CYTOKINE MESSENGER RNA EXPRESSION IN CALVES VACCINATED INTRANASALLY WITH MODIFIED-LIVE BOVINE RESPIRATORY SYNCYTIAL VIRUS (BRSV) PRIOR TO BRSV CHALLENGE

by

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(Under the direction of Amelia R. Woolums)

ABSTRACT

In order to investigate mechanisms of cellular immune responses in bovine respiratory syncytial virus (BRSV) infected and vaccinated calves, total RNA was isolated from various sites of 3 groups of calves: vaccinated with modified live (ML) BRSV intranassally (i.n.) followed by challenge (V/C); mock vaccinated followed by challenge (M/C); and mock challenge only (control). Expression of several cytokines was measured by reverse transcriptase-competitive-PCR (RT-cPCR). To normalize the amount of cytokine cDNA, HPRT was used as a housekeeping gene, and the ratio of HPRT cDNA/competitor was kept in the range of 0.6-1.5. Relative ratios were calculated by the formula: (cytokine cDNA/competitor) / (HPRT cDNA/competitor). Groups were compared with Kruskall-Wallis test and Dunn's post test for statistical analysis. Tumor necrosis factor-alpha (TNF-α) was measured in cranial lung and bronchoalveolar lavage fluid cells. Similar results were seen in both sites. Increased levels were present in M/C vs. V/C, and there was no difference between V/C and control. Interleukin-4 (IL-4) and interferon-gamma (IFN-γ) were measured in pharyngeal tonsil and tracheobronchial lymph node. Relatively increased IL-4 expression occurred in the pharyngeal tonsil in

M/C calves, and relatively increased IFN-γ expression occurred in the tracheobronchial lymph node in M/C calves. The results indicated a Th2 bias in pharyngeal tonsil and a Th1 bias in tracheobronchial lymph node in M/C which was diminished in V/C calves. Intranasal vaccination may have prevented disease following challenge in part through causing a more balanced cytokine response to challenge.

INDEX WORDS; BRSV, Cellular immune responses, Intranasal vaccination, Modified live virus vaccine, TNF-α, IL-4, IFN-γ, RT-cPCR.

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CHAPTER 1

INTRODUCTION

Bovine respiratory syncytial virus infection is a major cause of respiratory disease in calves during the first year of life. There is a high prevalence of seropositive animals in cattle populations indicating that exposure to BRSV is common.

Bovine respiratory syncytial virus and human RSV are antigenically and biochemically closely related and share common epidemiological, clinical and pathological characteristics (Van der poel et al., 1994). Human beings and cattle are considered to be natural hosts of RSV. BRSV is therefore not only an important pathogen in its own right, but is also an idea model for the study of HRSV.

Bovine respiratory syncytial virus infection is established in epithelial cells in the airways and alveoli in the cranioventral portion of the lung resulting in destructive changes, followed by inflammation and repair (Baker, 1991). The viral surface G and F glycoproteins are the two major neutralizing and protective antigens and they are therefore targets of vaccine development. Since RSV disease was discovered in 1955, many studies were attempted to determine mechanisms of pathogenesis and to develop safe and effective vaccines. Direct cytopathology may play a minor role and accumulated data indicate that immune-mediated mechanisms play a dominant role. Some important points regarding the immune response are as follows: Humoral immune responses are not enough to protect host from BRSV infection. Maternal antibodies may prevent effective immunization. Attenuated virus vaccination can induce high titers of IgG but low levels of neutralizing antibodies. Cellular immune responses may play an important role in

protection. $CD8^+$ T cells are crucial to viral clearance. γδ T cells seem not to be tightly associated with BRSV infection. $CD4^+$ T cells are important yet often unappreciated in antiviral immunity. However, cytokines produced by CD4 may also contribute to disease. It is commonly believed that a Th1 response, characterized in part by increased production of interferon-gamma (IFN-γ), is associated with protection; while a Th2 response, characterized in part by increased of interleukin-4 (IL-4), is associated with disease. Therefore study of cytokine responses to BRSV infection and vaccination will be useful to understand mechanisms of disease and protection.

Cytokine expression is tightly regulated, because the pattern of cytokine expression can determine the outcome of the immune response (O'Garra and Murphy, 1994). Interleukin-4 (IL-4) and interferon-gamma (IFN-γ) are known to be important in cross regulation of CD4 Th cell activation. Tumor necrosis factor-alpha (TNFα) is a proinflammatory cytokine initiating and modulating inflammatory responses. Therefore these three cytokines represent signals of distinct immune pathways.

A problem that has hampered RSV vaccine development is vaccine-enhanced disease. Formulation of vaccines and route of vaccination are two major factors that affect the host immune response. In one study in calves, MLV intranasal (i.n.) BRSV vaccination was more effective than killed vaccine i.n. or MLV vaccine administered intramuscularly (i.m.), even in the presence of maternal antibody (Kimman et al., 1989a). However, nothing is known about the cytokine response to i.n. BRSV vaccination. In the research described here, mRNA for IFN-γ, IL-4, and TNF-α was measured by reverse transcriptase-competitive PCR in various sites of calves vaccinated with MLV BRSV i.n. prior to challenge. Reverse transcriptase-competitive PCR (RT-cPCR) is an ideal method

to detect expression of these cytokine genes because it provides sufficient sensitivity and specificity to measure low level mRNA expression in small amounts of tissue.

CHAPTER 2

LITERATURE REVIEW

Epidemiology

Bovine respiratory syncytial virus is a viral agent often associated with severe epizootics of bovine respiratory disease especially in younger dairy and beef calves (Larsen, 2000), resulting in substantial economic losses to the cattle industry. It causes yearly outbreaks of respiratory disease in cattle all over the world. Seasonal periodicity of RSV infection is typical with highest incidences in autumn and winter. Stress such as that caused by movement, crowding and temperature changes are considered to play a role in bovine outbreaks (Van der poel et al., 1994). The morbidity is high (80%-100%) and the mortality is variable, ranging up to 20%. A higher seroconversion rate to BRSV (71.3%) than other respiratory viruses such as infectious bovine rhinotracheitis virus, bovine viral diarrea virus and parainfluenza virus type 3 (Martin and Bohac, 1986) leads to high prevalence of seropositive animals in the cattle population. Moreover, exposure to BRSV can induce T and B cell memory in young calves without causing seroconversion, probably due to the presence of maternal antibody inhibiting the calves from developing an antibody response (Sadbut and Roth, 2002). Taken together this information indicates that exposure to BRSV is very common. Also, there is a strong correlation between BRSV infection and the occurrence of respiratory disease. It has been demonstrated to act synergistically with bacteria in causing pneumonia (Baker, 1991). In one controlled study BRSV infection was significantly associated with feedlot acute respiratory distress syndrome (ARDS) (Collins et al., 1988).

The mode of transmission during the course of natural infection has not been defined, but direct contact is probably required. However experimental data suggest that aerosol transmission may also be possible over short distances (Mars et al., 1999). Congenital BRSV infection has never been reported. However, abortions during a BRSV outbreak have once been observed in cattle in Japan (Inaba et al., 1972). Transplacental transmission cannot be excluded since RSV antibodies have been detected in fetal bovine serum (Gould et al., 1978). Up until now, the persistence of human or bovine RSV in their natural hosts has never been demonstrated. Van der poel et al. (1997) failed to demonstrate direct evidence for persistent BRSV. But Valarcher et al. (2001) provided evidence for the persistence of small amounts of BRSV in the pulmonary lymph nodes of calves at least 71 days after infection. They demonstrated the in vivo persistent infection of B-lymphocytes by BRSV with active translation and transcription of structural genes more than 10 weeks after infection of the calves and also demonstrated that BRSV readily caused a persistent infection of B-lymphocyte cell lines in vitro for 6 months.

History

Respiratory syncytial virus was originally identified in 1955 after an outbreak of infection in chimpanzees. This was quickly followed by the demonstration that this virus represented a new human pathogen, after being detected in lung secretions from individual infants presenting with pneumonia and bronchiolitis (Morris et al., 1956; Chanock et al., 1957a,b). The new virus, initially named “Chimpanzee Coryza Agent”, caused characteristic syncytial masses in cell culture and hence was later renamed respiratory syncytial virus (Chanock et al., 1957a). In the early 1960’s, the importance of HRSV in respiratory disease of children worldwide was established. In the 1970’s, a

virus closely related to HRSV was isolated from calves during an epizootic of severe respiratory disease in Switzerland. It was named bovine RSV (BRSV). Since then, several surveys confirmed that BRSV is endemic in calf populations throughout the world (Larsen, 2000).

Bovine respiratory syncytial virus and human RSV share many epidemiological, clinical and pathological features (Van der poel et al., 1994). Both viruses' infections are common in the young, whereas infection in adults results in less severe disease. Maternal antibodies do not prevent infection, but may be protective to a certain degree. The two viruses cause similar lesions including lymphocytic bronchiolitis and bronchiolar epithelial necrosis, bronchiolar occlusion, parenchymal inflammation and alveolar exudation (Baker, 1991). Because of the similarities between BRSV and HRSV, information about HRSV will be included in this review.

Structure

Bovine respiratory syncytial virus and HRSV are members of the genus Pneumovirus, subfamily Pneumovirinae, family Paramyxoviridae (Murphy et al., 1995). They are similar in gene and protein compositions and are antigenically related at the level of the nucleocapsid (N), phosphoprotein (P), matrix (M), and fusion (F) proteins, with the major antigenic difference present in the attachment glycoprotein (G) (Lerch et al., 1989; Hacking and Hull, 2002). The RSV genome is composed of single stranded negative sense RNA of 15200 nucleotides encoding 11 proteins: envelope proteins (F, G, SH); nucleocapside proteins (L, N, P, M2-1); matrix proteins (M, M2); nonstructural proteins (1B, 1C). There is a great deal of information available about the nucleotide and amino acid sequences of HRSV. Human RSV is divided into two major antigenic

subgroups, A and B. The main differences between these two groups are located in the G protein, with other differences also occurring in the F protein and N protein (Mufson et al., 1985; Norrby et al., 1986). Studies indicate the A subgroup is more common and may be more pathogenic. In contrast, variability in dominance of supposed BRSV subgroups has not been reported so far. BRSV A, B and AB, an intermediate strain between A and B, have been proposed subgroups based on reactivity with monoclonal antibodies (mAbs) directed against the G protein. Subgroups A and AB were associated with severe respiratory disease (Furze et al., 1994; Schrijver et al., 1996). Baker et al. (1992) claimed to have distinguished two subgroups of BRSV based on differences in the molecular size of the P and F proteins. Subgroups within the G and F proteins of BRSV have also been characterized by serological analysis of a limited number of isolates and confirmed by phylogenetic analysis (Furze et al., 1994; Prozzi et al., 1997; Schrijver et al, 1996). Single amino acid changes at certain positions in the cysteine noose of the G protein have major structural and functional consequences and thereby determine the subgroup distinction. The biological significance of the appearance of different subgroups of BRSV has not yet been assessed, but some degree of cross protection is evident. The rate of evolution of the BRSV sequences varies according to the gene, with the G protein gene evolving more rapidly and being highly tolerant to the fixation of mutations, whereas the N and F protein genes appear to be under stronger selective structural constraints which limit their evolution (Valarcher et al., 2000). The ability to reconstitute infective RSV particles by introducing engineered viral genes into cells was a break-through in RSV research and is responsible for much of understanding of the function of the proteins in the virus

(Hacking and Hull, 2002). Use of this system has shown that the genes NS1, NS2, SH, M2-2 and G can be mutated while maintaining viral viability (Schmidt et al., 2001).

The surface F and G gycoproteins are the only viral components that induce RSV neutralizing antibody; they are therefore targets of vaccine development. The G protein is naturally synthesized both as a type II membrane-anchored glycoprotein and also as a smaller soluble form, which lacks the cytoplasmic domain and part of the membrane anchor sequence and is suggested to redirect the immune response away from infected cells, enhancing release of virus (Can, 2001). Vaccination with soluble G can induce eosinophilia in the primary immune response or following subsequent RSV challenge, with increased production of IL-5, IL-13 and eotaxin, but no IL-4 requirement (Graham et al., 2000). The G protein was not essential for in vitro cell passage, but acted as an accessory protein which increased the efficiency of the process (Teng and Collins, 1998). Monoclonal antibodies (MAbs) to the HRSV or BRSV G protein are subtype or strain-specific (Taylor et al., 1984; Furze et al., 1994; Melero et al., 1997). The BRSV peptide BG/174-187 derived from the G protein efficiently prevented BRSV-associated pneumonia in the gnotobiotic, RSV seronegative calves (Bastien et al., 1997). In the BALB/c mouse, sensitization with the G expressed by recombinant vaccinia induces CD4+ T cells that produce type 2 cytokines in vitro and are responsible for lung eosinophilia during secondary challenge in vivo (Hussell and Openshaw, 1999). The G protein does not generate a detectable MHC class I restricted CD8+ T cell response but does induce a strong memory G protein-specific CD4+ T cell response in the lungs characterized by a mixture of Th1 and Th2 cytokine-secreting cells (Srikiatkhachorn and Braciale, 1997a,b; Srikiatkhachorn et al., 1999).

The F protein is a type I, membrane-bound, glycoprotein that is responsible for the fusion of the viral and host cell membranes and for the generation of syncytia between infected cells. It is synthesized as a precursor (F_0), which consists of the F_2 domain, the cleavage peptide and the F_1 domain (Larsen, 2000). Fusion is thought to begin with an initial movement of the F_2 protein, and F_1 is destabilized, undergoes a conformational rearrangement, unmasking the fusion peptide and facilitating its insertion into the host cell membrane. Of the surface proteins, only the F has emerged to date as a target for therapeutic intervention, both as the epitope for monoclonal antibodies and vaccines and as a target for small-molecule antiviral agents (Meanwell and Krystal, 2000). Studies show that MAbs to the F protein are cross-reactive between HRSV and BRSV, indicating that B cell epitopes on the F protein of HRSV and BRSV are highly conserved (Taylor et al., 1992; Arbiza et al., 1992). The F protein induces both CD4+ and CD8+ T cells that are cytolytic and CD4+ T cells that produce type 1 cytokines (Hussell and Openshaw, 1999; Srikiatkhachorn and Braciale, 1997b). Immunization with an immunoaffinity-purified preparation of the RSV F glycoprotein was also shown to protect the lower respiratory tracts but not the upper respiratory tracts of rodents against RSV challenge (Walsh et al., 1987). However, some investigators showed that cotton rats immunized with purified F had altered immune responses (high levels of F antibody relative to RSV neutralizing antibody) and enhanced histopathologic changes in cotton rats challenged with wt RSV (Murphy et al., 1990). In a study of CD4+ T cell epitopes on F and G of BRSV, CD4+ T cell activation was not examined using assays other than proliferation and it is therefore possible that both the G and the F protein may contain epitopes that activate bovine T cells for cytokine production but not for proliferation. Studies to

analyze the cytokine responses of BRSV-specific T cells to F and G protein peptides are in progress (Fogg et al., 2001).

The function of the SH protein is not fully defined, but it probably enhances the F mediated fusion of membranes and thereby contributes to the formation of syncytial (Pastey and Samal, 1997). The function of the matrix protein is not clear, but its association with the membrane suggests that it may be involved in the interaction between the viral nucleocapsid and the cell membrane prior to assembly, maturation and budding of the virions. The M protein may also interact with the NS1 protein (Evans et al., 1996). In a study of the association of the RSV M protein with the plasma membrane, it was shown that the M's membrane binding is stabilized by the surface expression of the viral glycoproteins and that F appears to pull M into lipid rafts (Henderson et al., 2002). The M2 protein contains the immunodominant class I epitope for BALB/c mice. Incorporation of the M2 gene into vaccinia, or mucosal vaccination with the peptide epitope in conjunction with the adjuvant LTK63, primes strong CTL responses (Simmons et al., 2001). The N protein is a multifunctional protein that plays a central role in paramyxovirus replication. Using a yeast two-hybrid system, the BRSV N protein was shown to interact with both itself and the P protein (Krishnamurthy and Samal, 1998). Khattar et al. (2000) recently identified the regions of the N protein involved in transcription, replication, and encapsidation of viral RNA. The N protein is also an important antigen for RSV-specific human and murine cytotoxic T-lymphocytes and for murine helper T-lymphocytes (Bangham et al., 1986; Openshaw et al., 1988). Respiratory syncytial virus is unique among paramyxoviruses in having 2 genes coding for non-structural (NS) proteins preceding the nucleoprotein, which have anti-IFN-α, β activity.

The NS1 (1C) is a potent inhibitor of transcription and RNA replication (Atreya et al., 1998). The NS2 (1B) is not necessary for propagation of the virus in vitro (Buchholz et al., 1999); however, deletion of the NS1 or NS2 genes results in a marked reduction in replication efficacy in vivo (Teng et al., 2000; Whitchead et al., 1999).

Pathogenesis

Respiratory syncytial virus infects both ciliated and nonciliated cells in small airways, resulting in a bronchiolitis characterized clinically by wheezing. Bronchoalveolar lavage fluid reveals a predominance of polymorphonuclear leukocytes in respiratory secretions (Graham et al., 2002). Bovine respiratory syncytial virus replication is established in epithelial cells in the airways and alveoli in the cranioventral portion of the lung resulting in destructive changes, followed by inflammation and repair (Baker, 1991). Replication of BRSV takes place in the luminal lining of the respiratory tract. The virus is capable of cell-to-cell spread resulting in the generation of the characteristic syncytial giant-cells. Syncytia are observed in bronchiolar walls and in alveoli and such syncytia were always replicating BRSV (Viuff et al., 1996). The virus can also replicate in monocytes and lymphocytes (Keles et al., 1998). Although there was no evidence that BRSV replicated in bovine monocyte derived dendritic cells (MoDC) or influenced the expression of MHC class I, class II, or CD80/86, the virus did appear to affect MoDC survival (Werling et al., 2002).

The pathogenesis of RSV infection is not entirely clear. Historically, several hypotheses have been proposed (Baker, 1991). 1) It may involve a type 1 hypersensitivity reaction. The severe form of HRSV infection may be mediated by production of HRSV-specific IgE antibody. IgE bound to HRSV-infected nasopharyngeal cells has been

detected and HRSV-specific IgE antibody has been found in nasal secretions (Welliver et al, 1980; Welliver et al, 1984). The quantity of virus-specific IgE and histamine in nasopharygeal secretions at the time of HRSV infection correlates with the severity of infection (Bui et al., 1987). Limited investigations have been done on the role of RSV-specific IgE in BRSV infections. Stewart and Gershwin found a correlation between disease severity and the production of BRSV-specific IgE in experimentally infected calves, providing evidence that a type-1 hypersensitivity response can have a role in BRSV pathogenesis (Stewart and Gershwin, 1989a,b). 2). Serum antibodies may participate in an immunopathologic reaction through a mechanism similar to an Arthus reaction. Observations on the naturally occurring respiratory tract disease associated with BRSV and the results from experimental infections indicate that passively derived antibody does not prevent infections, but maternal antibody can decrease severity (Kimman et al., 1988; Stott et al., 1980). Although it is assumed that immune complex formation occurs in BRSV infection, no definitive evidence for involvement of type 3 hypersensitivity in the pathogenesis of BRSV infections has been demonstrated. 3) It may involve a type 4 hypersensitivity. The role of cell-mediated immunity in the pathogenesis of RSV was first suggested based on the observation that children vaccinated with an experimental formaldehyde inactivated RSV vaccine (FI-RSV) developed enhanced clinical disease upon subsequent infection. Increased blastogenesis responses were seen in children vaccinated with FI-RSV, suggesting involvement of type 4 hypersensitivity. More recent research in mice, humans, and cattle indicates that the balance of T helper cytokines produced during infection influences outcome (described below).

While accumulated data indicate that immune-mediated mechanisms play a dominant role in disease due to RSV, some aspects of the immune response are necessary for protection. The immune response to RSV infection is comprised of an innate response including actions of epithelial cells and alveolar macrophages, followed by activation of humoral and cellular specific immunity (Hacking and Hull, 2002). Respiratory epithelial cells are the first defense in innate immune response to virus. Alveolar macrophages play an important role against RSV as they may regulate the immune response, and release inflammatory cytokines during infection. The cellular immune response seems to be important in controlling the infection once initiated and for clearance of virus. Although the humoral response is not likely to be helpful in influencing the course of a primary infection once it has occurred, there is good evidence that protection from subsequent infections is mediated through antibodies (Hacking and Hull, 2002).

Also, BRSV enhances bacterial colonization and adherence and alters the specific and non-specific defense mechanism of the respiratory tract (Larsen, 2000).

Humoral Immunity

Despite decades of research on immunity and the immunopathological mechanisms involved in the pathogenesis of RSV infections, it has not been possible to prove a clear link between protection and level of actively produced or passively acquired antibodies in natural RSV infection. However, it is commonly believed that antibodies contribute to protection. Passively transferred monoclonal antibodies and polyclonal antibodies can protect the lungs of mice and cotton rats against RSV infection (Hemming and Prince, 1992). Studies of anti-μ-treated mice showed that although antibody was not required to terminate RSV replication in a primary infection, only partial immunity against challenge

was induced in the absence of antibody (Graham et al., 1991). In general, humoral immune responses involving secretory antibodies and serum antibodies appear to protect against infection of the upper and lower respiratory tract respectively, while cell-mediated responses directed against internal proteins appear to terminate infection (Dudas and Karron, 1998). In young individuals, antibody responses are limited because of immaturity of the immune system and interference by maternal antibodies (Brandenburg et al., 1997).

Respiratory syncytial virus infection induces much higher titers of antibodies that bind to RSV F or G proteins in ELISA than neutralize RSV in vitro. Subsets of RSV-specific antibodies neutralize virus and a smaller subset of these antibodies inhibit virus-mediated fusion in vitro (Crowe Jr, 2002). In response to RSV infection, antibodies against the F- and G-proteins are probably most important. Local antibodies in respiratory tract appear to be related to protection against reinfection; virus neutralizing antibodies correlate with protection against experimental infection in adults, and IgA coincides with virus clearance (Brandenburg et al., 2001). Serum RSV IgG protects the lower respiratory tract against infection while secretory IgA appear to play a major role in protection in upper tract (Crowe Jr et al., 2002). It has been argued that maternal antibodies are harmful or helpful. In the past it was suggested that maternal antibodies may form immune complexes to stimulate release of inflammatory mediators by neutrophils which could contribute to pathological process. However, in infants maternal antibodies have a protective role in the first month of life and passive antibody therapy is effective in preventing disease in high-risk children (Groothuis, 1995). There is also evidence that maternal antibodies, particularly antibodies to the G protein of RSV, have a

protective role in young children. (Murphy et al., 1986a). In calves maternal antibodies may be protective to a certain degree, since the severity and incidence of disease in calves less than 3 months of age seem to be inversely related to the serum level of BRSV specific antibodies (Kimman et al., 1988; Stott et al., 1980).

Experiments on the kinetics of the antibody responses to BRSV indicated that antibody detected by fluorescent antibody was detected on PID 3 with maximum titers appearing approximately on PID 10; low neutralizing antibody was detected on PID 3 and titers peaked approximately 4.5 weeks after inoculation and then decreased (El Azhary et al., 1980). Most calves experienced an increase in the specific IgM and IgG1 titers about 6-10 days; the IgM titer was transient showing positive titers for only 5-10 days, while specific IgG1 was present for a longer time. IgA was detected concomitantly with IgM but at a lower level, and production of IgG2 was detected from 3 weeks after infection (Uttenthal et al., 2000). In a bovine model of FI-BRSV vaccine enhanced disease, vaccination did not induce neutralizing antibodies, but viral specific IgG was detected (Gershwin et al., 1998). Although both attenuated live virus and inactivated-virus induced antibodies that recognized the F protein, only live virus induced neutralizing antibodies (Ellis et al., 1992). In many studies, the neutralizing antibodies persisted in serum for months (Larsen, 2000). IgM and IgA were the only classes of antibodies found in secretions from the eye, nose and lungs of calves 8-10 days after infection (Kimman et al., 1987). Protection is not clearly associated with the presence of IgA at the mucosae at the time of challenge but with the ability to mount a rapid and strong response. A peculiarity of the IgA mucosal response is the apparently long duration (Kimman and Westenbrink, 1990). Virus administered intramuscularly to

seronegative calves or infection of calves with maternal antibodies failed to induce a primary mucosal response, but did prime for a local memory response as revealed by a strong, rapid and probably protective response at rechallenge. But this protection was not so much associated with the amount of mucosal IgA as with the speed of the mucosal IgA response. (Kimman et al., 1989a). In infants, the development of RSV-specific IgA in nasal secretion correlated temporally with disease clearance (McIntosh et al., 1978).

The mechanisms by which maternal antibody affects antibody production by B cells are not clear. It is generally thought that immunosuppression by passive antibody results from antigen being bound and neutralized. In cattle, pre-existing maternal antibodies were reported not to protect against BRSV infection but did reduce the disease severity, since the severity and incidence of disease in calves less than 3 months of age was inversely related to the serum level of maternal derived BRSV-antibodies (Belknap et al., 1991; Kimman et al., 1988; Stott et al., 1980). In the presence of maternal antibodies, mucosal and systemic IgA reponses are inhibited in calves, and serum BRSV-IgG responses are suppressed to a greater degree than BRSV-IgM (Kimman et al., 1987).

One possible mechanism by which RSV can induce infant allergies and asthma could be through increased IgE and IgG4 production. Increased expression of CD23 in B cells in RSV-infected patients suggests that CD23, a low-affinity IgE receptor, could play a role in IgE regulation and pathogenesis (Rabatić et al., 1997). Wellivier et al. (1981) measured high concentrations of histamine and RSV-specific IgE in the nasopharyngeal secretions of children who had the most severe form of the disease. Calves responding to BRSV infection with virus-specific IgE production develop more severe disease than those that do not (Stewart and Gershwin, 1989a).

Cellular Immunity

BRSV may adversely affect the normal functions of various inflammatory cells such as macrophages, neutrophils and lymphocytes (Sharma and Woldehiwet, 1991), although bovine alveolar macrophages as well as human or murine alveolar macrophages exhibit a high intrinsic resistance to RSV (Schrijver et al., 1995). Both HRSV and BRSV significantly depress the proliferative responses of PBMC to phytohaemagglutinin (PHA) in vitro (Preston et al., 1992). Cellular immunity to RSV involves both the innate and adaptive immune responses and includes NK cells, macrophages, and $CD4^+$ and $CD8^+$ T cells and their products. Although T cells are important in recovery from RSV infection, there is evidence that they also contribute to the pathogenesis of disease (Cannon et al., 1988; Alwan et al., 1992). Cattle can acquire T-cell responsiveness to BRSV while remaining seronegative to the virus, and these calves with T-cells primed against BRSV respond more rapidly and vigorously to vaccination than naïve animals (Sadbut and Roth, 2002). In mice, CTL are involved in both HRSV clearance from lung and lung pathology.

Following BRSV infection there was a recruitment of $CD8^+$ T cells into the trachea and lung, which peaked on day 10 after infection (Mcinnes et al., 1999). It has been demonstrated that the F, N and M2 proteins are recognized by BRSV-specific bovine $CD8^+$ T cells in MHC class I defined cattle (Gaddum et al., 2003). $CD8^+$ cells in BRSV-infected calves may exert an important effect by acting as cytotoxic T cells. However, Ostler et al (2002) identified that it was IFN-γ, not CD95 ligand or TNF or perforin, that was as a key molecule mediated virus clearance and immunopathology by $CD8^+$ T cells during RSV infection. Several studies revealed a significant $CD8^+$ T-cell response in peripheral blood of calves and lambs (Thomas et al., 1996; Sharma et al, 1990) after

experimental infection with BRSV. Depletion of $CD8^+$ T cells in experimentally infected calves affected neither the serum nor local antibody responses to BRSV and resulted in delayed viral clearance and increased the severity of lung consolidation, whereas depletion of $CD4^+$ cells suppressed the antibody response (Taylor et al., 1995; Thomas et al., 1996).

$CD4^+$ T cells play an important yet often unappreciated role in antiviral immunity by providing help for B cells in generating antiviral antibodies as well as secreting cytokines. However studies using the BALB/c model have demonstrated that $CD4^+$ T cells can also prove detrimental to the host by mediating immunopathology and exacerbating disease (Varga et al., 2002). In vitro stimulation of PBMC with different RSV antigen preparations showed a type 1 like cytokine response upon stimulation with live RSV and RSV-F protein whereas a type 2 like response was found after stimulation with FI-RSV or RSV-G protein (Jackson and Scott, 1996). These results indicated that RSV infections may induce both type 1 and type 2 like T cell responses. Furthermore, significant proliferative responses of $CD4^+$ T cells and increased IL-2 and IFN-γ concentrations were observed in calves following vaccination with modified live or inactivated vaccines (Ellis et al., 1992).

Passive transfer of RSV-specific $CD8^+$ CTL lines or clones or $CD4^+$ lines caused clearance of virus from the lungs of RSV-infected mice but resulted in a lethal pulmonary disease, and $CD4^+$ T cells cause more severe pathological changes than $CD8^+$ T cells (Cannon et al., 1988; Alwan et al., 1992). It seems that in the mouse model, $CD8^+$ cells are potent downregulators of Th2 responses and disease severity, presumably through secretion of IFN-γ. In contrast, $CD4^+$ cells exhibit immunopathogenic potency, with the

most pronounced effect on weight loss when they are of the Th2 phenotype (van Schaik et al., 2000). In calves $CD4^+$ cells were activated during BRSV infection as indicated by changes in expression of CD45R, CD45RO, L-selectin and IL-2 receptors (Mcinnes et al., 1999).

In 1986, Mosmann et al reported that most cloned lines of murine $CD4^+$ T cells could be classified into two groups, Th1 and Th2, based on the cytokines they produced and their related functional activities (Mosmann et al., 1986). However, this paradigm is an oversimplification and may not be accurate in the bovine. Studies with bovine Th cell clones and immunoregulatory cytokines indicated that Th cell clones coexpress IL-4 and IFN-γ, and polarized cytokine profiles were rarely observed (Brown et al., 1998). However, bovine IL-4 regulated IgG1 expression and IFN-γ regulated IgG2 expression (Estes and Brown, 2002) which is a pattern similar to that occurring in mice. Gershwin et al demonstrated both Th1 and Th2 cytokine profiles in pulmonary lymph before and during BRSV infection indicating that calves do not have highly polarized Th1 and Th2 responses of syngeneic mice infected with RSV (Gershwin et al., 2000)

Another relevant point regarding the bovine cell-mediated immune response is that a difference in the phenotypic composition of bronchoalveolar and peripheral blood lymphocytes in cattle has been observed. BAL T cells were predominately $CD8^+$ characterized by low expression of CD45R and activated $CD25^+$ while peripheral blood T cells were predominately $CD4^+$ and high proportion of γδ T cells. This confirms that selective lymphocyte migration to the normal bovine lung does occur (McBride et al., 1997).

It has been suggested that γδ T cells may present a first line of defense against pathogens and alterations in γδ T-cell numbers do occur during some respiratory virus infections (Carding et al., 1990; Ogasawara et al., 1994). However, previous studies have shown that the course of BRSV infection in calves is not affected by depletion of $WC1^+$ γδ T cells (Taylor et al., 1995). Changes in γδ T-cell numbers were not detected in BAL from HRSV-infected mice (Openshaw, 1991) or in peripheral blood, lung, BAL, trachea and BLN from BRSV-infected gnotobiotic calves (Mcinnes et al., 1999). Taken together these observations indicate that γδ T cells do not have a major role in RSV infection.

Neutrophils appear to be the predomiant cell in BAL from infants with HRSV (Everard et al., 1994). They may play an important role during early phases of RSV infections, by acting against RSV infection at the site of viral replication, and by attachment to RSV-infected cells and subsequent release of mediators. Faden et al (1984) reported that adherence of neutrophils to RSV-infected cells is directly proportional to the extent of RSV replication. RSV-specific antibodies have been shown to enhance the adherence of PMN to human RSV-infected cells in a dose-dependent manner in vitro. Current evidence indicates that viral infection induces neutrophil migration into the inflammatory sites; neutrophil-epithelial adhesion is increased; neutrophil apoptosis is up-regulated in RSV-infection (Wang and Forsyth, 2000).

Overall, many studies have demonstrated the importance of a strong $CD8^+$ T-cell response in the clearance of BRSV in calves. $CD4^+$ T-cell may contribute both to pathogenesis and also to recovery from disease. The role of γδ T-cell does not appear to be significant.

Cytokine Responses

Cytokines are small proteins secreted by a variety of cells (NK cells, T-cells, macrophages, epithelial cells, B-cells etc). They affect cell behavior in a variety of ways and play key roles in controlling the growth, development, and functional differentiation of lymphocytes and other cells. In this regard they act as effector molecules of activated cells. Many cytokines bind to receptors that use a particularly rapid and direct signaling pathway to effect changes in gene expression in the nucleus (Janeway Jr et al., 2001). Thus, cytokines are immunomodulatory proteins that have an important role in the development of the repertoire of immune cells that respond to infection. They also provide important information about the immune response. Cytokine production by T cells appears to be one of the key factors determining the outcome of BRSV infection in calves. Studies of the cytokine response during BRSV infection should contribute to a better understanding of the pathogenesis and immune response to BRSV disease.

Cytokines are involved in cell-cell communication and activation and have been classified into Th1 and Th2 subfamilies. Cytokine expression is tightly regulated because the pattern of cytokine expression can determine the outcome of the immune response (O'Garra and Murphy, 1994). It is generally believed that type-1 or type-2 cytokine production by particular lymphocyte subset determines the clinical outcome of different disease (Mosmann and Sad., 1996). IL-4 and IFN-γ are two important cytokines that play a major role in the regulation of cellular immune responses.

Because the balance of Th1/Th2 cytokines has been associated with outcome following RSV infection, quantitation of cytokine IFN-γ/IL-4 expression is important in the analysis of the immune response to RSV infection and vaccination. The role of

different antigens of RSV, T cell subsets and cytokines in influencing the nature of host's antiviral immune response and outcome of infection has been the subject of intense recent study. In a mouse model of RSV vaccination followed by challenge, an enzyme-linked immunspot assay demonstrated that $CD4^+$ T cells were responsible for the production of IL-4, while many cell types secreted IFN-γ (Tang et al., 1997). In a murine mode of RSV infection, mice which had been sensitized to RSV F protein, developed a protective immune response predominantly driven by Th1 cells, involving the production of IL-2 and IFN-γ. In contrast, those sensitized to RSV G protein developed strong Th2 responses against the virus which exacerbated disease, involving IL-4 and IL-5 with resultant local accumulation of inflammatory cells, particularly eosinophils. The F protein primed for a strong CTL response, unlike the G protein which failed to elicit a detectable CTL response unless presented in association with M2 (Srikiatkhachorn and Braciale, 1997a). In infants, after in vitro restimulation of peripheral blood cells, intracellular IL-4 and IFN-γ were measured by flow cytometry. The cells from RSV-infected infants produced more IL-4 and less IFN-γ than those from healthy controls. IL-4 production was more frequent in CD8 than in CD4 cells, and bias toward IL-4 production was greatest in infants with mild infections, whereas IFN-γ production increased with disease severity (Bendelja et al., 2000). In contrast, in a study of the cytokine mRNA response to RSV in human PBMC, Th1 cytokine-specific mRNA levels increased, while no detectable Th2 cytokine-specific mRNA (Anderson et al., 1994). Certain cytokine gene polymorphisms moderate immune responses and illness severity in adults experimentally exposed to RSV (Gentile et al., 2003). The IFN-γ genotype was directly related with the frequency of subjects having at least a four-fold increase in RSV-specific serum IgG and TNF-α

genotype was inversely associated with the frequency of subjects having at least a two-fold increase in RSV-specific nasal IgA.

T helper 2 immune responses mediated by the secretion of IL-4, IL-5 and IL-13 are key in the pathogenesis of asthma. Interleukin-4 and IL-13 mediate B cell isotype switching and production of IgE. The binding of allergen-specific IgE to mast cells results in allergic sensitization. Interferon-gamma production by Th1 cells can inhibit Th2 differentiation; however it may also enhance established allergic disease by increasing inflammatory responses. (Lewis, 2002). It has been suggested that RSV bronchiolitis is related to the subsequent development of asthma because increased IL-4/IFN-γ ratios are sometimes present in RSV infection (Roman et al, 1997; Bendelja et al, 2000).

IFN-γ, a type II IFN, is made by NK and T cells during the immune response to viral infection and has many biological effects. The properties of IFN-γ include direct antiviral activity, help in the generation of antigen presentation through induction of expression of MHC class I and II molecules, and activation of NK cells. In addition, IFN-γ has a role in the regulation of the switch of antibody isotypes, including the switch in expression by B cells from IgM to IgG2a (Boehm et al., 1997). Agents that trigger IL-12 production by macrophages promote IFN-γ synthesis. IFN-γ induces transcription of genes using multiple mechanisms, some of which require the synthesis of new cellular proteins (Sen, 2001). In general, IFN-γ is considered to be a key cytokine in inducing protective responses against viral pathogens. However, IFN-γ production by Th1 cells may also augment Th2-dependent pathology in murine models of asthma (Hofstra et al., 1998). Although IFN-γ has important protective effects in RSV infection, a role for IFN-γ

has also been mentioned in the pathogenesis of RSV disease. It is known to be a potent activator of mononuclear phagocytes, neutrophils, and mast cells, causing these cells to release inflammatory mediators (Boehm et al, 1997). Interferon-gamma knockout mice or mice administrated with anti-IFN-γ antibody developed less severe disease following RSV infection than controls. Paradoxically, administration of recombinant IFN-γ had a suppressive effect on disease. Therefore, it was concluded that exogenous IFN-γ alleviates the asthma-like inflammation while endogenous IFN-γ contributes to disease (Hessel et al., 1997; Hofstra et al, 1998). Finally, IFN-γ receptors have been demonstrated on eosinophils, and in vitro studies have shown activation of eosinophils by IFN-γ (Valerius et al., 1990); thus any enhancement of RSV disease by IFN-γ could be due to these effects on eosinophils.

IL-4 is mainly produced by subsets of the T lymphocyte lineage, mast cells and basophils. Increased levels of IL-4 have been detected in biological samples from several disease states including bronchial asthma and allergy (Mire-Sluis and Thorpe, 1998). Interleukin-4 is known to stimulate B cells to preferentially make IgG4 and IgE, to inhibit Th1 production of IFN-γ, and to increase expression of its own receptor. Interleukin-4 is often associated with the secretion of IL-5, which increases IgA production and induces eosinophilia; IL-6, which drives B cell differentiation; and IL-10, which further inhibits IFN-γ production by Th1 (Renz et al., 1991). In addition, IL-4 has a chemotactic effect on eosinophilis (van Schaik et al., 2000). It is not known whether this set of events is intrinsically harmful to the host, but once the Th2 pattern of cytokine production is initiated it is self-perpetuating because of the IL-4 autocrine effects and the inhibition of the complementary Th1 cells (Street and Mosmann, 1991). The presence or absence of

IL-4 in disease has resulted in different strategies to increase IL-4 levels or inhibit its activity depending on the disorder (Mire-Sluis and Thorpe, 1998). Increased IL-4/IFN-γ ratios have been mentioned in RSV infected infants (Roman, et al, 1997). Additionally, IL-4 was detected only in PBMC from BRSV infected gnotobiotic calves but not in cells from uninfected animals (McInnes et al, 1998), indicating that IL-4 expression is associated with RSV infection.

Little is known about inflammatory mediators in BRSV disease. Tumor necrosis factor-alpha is a proinflammatory cytokine, produced by stimulated monocytes and macrophages, with many biological activities including initiation and modulation of inflammatory responses. It can be directly toxic to pulmonary endothelial cells, acting synergistically with other endogenous mediators or endotoxin; it may also act indirectly by stimulating the recruitment, adherence and degranulation of neutrophils in lung tissues to induce acute lung injury (Goldblum et al., 1989). Tumor necrosis factor-alpha and IL-1β are known to promote transendothelial neutrophil passage, augmenting neutrophil sequestration in the lung (Moses et al., 1989). Therefore, the biological effects induced by TNF-α could explain the presence of purulent exudates, edema and atelectasis in the BRSV-infected lung. Tumor necrosis factor-alpha seems to be an important cytokine to look for in the lungs of BRSV-infected calves. Large amount of TNF-α are produced in the BAL fluids of calves on day 7 after BRSV infection with severe lung lesions and clinical signs, but no infectious BRSV could be isolated from the lungs of six out of eight BRSV-infected calves (Røntved et al., 2000). These findings indicated that TNF-α may involved in both the pathogenesis of the disease and the immune defense directed against the BRSV infection.

In summary, some cytokines have been associated with protection against disease due to RSV, while others have been associated with pathogenesis. Studies of the cytokine response to RSV will be important in evaluating candidate vaccines.

Vaccination

Despite 45 years of HRSV research there is still no completely effective treatment nor any immediate prospect of a vaccine for HRSV. There are two major problems affecting the development of an HRSV vaccine: First, prior immunity can enhance the severity of natural disease; second, natural infection does not produce solid cross-reactive or even specific protection against reinfection (Crow, 1998). Any immunization would need to be done early in life, as the disease is most severe in the very young. A living vaccine that expresses a surface determinant recognized by neutralizing antibody might be rendered less effective by maternal derived immunoglobulin. Early trials with formalin-inactivated vaccines had disastrous consequences, with many children developing more severe disease when subsequently infected with the virus (Doherty, 1994). Several commercial BRSV vaccines are currently in use in cattle (Baker et al., 1997). However in rare cases vaccine-enhanced disease has also been reported, one associated with killed virus (Gershwin et al., 1998) and one associated with MLV (Kimman et al, 1989b). The successful development of an RSV vaccine will require a better understanding of the pathogenesis of primary infection, the factors that affect susceptibility to reinfection, and the immunopathology of enhanced illness in children immunized with a non-replicating RSV candidate vaccine.

A key impediment to the development of RSV vaccines is a lack of understanding of the enhanced disease that occurred in children who received a FI-RSV vaccine.

Serological studies showed that recipients of FI-RSV developed high titers of serum antibodies to RSV F protein as measured by ELISA with poor neutralizing activity and fusion-inhibiting activity (Murphy et al., 1986b; Murphy and Walsh, 1988). In mice, priming immunization with FI-RSV followed by challenge induced a Th-2-like response (Waris et al., 1996; Graham et al., 1993). The depletion of $CD4^+$ T cells (Connors et al., 1992) or IL-4 or IL-10 (Tang and Graham, 1995) before challenge abrogated the enhanced histopathologic changes in mice previously immunized with FI-RSV. These findings suggested that the FI-RSV did not prime for $CD8^+$ cytotoxic T-cell responses and the viral infection produced an unchecked cytopathic effect in the lower respiratory tracts of infants. In addition, FI-RSV vaccination mediated a Th2 response with increased IL-4, IL-5, IL-10, and an influx of lymphocytes and eosinophils (Dudas and Karron, 1998).

The effect of FI-RSV vaccination has also been studied in cattle. In an early study, Mohanty et al demonstrated that FI-BRSV vaccination elicited a moderate level of serum antibodies and did not cause exacerbation of a mild respiratory tract disease after challenge infection of these calves (Mohanty et al., 1981). West et al found that vaccination with FI-BRSV vaccine resulted in more rapid onset of clinical disease, mild to moderate peribronchial eosinophil infiltrate, but reduced pulmonary pathology in most calves (West et al., 1999). But in another study, there was significantly greater disease FI-BRSV vaccinated calves followed by BRSV infection and histological lesions in calves that were in some ways similar to those of affected children. Only IgG was detectable by ELISA, no neutranizing antibodies were induced (Gershwin et al, 1998), and IFN-γ production by PBMC was decreased (Woolums et al., 1999).

Enhancement of BRSV-associated respiratory tract disease is not a generic response to all inactivated virus vaccines. Ellis et al (2001) demonstrated that an inactivated BRSV vaccine provided clinical protection from experimental infection and efficacy was similar to modified-live BRSV vaccines. In this study, vaccinated calves had increased BRSV-specific IgG, and virus neutralizing antibody titers and IFN-γ production. Virus neutralizing antibody titers were consistently less than IgG titers. In the 1980s, British studies revealed that an inactivated vaccine was more effective than live attenuated virus or natural infection in providing protection against BRSV. The inactivated vaccine was one that consisted of glutaraldehyde-fixed bovine nasal mucosal cells that were persistently infected with BRSV (Stott et al., 1984; Taylor et al., 1989). This vaccine protected against an inflammatory response in the lungs of calves with decreased macrophages and increased neutrophils (Taylor et al., 1989). This vaccine was not marketed outside Europe and has not been tested in North American calves.

When compared with killed immunogens, modified-live virus (MLV) vaccines generally have the advantages of longer duration of immunity and stimulation of cellular effector mechanisms, in addition to humoral responses (Tizard, 1987). A previously study documented that MLV-BRSV reduced respiratory tract disease in calves (Bohlender, 1984). Another study showed that MLV-BRSV effectively stimulated humoral responses in cattle and boosted at least a 16-fold increase in neutralizing antibody (Ellis et al., 1990) which has been associated with reduced risk of treatment for respiratory tract disease (Martin and Bohac, 1986). In another study of the efficacy of commercial MLV BRSV vaccines, clinical efficacy was associated with reduced viral shedding, reduced pulmonary pathology, vaccine-induced immune responses and enhanced post challenge

antibody responses (West et al., 2000). Although both activated and inactivated vaccines induced production of antibodies that recognized the F protein, the ratio of neutralizing antibody titer to IgG concentration was lower for calves that received an inactivated virus vaccine than for calves that received the MLV vaccine. Both vaccines induced lymphocyte proliferative responses to BRSV (Ellis et al., 1995). In an experiment with mice, vaccine antigen formulation appeared to be the key factor influencing the nature of the RSV-induced immune response. Killed virus priming was associated with increased IL-4 mRNA in the lung, while live virus priming intramuscularly resulted in relatively greater expression of IFN-γ after RSV challenge (Tang et al., 1997).

As previously noted, the formulation and delivery of a vaccine can influence the nature of the immune response to challenge. Challenge of mice primed with live RSV by parenteral or mucosal routes induced a Th1-like pattern of cytokine mRNA expression (Graham et al., 1993). Li et al. (1998) engineered an optimized plasmid DNA vector expressing the RSV F protein. They found that the route of vaccination appeared crucial: intramuscular priming produced a Th1 response, whereas intradermal priming favored Th2 development. When Remco et al. administered a vaccine composed of a plasmid carrying a synthetic gene encoding for the BRSV G protein by different methods, they found that intradermal application with a needleless injector was better than intramuscular or intradermal vaccination at reducing virus shedding and increased serum antibodies against G protein following challenge (Schrijver et al., 1998). Intramuscular BRSV priming of calves with maternal antibodies or colostrum-deprived calves did not result in mucosal memory and protection (Kimman et al., 1989a). This observation was also found in a field trial (Zygraich, 1989). A similar immunization induced some

resistance to infection in cotton rats (Prince et al., 1979), but was not effective in children (Belshe et al., 1982). However, MLV BRSV i.n. vaccination was protective even in the presence of maternal antibody (Kimman et al., 1989a). This has also been shown for live attenuated HRSV vaccines, administered as nasal drops (Dudas and Karron, 1998).

The goal of RSV vaccination is not to prevent RSV infection but rather to prevent RSV-associated lower respiratory tract illness (Dudas and Karron, 1998). The surface F and G proteins are the only viral components that induce RSV neutralizing antibody; they are therefore important targets of vaccine development (Dudas and Karron, 1998). Synthetic peptides corresponding to the amino acid region 174-187 of the G glycoprotein from HRSV, containing serine substituted for cysein at position 186, completely protect immunized mice against RSV infection (Trudel et al., 1991; Simard et al., 1997). Similarly, this peptide of bovine G protein coupled with keyhole limpet hemocyanin (KLH), completely protected mice from BRSV infection and reduced pneumonic lesions in vaccinated gnotobiotic calves, although none of calves were protected from upper respiratory tract disease (Bastien et al., 1997). Purified F protein given in Freund's adjuvant i.m. failed to induce VN antibodies following vaccination or experimental challenge in a majority of calves (Nelson et al., 1992). Immunization of lambs with recombinant baculovirus-expressed F (Bac-F) protein resulted in significant humoral and cellular immune responses. Bac-F also induced a significant anamnestic responses and inhibited virus shedding after BRSV challenge infection (Sharma et al., 1996). Little is known about DNA vaccination in cattle. A DNA plasmid vaccine coding for the G protein of BRSV could partially protect calves against BRSV infection (Schrijver et al., 1997).

RT-cPCR

As noted above, quantification of cytokine expression is necessary to completely analyze the immune mechanisms of BRSV vaccination and infection. In the laboratory, several methods have been applied for the measurement of cytokine expression including Northern blot analysis, ELISA, RNase protection assay (RPA) and enzyme immunoassay (EIA). Both Northern blotting and RPA are time-consuming, laborious and expensive. Harvested tissues may not be large enough to measure cytokines by EIA at the protein level in a single sample (Deng et al., 2003). Northern blot analysis and dot or slot blot analysis typically require several micrograms of total RNA with detection limit of approximately 10^5-10^7 molecules (Ferre et al., 1994). Most importantly, the above methods are not sensitive enough to detect some cytokines expressed at a relatively low level, such as IL-4 (Deng et al., 2003). Real-time PCR methods allow measurement of mRNA expression of cytokines faster and more accurate (Stordeur et al., 2002), but they are also expensive.

Reverse-transcriptase (RT)-PCR provides sufficient sensitivity and specificity to measure low level mRNA expression in small amounts of tissue. It can be performed with small quantities of total RNA and can detect transcripts representing as little as 6 pg of total RNA in a single cell (Fuqua et al., 1990). Zarlenga developed RT-competitive PCR to quantify bovine cytokine gene expression. Competitor molecules for the selected cytokines were constructed. These competitors share primer sequences with the target cDNA, and are co-amplified during PCR. However, the competitors are constructed so the resulting amplicon is of slightly smaller size than the target cDNA. This method is fast and accurate and minimizes differences in the efficiency of the PCR amplification

between the competitor and the cDNA (Canals et al., 1996). This assay is based on competitive co-amplification of a specific target template sequence together with known amounts of a competitor in the same test tube (Zimmerman and Mannhalter, 1996). A model of competitive PCR is presented in Figure 1. A serial dilution of competitor is added to a constant amount of cDNA. Following amplification, PCR products are analyzed by gel electrophoresis. Upper bands are target cDNA and lower bands are competitor. The amount of cytokine gene can be deduced from the known concentration of the competitor (Zimmerman and Mannhalter, 1996). However, two conditions must be met: one, the molar quantity of the competitor must be known. It can be measured by UV spectrophotomrtry; two, the amplification efficiency of the competitor and target must be identical. This is true because two DNAs share same primers (Anonymous). To ensure comparable RNA in a serials of samples, housekeeping genes are usually quantitated in addition, and the copy number of each cytokine cDNA is normalized to a fixed copy number of housekeeping gene cDNA (Montagne et al., 2001). It has been demonstrated that quanitative measurements of PCR-amplified products are most accurate when ratios of cDNA and competitor are equal or similar (Arnold et al., 1992; Raeymaekers, 1993). Zarlenga has indicated that for the hypoxanthine phosphoribosyl transferase (HPRT, housekeeping gene) ratios (cDNA/competitor) to be meaningful, they must be between 0.6-1.5. Outside of this range significant amplification bias is a problem. In order to get a good standardization of the cDNA content, normalization should be done in a range where HPRT signal will not be artificially selected (Zarlenga, personal communication).

In RT-cPCR, amplification over many cycles allows the PCR product to be visualized and quantitated by ethidium bromide staining (Gilliland et al., 1990). Since the

RT-PCR technique is based on an exponential amplification of the target sequence, it is an extremely sensitive technique. Because of these characteristics, RT-cPCR was chosen for measurement of cytokine mRNA in the research presented here.

CHAPTER 3

LITERATURE CITED

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CHAPTER 4

CYTOKINE MESSENGER RNA EXPRESSION IN CALVES VACCINATED INTRANASALLY WITH MODIFIED-LIVE BOVINE RESPIRATORY SYNCYTIAL VIRUS (BRSV) PRIOR TO BRSV CHALLENGE [1]

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[1] Cytokine messenger RNA expression in calves vaccinated intranasally with modified-live bovine respiratory syncytial virus (BRSV) prior to BRSV challenge, Miao, C., Brown, C., Brown Jr, J.C., Williams, S., Scott, M., Zarlenga, D.Z., Woolums, A.R. Submitted to *Veterinary Immunology and Immunopathology*, 03/20/2003.

1. Abstract

The objective of this study was to characterize the cellular immune response as measured by cytokine mRNA expression in various sites of calves vaccinated intranasally (i.n.) with modified live (ML) bovine respiratory syncytial virus (BRSV) prior to BRSV challenge. Calves received live high passage BRSV i.n. (V/C, n=4) or mock vaccine i.n. (M/C, n=5) followed by challenge; a third group receiving only mock challenge (control, n=3) was also included. Clinical signs were evaluated daily; calves were euthanized at day 7 postchallenge. Total RNA was isolated from cranial lung, bronchoalveolar lavage fluid (BAL) cells, pharyngeal tonsil and tracheobronchial lymph node. Cytokine mRNA was quantified by reverse transcriptase-competitive PCR (RT-cPCR). Message for TNF-α was measured in lung and BAL cells; message for IL-4 and IFN-γ was measured in pharyngeal tonsil and tracheobronchial lymph node. Hypoxanthine phosphoribosyl transferase (HPRT) was used as the housekeeping gene. Differences between groups were evaluated with the Kruskall-Wallis test followed by Dunn's post test. Vaccinated calves were protected from severe disease following challenge. TNF-α mRNA levels in cranial lung and BAL were higher in the M/C group than in the control group ($p<0.05$), they were also higher than in group V/C although the difference was not statistically significant. There was no significant difference between the control and the V/C groups. In the pharyngeal tonsil, there was no difference in IL-4 and IFN-γ mRNA levels between the control and the V/C groups. IL-4 and IFN-γ mRNA levels and IL-4/IFN-γ ratios were higher in the M/C group than in controls ($p<0.05$); there was no significant difference between M/C and V/C groups. In the tracheobronchial lymph node, there was no significant difference in IL-4 message expression between groups. Interferon-gamma

mRNA expression was decreased in the V/C group compared to the M/C group ($p<0.05$); there was no significant difference in IFN-γ mRNA expression between the M/C and the control groups. No significant differences were observed in the IL-4/IFN-γ expression ratios between groups. These results suggest that TNF-α expression in lung and BAL is associated with disease caused by BRSV. BRSV infection caused a Th2 bias in pharyngeal tonsil and a Th1 bias in tracheobronchial lymph node. Protection due to i.n. vaccination led to decreased pulmonary inflammation as measured by TNF-α expression in lung and BAL, and changes in Th1/Th2 balance in the pharyngeal tonsil and tracheobronchial lymph node.

Key words: BRSV; Cytokines; RT-cPCR; Modified live virus vaccine; Intranasal vaccination; Cellular immune response

Abbreviations:

BRSV: bovine respiratory syncytial virus

RT-cPCR: reverse transcriptase-competitive PCR

M/C: mock vaccinated, BRSV challenged

V/C: modified live BRSV i.n. vaccinated, BRSV challenged

BAL: bronchoalveolar lavage

HPRT: hypoxanthine phosphoribosyl transferase

MLV: modified live virus

2. Introdution

Respiratory syncytial virus, a pneumovirus within the paramyxoviridae family, is a leading cause of respiratory disease in human infants and calves (Heilman, 1990; Van der

poel et al., 1994; Larsen, 2000). Bovine respiratory syncytial virus (BRSV) and human RSV are antigenically and biochemically closely related and share common epidemiological, clinical and pathological characteristics (Van der poel et al., 1994). The pathogenesis of RSV infection is not clear, but direct viral cytopathology may play a minor role and the majority of injury may result from the host immune/inflammatory response (Varga and Braciale, 2002). Only partial immunity is achieved despite repeated infection.

The role of cell-mediated immune responses to BRSV infection is not clearly defined. CD8 cells apparently play an important role in protection against disease, possibly through BRSV specific cytotoxic activity (Gaddum et al., 1996). They may also influence the response by producing cytokines such as interferon-gamma. The role of CD4+ T cells is more complicated and uncertain. A hypothesis that RSV bronchiolitis may be the result of relatively increased production of Th2-type cytokines has become popular. Interleukin-4 (IL-4) secreted by Th2 cells is considered to be associated with disease, possibly in part because it induces immunoglobulin production by B cells with isotype switching towards IgE (Gershwin et al., 2000). Interleukin-2 and IFN-γ secreted by Th1 cells provide help for generation of CTL and thus are associated with protection (van Schaik et al., 2000b). However, studies in human infants with RSV (Brandenburg et al., 2000) or in mice with RSV (van Schaik et al., 2000a) provided evidence that IFN-γ production may contribute to RSV-induced wheezing. This has not been reported for BRSV, and in fact research suggests that IFN-γ production is associated with protection against BRSV infection (Woolums et al., 1999a; West et al., 2000**).** Because it has been shown that type1/type2 cytokines imbalances may play an important role in the

pathogenesis of RSV disease (van Schaik et al., 2000b), studies of cytokine responses to BRSV will be helpful to clarify the role of T cell cytokines in the pathogenesis of disease in cattle.

Studies of the cytokine responses to BRSV vaccination should aid in the development of safe and effective vaccines. Although many BRSV vaccines are marketed in the USA, in rare cases vaccine-enhanced disease has also been reported, one associated with a killed virus vaccine (Gershwin et al., 1998) and one associated with a modified live virus (MLV) BRSV vaccine (Kimman et al., 1989a). There is still no licensed RSV vaccine for humans (Crowe Jr, 2002). Because infection is widespread and disease can be severe, there is a clear requirement for effective vaccines against both HRSV and BRSV. Much research has been carried out to identify the ideal form of vaccine and route of vaccination. Graham et al. (1993) found that challenge of mice primed with live RSV by nasal routes induced a Th1-like pattern of cytokine mRNA expression which was associated with protection against challenge. In one study in calves, modified live virus (MLV) i.n. BRSV vaccination was more effective than killed vaccine i.n. and MLV vaccine administrated intramuscularly (i.m.), even in the presence of maternal antibody (Kimman et al., 1989b). While i.n. vaccination may be a safe and effective means of protecting calves against BRSV challenge, little research has focused on i.n. BRSV vaccination. Specifically, the impact of i.n. vaccination on cytokine expression has not been studied.

The objective of this study was to characterize the cellular immune response as measured by cytokine mRNA expression in various sites of calves vaccinated i.n. with MLV BRSV prior to BRSV challenge. Interleukin-4 and IFN-γ are known to be

important in cross regulation of CD4+ TH cell activation (Street et al., 1991). Tumor necrosis factor-alpha (TNF-α) is a cytokine with many biological activities including initiation and modulation of inflammatory responses (Kumkel et al., 1990; Vilček et al., 1991) and is implicated in the pathogenesis of disease due to RSV (Hayes et al., 1994; Røntved et al., 2000). Therefore these three cytokines were measured as representative signals of distinct immune pathways. The first hypothesis tested was that following BRSV challenge, mRNA expression for TNF-α and IL-4 would be increased, and mRNA expression for IFN-γ would be decreased. The second hypothesis tested was that MLV i.n. vaccination would lead to decreased mRNA expression for TNF-α and IL-4, and increased mRNA expression for IFN-γ (Table 1).

3. Materials and Methods

3.1. Animals and Sample Collection

Twelve conventionally reared calves 8-12 weeks old were divided into three groups held in isolation. Due to the length of time required for sample collection, calves were studied in pairs, with vaccinated calves separated from mock vaccinated calves to prevent spread of the vaccine BRSV to unvaccinated calves. Details of vaccination are described elsewhere (Woolums et al, submitted). Briefly, calves were vaccinated at day 0 with 2 ml live high passage low virulence BRSV (VT-794, American Type Culture Collection, Manassas, VA) i.n. at 1×10^{7} $TCID_{50}$/ml. Calves were challenged at day 30 post vaccination via aerosol exposure as previously described (Woolums et al., 1999b); each calf received 6 ml ($5 \times 10^{4} - 1 \times 10^{5}$ $TCID_{50}$ units/ml) of a low pass, high virulence isolate (gift of Dr. L. Gershwin) by aerosol. Four calves were vaccinated and challenged

with BRSV (V/C). Five calves received 2 ml mock vaccine (spent tissue culture media) i.n. followed by BRSV challenge (M/C). The number of calves in the V/C and M/C groups was initially equal, but due to the development of unrelated disease between the time of vaccination and challenge in some calves, the final numbers are unequal. Three calves received mock challenge (6 ml spent tissue culture media) as a negative control for challenge. Clinical signs were evaluated as previously described (Woolums et al., submitted). Calves were euthanized at day 7 postchallenge with an intravenously administered dose of barbiturate. A 2x2x1 cm section of the right cranial lung, a portion of the pharyngeal tonsil and a portion of a tracheobronchial lymph node were removed promptly and quickly frozen in liquid nitrogen before storage at -80˚C. For bronchoalveolar lavage (BAL), a snugly-fitting adapter (#501, 5-in-1 Tubing Connector, Busse Hospital Disposables, Hauppauge NY) was inserted into the left cranial bronchus and 500 ml of sterile isotonic saline was infused by moderate manual pressure through an intravenous delivery set attached to the adapter. After infusion, fluid was allowed to run through the delivery set into sterile glass bottles; in general 25-50% of the total volume infused was recovered. The fluid was stored on ice and returned to the laboratory within one hour, when a manual cell count was done on a hemocytometer. Total cells recovered ranged from $2 \times 10^7 - 7 \times 10^8$. Cells were collected by centrifugation at 200 g and the cell pellet was suspended in 15 ml Trizol (Invitrogen, Carlsbad, CA) sheared by 4-5 passes through an 18 gauge needle, and stored at –80˚C until RNA was isolated.

3.2. Total RNA Isolation

Total RNA was extracted from tissue or cell samples by homogenization with Trizol (Invitrogen, Carlsbad, CA). RNA precipitates were washed with 75% ethanol, dried, and resuspended in DEPC-treated water. In order to prevent contamination by genomic DNA, each RNA sample was treated with Rnase Free DNase I (Roche Diagnostics GmbH, Mannhei, Germany) for 30 minutes in a water bath at 37˚C. DNase I treatment was followed by Trizol-chloroform extraction. RNA was quantified by absorbance measurements at 260nm and the purity was assessed by the 260:280nm ratio. Values ranged from 1.8-2.0. Total RNA was run in agarose gel electrophoresis. Clear bands corresponding to 18S and 28S RNA could be identified from total RNA (Figure 2), suggesting minimal degradation of total RNA had occurred between euthanasia and RNA isolation.

3.3. RT-cPCR Conditions

RNA was incubated at 65˚C for 5 minutes, then reverse transcription was performed using oligo-dT primers and murine leukemia virus Reverse Transcriptase (MuLV RT) at 42˚C for 45 minutes and 95˚C for 3 minutes. Generated cDNA was kept on ice prior to use.

Competitor molecules for bovine TNF-α, IL-4, IFN-γ and HPRT were provided by Dr. Zarlenga. Plasmid DNA was isolated with Concert Rapid Plasmid Miniprep System (GibcoBRL, Paisley, UK). Three serial dilutions of competitor molecules were made and 5ul of each dilution was added to three different tubes. For competitive-PCR, 5 ul target cDNA, 0.1ul DNA polymerase (0.5U), and a master mix containing 25mM $MgCl_2$, 10×

PCR buffer II and primers shared by both DNAs was added to each tube for a final volume of 25ul. The mixture was cycled 35 times at 95˚C for 15 sec, 60˚C for 20 sec, 70˚C for 20 sec followed by a terminal extension phase of 72˚C for 7 min. For each cytokine both DNA species hybridize competitively to the same primers (Table 1) (Canals et al., 1997). Target cDNA of unknown concentration was deduced from the concentration of competitor DNA.

3.4. Gel Electrophoresis and Analysis of Products

PCR products from each tube were separated on a 1.8% agarose gel and stained with ethidium bromide, in parallel with a 100-bp DNA molecular weight marker (Figure 3). Electrophoresis was performed in Tris-Acetate EDTA (TAE) buffer. Ethidium bromide stained gels were examined under UV illumination to demonstrate visible reaction products. Analysis of PCR reaction products was performed using Gel expert soft ware (Nucleo Tech, San Mateo, CA) and Microsoft Excel (Microsoft Corporation, Seattle, WA).

The amount of cytokine mRNA was calculated by the relative ratio:

$$\frac{\textit{intensity cytokine cDNA / intensity cytokine competitor}}{\textit{intensity HPRT cDNA / intensity HPRT competitor}}$$

For TNF-α detection (Figure 3A) HPRT competitor DNA was at 6000 fg and TNF-α competitor DNA was at 100 fg. For IL-4 and IFN-γ detection (Figure 3B), HPRT competitor was at 46.9 fg, IL-4 competitor DNA was at 25 fg and IFN-γ competitor was at 50 fg.

3.5. Statistical Analysis

Relative ratios were evaluated using Kruskall-Wallis test and Dunn's post test (GraphPad prism version 3.02 for windows, Graph Pad Software, San Diego, CA). The difference between each group was considered significant if the p value was < 0.05.

4. Results

4.1. Clinical data

Results of clinical data are presented elsewhere (Woolums et al., submitted). Briefly, following BRSV challenge, temperatures, respiratory rates, and clinical scores were significantly higher in M/C calves as compared to V/C and control calves. There was no difference in temperatures, respiratory rates, or clinical scores between V/C and control calves.

4.2. mRNA for TNF-α in cranial lung

Figure 4 shows that experimental BRSV infection resulted in a significant increase of TNF-α mRNA compared to control animals ($p< 0.05$). Vaccinated calves had similar TNF-α mRNA levels as the control calves. TNF-α in the V/C group was lower than that in M/C although it was not statistically significant ($p>0.05$).

4.3. mRNA for TNF-α in BAL cells

Due to loss of a few samples, there were only three BAL samples from the V/C group and four samples from the M/C group. Figure 5 shows that the TNF-α level in M/C was significantly higher than that in controls ($p<0.05$). TNF-α expression in V/C was

very close to the level of TNF-α in control. Although the difference was not significant between V/C and M/C, there was a trend toward a lower level of TNF-α in V/C calves. Evaluation of the correlation between the relative ratios for TNF-α in the lung and BAL of individual calves indicated that expression of TNF-α was similar in lung and BAL.

4.4. mRNA for IL-4 and IFN-γ in pharyngeal tonsil

Due to loss of two samples, pharyngeal tonsil RNA samples were available for only 2 V/C calves. Cytokine expression in the pharyngeal tonsil is presented in Figure 6. Interleukin-4 message in the pharyngeal tonsil was significantly increased in the M/C group compared to the control group ($p<0.05$) (Figure 6A). The V/C group had a relatively low level of IL-4 compared to the M/C group, although the difference was not statistically significant. Similarly, IFN-γ message in M/C group was significantly higher than that in control calves ($p<0.05$); IFN-γ levels were also higher in the M/C group than that in the V/C group, but the difference was not statistically significant. (Figure 6B). The ratio of IL-4 to IFN-γ was calculated as a measure of relative expression of Th2 vs. Th1 cytokines. Relative expression of IL-4 and IFN-γ was different between groups (Figure 6C), with a higher ratio seen in the M/C group than in both the control group ($p<0.05$) and V/C group ($p>0.05$).

4.5. mRNA for IL-4 and IFN-γ in tracheobronchial lymph node

Cytokine expression in the tracheobronchial lymph node is shown in Figure 7. There was no significant difference between each group with respect to IL-4 expression in the tracheobronchial lymph node (Figure 7A). In the M/C group, in general IL-4

expression was decreased following BRSV challenge, with the exception of one outlier. Figure 7B shows that there was no significant difference in IFN-γ expression between the control and experimental groups, but the V/C group had significantly lower expression of IFN-γ as compared to the M/C group ($p<0.05$). The ratio of IL-4/IFN-γ expression in each group are displayed in Figure 7C. Although there was no significant difference between each group, with the exception of the outlier M/C animals had a trend toward a decreased ratio.

5. Discussion

Cytokine production by T cells is one of the key factors determining the outcome of RSV infection in the murine model (Alwan et al., 1994; Tang et al., 1994; Hussell et al., 1996;). We examined the ex vivo cell-mediated immune response in calves following BRSV infection and MLV i.n. BRSV vaccination prior to challenge in order to gain information about the pathogenesis of BRSV disease and the impact of vaccination on cytokine expression in various sites. To our knowledge, this is first study to provide data comparing cytokine gene expression in various sites of i.n. vaccinated and infected calves. A significant reduction of clinical scores in the vaccinated group compared to the infected group indicated that MLV i.n. BRSV vaccination effectively protected calves from BRSV infection. Semiquantitative RT-cPCR was used to determine the levels of mRNA rather than ELISA for corresponding translated protein products because technique for doing so is more sensitive, and also because the stimulus-induced cytokine responses obtained after in vitro exposure to antigen or mitogen may not accurately reflect those occurring in vivo (Than et al., 1997).

The relationship between the development of pulmonary pathology and the presence of different T cell subsets and the roles of various cytokines in the respiratory tract during BRSV infection remains obscure. TNF-α may be involved in both pathogenesis of disease and protection against RSV infection. Several studies in humans and mice with RSV infection showed that the presence of TNF-α was associated with disease (Matsuda et al., 1995; Hayes et al., 1994) but also possessed an antiviral effect in human cells in vitro (Cirino et al., 1993; Merolla et al., 1995) as well as in mice in vivo (Neuzil et al., 1996). Our results indicating that TNF-α mRNA expression was increased in the lung of BRSV infected calves are consistent with those demonstrated by Røntved et al. (2000), who quantified TNF-α with ELISA in calves experimentally infected with BRSV. Although not statistically significant in our study, TNF-α mRNA expression was decreased in vaccinated calves, which may be related to decreased pulmonary inflammation in vaccinated calves. Few studies have evaluated cytokine expression by BAL cells during BRSV infection. The activity of cells from within the respiratory tract is likely to be most representative of the lung response to BRSV. The results from BAL should be representative, since the lung is usually extensively infected in naturally occurring cases (Viuff et al., 1996). We found that results from analysis of RNA from cells obtained by BAL agreed with results obtained from total lung RNA; high amounts of TNF-α were detected in M/C calves while low levels were present in the V/C group. These results also indicate that BAL accurately predicts lung cytokine expression, suggesting that in future studies ante mortem BAL could accurately represent lung TNF-α expression.

Evidence that differential utilization of T helper subsets can occur after vaccination is documented in mouse models of RSV (Graham et al., 1993; Tang et al., 1997;). In general, immunization that leads to a dominant IL-4 response from T cells is associated with disease severity, while immunization which leads to dominant IFN-γ production is protective (van Schaik et al., 2000b). During RSV infection, production of IL-4 and IL-10 may enhance disease severity by inhibiting the development of Th1 cells, and promoting IgE production (Janeway Jr et al., 2001). Studies in infants infected with RSV have also indicated a role for Th cytokines in disease outcome. Roman et al. (1997) reported decreased production of both IFN-γ and IL-4 by cultured PBMC from infants with acute RSV bronchiolitis as compared to healthy control infants. Since IFN-γ levels were more depressed than the levels of IL-4, the authors concluded that this represented an imbalance in cytokine production favoring a Th2 profile. Similarly, in calves, mRNAs for IFN-γ, IL-2, IL-4 and IL-10 were detected in pulmonary and peripheral blood mononuclear cells from BRSV infected calves; only mRNA for IFN-γ was detected in uninfected calves (McInnes et al., 1998). Graham et al. (1993) indicated that the formulation and route of delivery of vaccine could influence the pattern of cytokine expression in mouse lung upon RSV challenge. Challenge of mice primed with live RSV by parenteral or mucosal routes induced type 1 cytokine mRNA expression bias. In cattle, MLV i.m. BRSV vaccination induced high levels of IFN-γ production detected by ELISA in plasma collected from whole blood cultures (West et al., 2000).

This report is the first to describe measurement of cytokine mRNA in pharyngeal tonsil of BRSV-infected calves. Although both IL-4 and IFN-γ expression was increased in the pharyngeal tonsil of M/C calves, IL-4 was increased relatively more. This result

supports the concept that Th2 cytokine bias is associated with BRSV-induced disease. Another interpretation is that increased IL-4 expression was related to induction of a mucosal antibody response by cells in the pharyngeal tonsil. Th2 cytokines such as IL-4 and IL-5 are important in the induction of IgA production (van Schaik et al., 2000b). If IL-4 expression in the pharyngeal tonsil was related to the development of a protective mucosal antibody response in vaccinated calves in this study, it may be that IL-4 mRNA expression was relatively increased earlier than d. 7 post challenge, when samples were collected.

Cytokine message expression in the tracheobronchial lymph node was different than in the pharyngeal tonsil in this study. Interleukin-4 expression was not significantly different between each group and, with the exception of an outlier, was relatively low following BRSV challenge even in the calves immunized with MLV i.n. vaccine. Levels for IFN-γ message were not significantly different between control calves and experimental calves, and IFN-γ expression was significantly lower in the V/C group than in the M/C group. There was no significant difference in ratio of IL-4/IFN-γ between groups. In general, the IL-4/IFN-γ ratio was relatively low in the M/C group, with the exception of one outlier. All these results indicate that there is a trend toward association of Th1 cytokine bias with disease in tracheobronchial lymph node.

In our previous study, BRSV-specific IFN-γ production was increased in tracheobronchial lymph node and pharyngeal tonsil of V/C calves and in PBMC of M/C calves (Woolums et al., submitted). This was in contrast to the results described here, where levels of mRNA for IFN-γ were lower in the tracheobronchial lymph node of V/C calves compared to M/C calves. This contrast is similar to what has been found in other

studies, when evaluation of cytokine profiles at both protein (by ELISA) and mRNA levels has produced conflicting results. In one study where protein was measured, mitogen-stimulated PBMCs from RSV-infected infants were found to produce a higher ratio of IL-4 to IFN-γ protein than controls (Roman et al, 1997). In contrast, in a study where cytokine mRNA was measured, the PBMC obtained from RSV^+ healthy infants upon restimulation with whole RSV had an increased mRNA level for type –1-associated cytokines, i.e. IFN-γ, with no detectable type-2 cytokine-specific mRNA, i.e. IL-4 (Anderson et al., 1994). In a third study, after in vitro restimulation of peripheral blood cells from RSV-infected infants, intracellular IL-4 and IFN-γ protein levels were measured by flow cytometry. The RSV-infected infants produced more IL-4 and less IFN-γ than those from healthy controls. IL-4 was more frequent in CD8 than CD4 cells, and the bias toward IL-4 was greatest in infants with mild infections, whereas IFN-γ increased with disease severity (Bendelja and Gagro, 2000).

In the calves described here, the one outlier in cytokine response in the tracheobronchial lymph node in the M/C group may best be explained by individual variation in response to BRSV challenge.

If i.n. vaccination led to protection through induction of a relative Th1 bias, this was not supported by the results seen in the tracheobronchial lymph node. These results may be explained by three possibilities. The first possibility is that although IFN-γ is considered to be a key cytokine in inducing protective responses against BRSV, it is also associated with BRSV pathogenesis, as has been reported in studies of human infants with RSV and RSV infected mice. When van Schaik et al. (2000a) addressed the role of IFN-γ in studies of IFN-γ knock-out mice and of mice depleted of IFN-γ by in vivo

administration of a neutralizing antibody, they suggested a protective role of IFN-γ in RSV infection in terms of limiting viral replication and inflammatory responses, but also a pathogenic role in contributing to airway obstruction. IFN-γ receptors have been demonstrated on eosinophils, and in vitro studies have shown activation of eosinophils by IFN-γ (Valerius et al., 1990). This may be related to the observation that IFN-γ knock-out mice developed less hyperreactivity than wild-type mice. Paradoxically, administration of recombinant IFN-γ attenuated RSV replication in mice without compromising immunogenicity (Bukreyev et al., 1999) and had a suppressive effect on airway hyperresponsiveness. It was concluded that exogenous IFN-γ alleviates the asthma-like inflammation while endogenous IFN-γ contributes to disease. (Hessel et al., 1997; Hofstra et al., 1998). Analysis of RSV stimulated T cell cultures established from PBMC of RSV-infected children showed that, regardless of clinical severity, the responses were dominated by the production of IFN-γ, and that only low levels of IL-4 and IL-10 were detectable (Brandenburg et al., 2000) in the pathogenesis of RSV disease. In summary, studies of the role of IFN-γ have given mixed results.

A second possibility is that the kinetics of expression of cytokines, which are known to be produced and degraded rather quickly, impacts the interpretation of our results. We euthanized animals at day 7 postchallenge. As discussed earlier in the context of IL-4 expression in the pharyngeal tonsil, IFN-γ mRNA may have been produced at a high level before day 7 in the tracheobroncial lymph node of vaccinated calves. This is supported by other work from our laboratory that showed that the IFN-γ protein production by cells from the tracheobronchial lymph node was increased in vaccinated calves as measured by ELISA (Woolums et al., submitted). Future studies with samples

collected at various times post challenge might better clarify changes in cytokine expression in BRSV vaccinated, infected calves.

A third possibility is that the tracheobronchial lymph node contains relatively naïve T cells, and that cytokine message expression in the lymph node is not representative of the response to the virus in the lung. After activation by BRSV vaccination, Th1 cells producing IFN-γ may migrate out of the traceheobronchial lymph node and into the lung more rapidly in vaccinated calves than in nonvaccinated calves. This hypothesis will be tested in our ongoing work to detect IL-4 and IFN-γ expression in cranial lung and BAL collected post challenge from the calves in this study.

A better understanding of immune/inflammatory responses to BRSV and cellular immune responses induced by MLV i.n. BRSV vaccination will provide clues to aid in the development of effective and safe HRSV and BRSV vaccines. Polar cytokine production of type1or type2 induced by BRSV infection may dictate the disease outcome in calves. The change in relative balance of cytokines caused by MLV i.n. BRSV vaccination may decrease or prevent disease. There is no dispute that the balance between local levels of cytokines is an important determinant in many responses. However, it should be noted that a rigid interpretation of the Th1/Th2 paradigm may be too simple to encompass the variety of immunologic events that occur in response to BRSV challenge. In addition to T helper cells, it is likely that other types of cells, such as CD8+ cytotoxic T lymphocytes, γ/δ T cells, natural killer (NK) cells, B cells, and epithelial cells also contribute to host response and thereby influence the modulation of subsequent immune events (Eeard et al., 1993; Scharton et al., 1993; Ferrick et al., 1995; Shanker et al., 1995).

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CHAPTER 5

CONCLUSIONS

Bovine respiratory syncytial virus (BRSV) is commonly associated with severe epizootics of respiratory disease in calves. Incomplete understanding of the pathogenesis of BRSV infection has slowed development of safe and effective vaccines. Vaccine-enhanced disease has also been reported occasionally. Although several commercial vaccines are able to protect calves against experimental challenge, and one study indicated that modified live BRSV administered intranasally (MLV BRSV i.n.) was more effective than vaccine given intramuscularly or killed vaccine given i.n., little information is available regarding the immunologic mechanisms associated with safe and effective vaccination. Many studies have indicated that cellular immune response is important in BRSV disease, but details regarding the components of the immune response that provide protection are still largely unknown. The purpose of this study was to investigate expression of several cytokines in various sites of calves following MLV BRSV i.n. vaccination followed by challenge.

In this study, levels of TNF-α mRNA was significantly increased in the lung in M/C calves compared control animals. This result is consistent with a previous study that detected TNF-α protein with ELISA in BRSV-infected calves. Taken together, these results show that pulmonary inflammation leads to increased TNF-α gene expression during disease due to BRSV. On the other hand, TNF-α levels were relatively lower in the V/C group than those in the M/C group and were close to levels in control animals. This suggests that MLV i.n. BRSV vaccination effectively protects calves from

inflammation caused by BRSV in the lung, and decreased pulmonary inflammation results in decreased levels of TNF-α.

TNF-α mRNA was also detected in RNA from cells obtained by BAL. The result was similar to that deduced from the lung. These results not only confirm the efficacy of MLV BRSV i.n. vaccination to decrease pulmonary inflammation due to BRSV infection, but also demonstrates that in future studies, ante mortem BAL may accurately represent lung TNF-α expression.

Little information is available regarding cytokine responses in pharyngeal tonsil and tracheobronchial lymph node of cattle. In this study, mRNA for both IL-4 and IFN-γ was measured. In the pharyngeal tonsil, levels of IL-4, IFN-γ and ratio of IL-4/IFN-γ were all significantly higher in the M/C group than those in control animals. Expression of mRNA for these cytokines in the V/C group was close to those in the control group. These results indicate that BRSV infection causes IL-4 to increase to a greater degree than IFN-γ in the pharyngeal tonsil and support the concept that Th2 cytokine bias is associated with disease. Vaccination with MLV i.n. BRSV prevents this biased response. Another interpretation is that IL-4 mRNA expression in the pharyngeal tonsil is associated with a protective mucosal antibody response to BRSV challenge, If this is the case, it may be that IL-4 levels were increased in the V/C group earlier than day 7 postchallenge. Because of the limited number of samples evaluated, studies with more animals will be needed to confirm these results.

In contrast to the pharyngeal tonsil, the cellular immune response in tracheobronchial lymph node was different than expected. In general, after BRSV challenge, IL-4 was decreased with the exception of an outlier in the M/C group. IFN-γ

was significantly higher in the M/C group than in the V/C group, and was similar to control animals. The ratio of IL-4/IFN-γ trended to be decreased in the M/C group. Vaccinated calves had similar levels of cytokine ratios as the control animals. It is hard to make a conclusion regarding these results in light of previous work which showed that IFN-γ expression is associated with protection against disease due to BRSV. Three possible interpretations were analyzed in the discussion. Future work with IL-4 and IFN-γ mRNA measurement in the lung and BAL will further clarify the role of cytokine expression in the calves in this study.

Table 1. Hypotheses tested regarding cytokine mRNA expression

The hypotheses tested in the experiments described in section 2 (introduction), and outlined here. TNF-α mRNA and IL-4 mRNA were expected to be increased in the M/C group and decreased in the V/C group; while IFN-γ mRNA was expected to be increased in the V/C group and decreased in the M/C group.

	Animal Group		
mRNA	Control	M/C	V/C
TNF-α	—	↑	↓
IL-4	—	↑	↓
IFN-γ	—	↓	↑

Table 2. Primer sequence

Cytokine competitors, size (bp) of the amplicon from cDNA and competitor, and forward (F) and reverse (R) primer sequences used in the RT-cPCR

Cytokine Competitor	Size (bp)	Primer sequence 5' → 3'
HPRT	T: 230 C: 186	F: GGA GAT GAT CTC TCA ACT TTA ACT GG R: CAT TAT AGT CAA GGG CAT ATC CCA C
TNF-α	T: 410 C: 350	F: CAA GAA TTC AGG TCC TCT TCT CAA GCC TCA AGT AAC R: TTT GGA TCC CGG CAG GTT GAT CTC AGC ACT GAG G
IL-4	T: 400 C: 310	F: ATG GGT CTC ACC TAC CAG CTG R: CAC TTG GAG TAT TTC TCC TTC ATA ATC G
IFN-γ	T: 440 C: 310	F: TAT GGC CAG GGC CAA TTT TTT AGA GAA ATA G R: TTA CGT TGA TGC TCT CCG GCC TCG AAA GAG

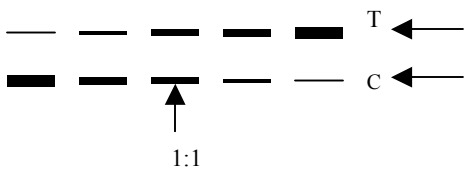


Figure 1. Schematic diagram of competitive PCR

Figure 1: Schematic diagram of competitive PCR utilizing a competitor DNA fragment (C), that differs in size from the target sequence (T). Copy number in cDNA equals the copy number in the competitor where a 1:1 ratio is seen.

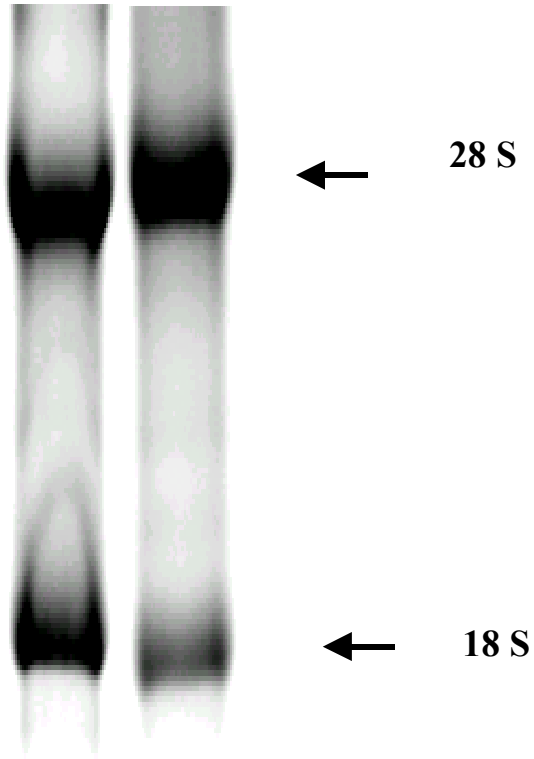
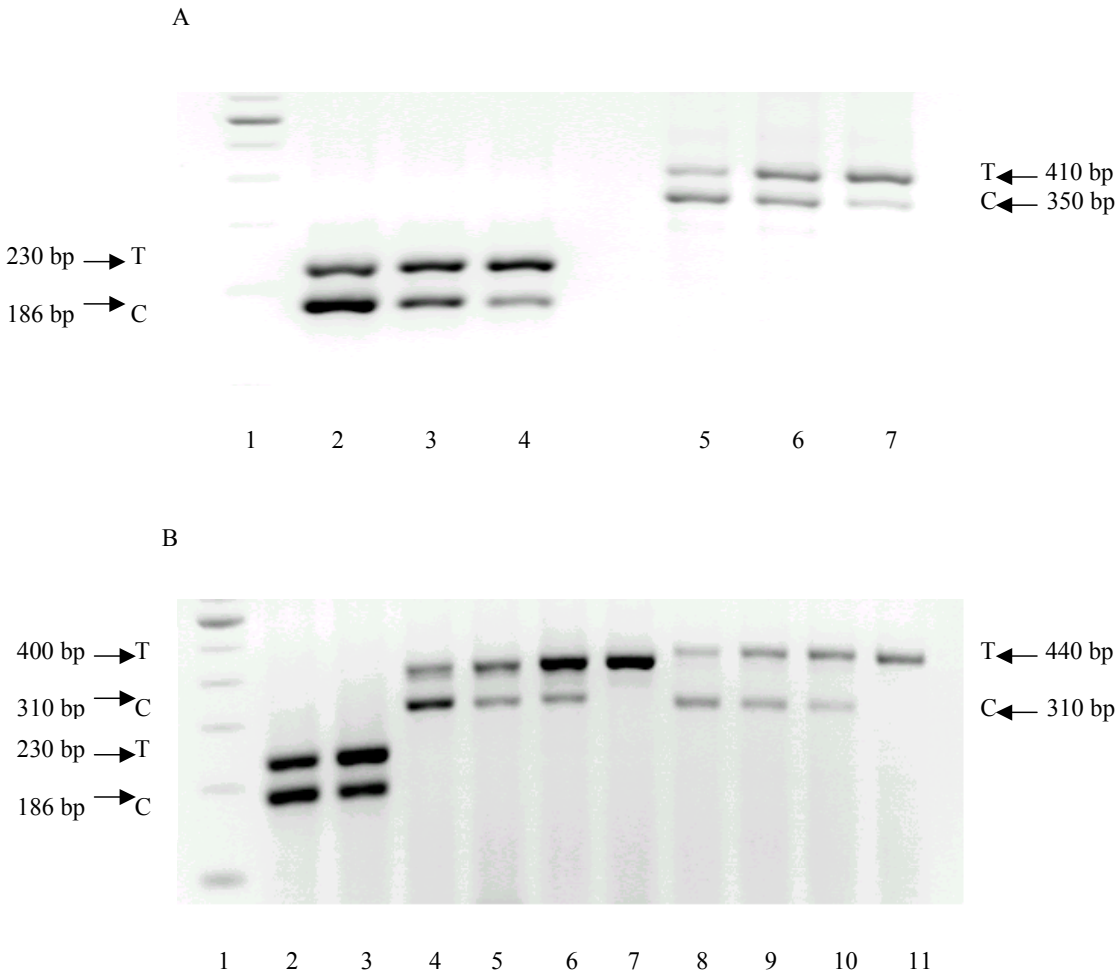


Figure 2. Total RNA from lung tissue in controls

Figure 2: Total RNA isolated from lung tissue from normal calves as described in section 3.2. Clear bands corresponding to 18S and 28S RNA can be seen, suggesting minimal degradation of total RNA occurred between euthanasia of calves and RNA isolation from tissues.



T: target cDNA; C: competitor

Figure 3. Analysis of cytokine expression

Figure 3: Analysis of TNF-α (A) and IL-4 and IFN-γ (B) mRNA expression in one representative calf by RT-cPCR. A: Lane 1: 100 bp ladder. Lanes 2-4: 12000, 6000, 3000 fg HPRT competitor. Lanes 5-7: 200, 100, 50 fg TNF-α competitor. B: Lane 1: 100 bp ladder. Lanes 2-3: 187.5, 93.75 fg HPRT competitor. Lanes 4-7: 200, 100, 50, 0 fg IL-4 competitor. Lanes 8-11: 400, 200, 100, 0 fg IFN-γ competitor.

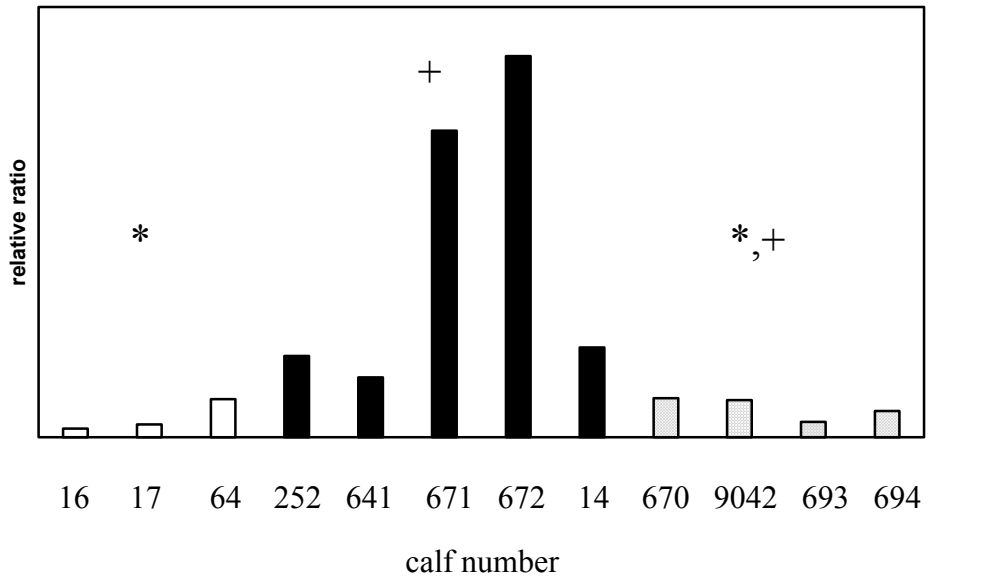


Figure 4. mRNA for TNF-α in cranial lung

Figure 4: Relative ratio of TNF-α mRNA (calculated as described in Section 3.4) from cranial lung from each calf in the nonvaccinated, mock challenged group (Control, n = 3); mock vaccinated, BRSV challenged group (M/C, n = 5); and the i.n. vaccinated, BRSV challenged group (V/C, n = 4). Groups with different symbols are significantly different ($p < 0.05$).

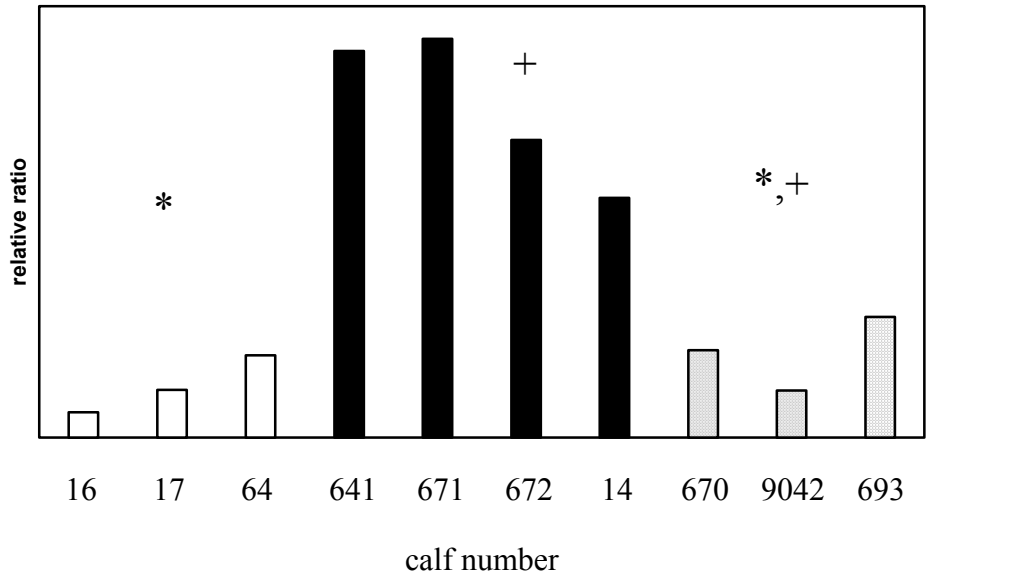


Figure 5. mRNA for TNF-α in bronchoalveolar lavage fluid cells

Figure 5: Relative ratio of TNF-α mRNA from bronchoalveolar lavage fluid (BAL) cells from calves in groups as described for Figure 4. For the control group, n = 3; for the M/C group, n = 4; and for the V/C group, n = 3. Groups with different symbols are significantly different ($p < 0.05$).

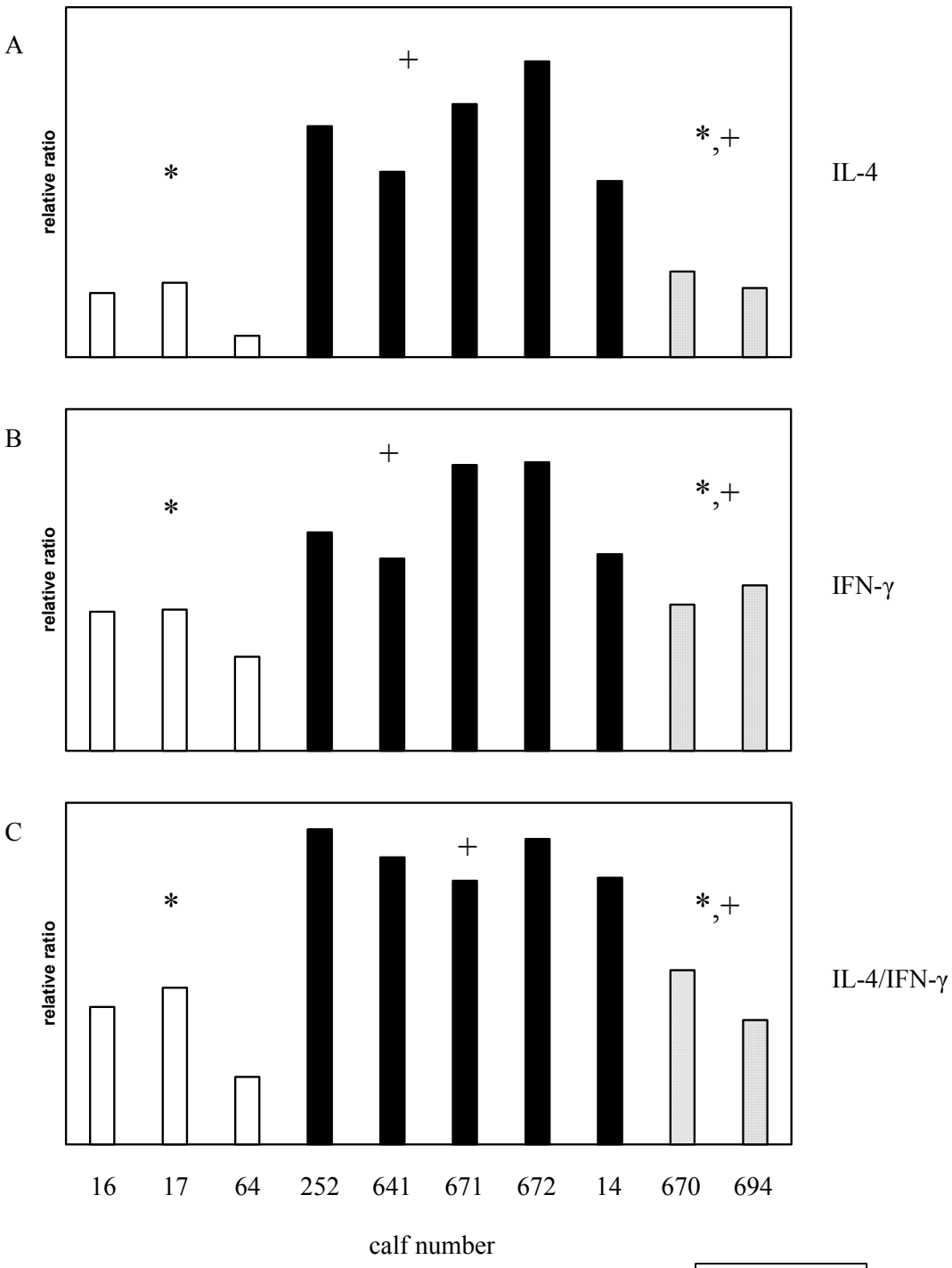


Figure 6. mRNA for IL-4 and IFN-γ in pharyngeal tonsil

Figure 6: Relative ratio for IL-4 mRNA (A), IFN-γ mRNA (B), and the ratio of the relative ratios for IL-4/IFN-γ for each sample (C) from the pharyngeal tonsil from each calf in each group as described in the legend for Figure 4. For the control group, n = 3; for the M/C group, n = 5; and for the V/C group, n = 2. Groups with different symbols are significantly different ($p < 0.05$).

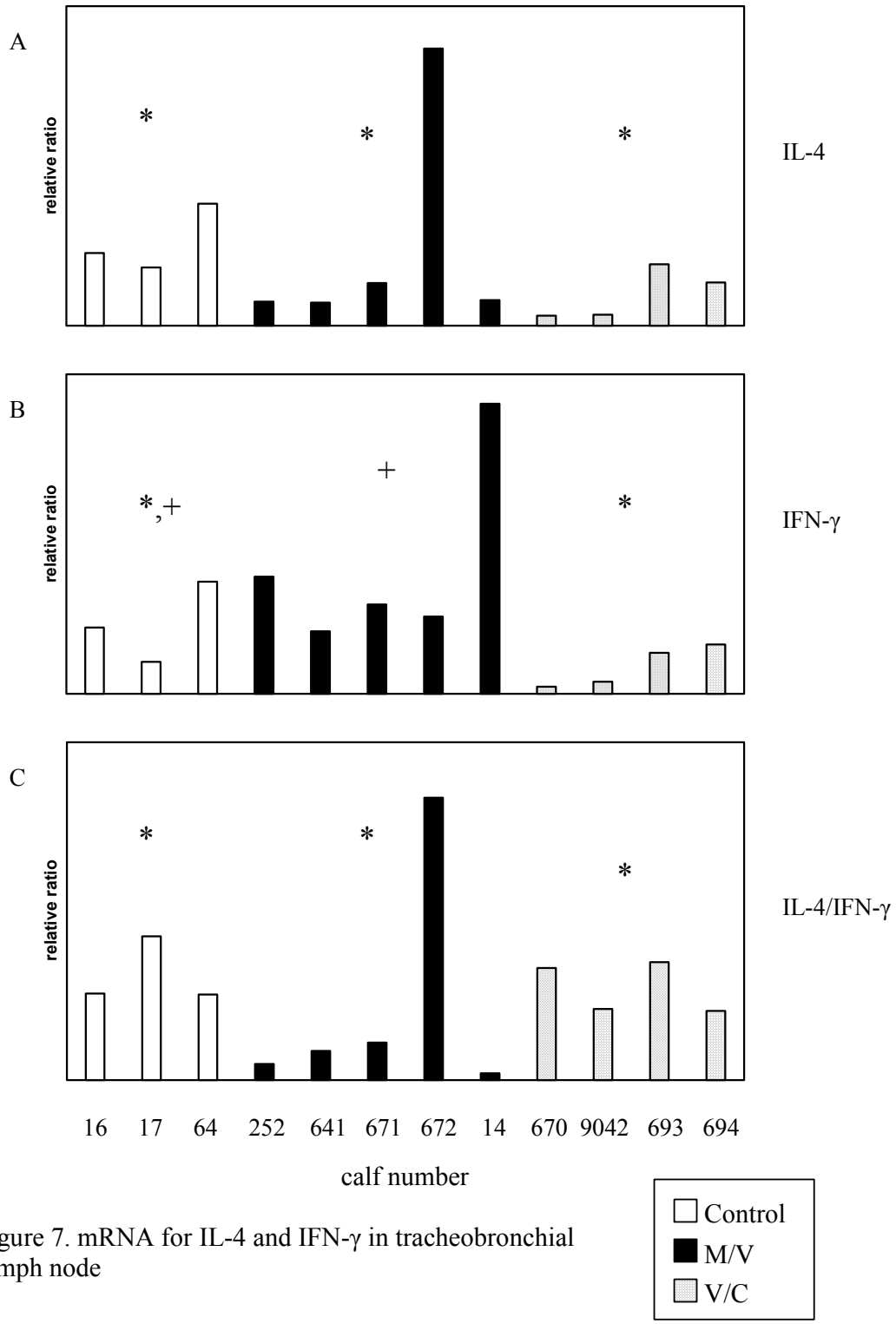


Figure 7. mRNA for IL-4 and IFN-γ in tracheobronchial lymph node

Figure 7: Relative ratio for IL-4 mRNA (A), IFN-γ mRNA (B), and the ratio of the relative ratios for IL-4/IFN-γ for each sample (C) from the tracheobronchial lymph node from each calf in each group as described in the legend for Figure 4. For the control group, n = 3; for the M/C group, n = 5; and for the V/C group, n = 4. Groups with different symbols are significantly different ($p < 0.05$).