investigations which have indicated that the proteins of respiratory syncytial viruses maintain their antigenicity under common conditions of transport (McIntosh, 1982).

The suitability of nasal swabs, in this experiment, as specimens for the detection of BRSV infection by the PCR technique is in agreement with a previous investigation (Vilcek, 1994), where a similarly high sensitivity was reported. There were more discrepancies between the results of this assay and both the NP ELISA or virus isolation, than between each other, but the kappa values indicate a high degree of agreement between all assays (Martin, 1987a). Some of these differences, particularly where virus shedding was minimal, may be explained by the two assays being done on identically collected, but different swabs, but the possibility of false negatives early in the infection cannot be ruled out. In this experiment, the PCR technique may have had reduced sensitivity relative to the other two assays early in the infection, but had apparently greater sensitivity later in the infection. Shedding was detected in 3 calves through to day 8, in one case at high levels. This calf was clinically the most severely affected of the remaining calves, but was BRSV negative on day 8 in both the NP

ELISA and by virus isolation. It is possible that high levels of nasal antibody may either neutralize virus infectivity, or block detection by the NP ELISA. It is also worth noting the PCR results obtained in this experiment were performed on specimens subjected to normal field transportation conditions (overnight ground transport on ice packs), but controlled freeze-thaw conditions were not investigated.

In conclusion, we have presented two alternatives to virus isolation for the detection of BRSV in clinical specimens that should be applicable in most research or diagnostic laboratories. Both the nucleoprotein ELISA and the PCR technique have a similar sensitivity, comparable to, or exceeding, virus isolation done under ideal conditions, but without the stringent specimen handling requirements of the latter.