Bovine T cell responses to bovine respiratory syncytial virus

and a vaccine vector candidate

by

Matthew Robert Sandbulte

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Immunobiology

Program of Study Committee:
James A. Roth, Major Professor
Douglas E. Jones
Randy E. Sacco
Steven D. Sorden
Michael J. Wannemuehler

Iowa State University
Ames, Iowa
2003

Copyright © Matthew Robert Sandbulte, 2003. All rights reserved.
Graduate College
Iowa State University

This is to certify that the doctoral dissertation of
Matthew Robert Sandbulte
has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

Major Professor

Signature was redacted for privacy.

Für the Major Program
# Table of Contents

Chapter 1. General Introduction 1
   - Introduction to Subject 1
   - Aims of the Present Studies 2
   - Organization of Dissertation 3

Chapter 2. Review of Literature on Bovine Respiratory Syncytial Virus (BRSV) and Bovine T Cells 4
   - Characteristics of BRSV and Resulting Disease 4
   - BRSV Immunity 8
   - BRSV Vaccines 18
   - Difficulties with Vaccines for BRSV and Other RSVs 23
   - Characteristics and Functions of Bovine T Cells 29
   - Mechanisms of T Cell Activation 33
   - References 37

Chapter 3. Methods for Analysis of Cell-Mediated Immunity (CMI) in Domestic Animals Species 48
   - Introduction 48
   - Classical CMI Methods 51
   - Contemporary CMI Methods 52
   - Considerations for Experimental Design 67
   - Conclusion 68
   - References 68

Chapter 4. T Cell Populations Responsive to BRSV in Seronegative Calves 73
   - Abstract 73
   - Introduction 74
   - Materials and Methods 76
   - Results 81
   - Discussion 91
   - Acknowledgements 93
   - References 93

Chapter 5. Priming of Multiple T-Cell Subsets by Modified-Live and Inactivated BRSV Vaccines 96
Chapter 6. T Cells from Apparently Naïve Cattle Are Frequently Activated by Modified Vaccinia Virus Ankara (MVA)

Abstract 122
Introduction 123
Materials and Methods 124
Results 130
Discussion 139
Acknowledgements 143
References 143

Chapter 7. In Vitro Analysis of BRSV-Specific T Cell Priming in Vaccinated Cattle by an Enhanced Assay for Activation and by ELISA for Interferon-γ Production

Abstract 145
Introduction 146
Materials and Methods 148
Results 153
Discussion 159
Acknowledgements 163
References 163

Chapter 8. General Conclusions

Discussion 166
Recommendations for Further Research 169

Acknowledgements 172
Chapter 1. General Introduction

Introduction to the Problem

Bovine respiratory syncytial virus (BRSV) is a common and economically important agent of respiratory disease in cattle. Infection with BRSV alone can induce acute respiratory disease, and it is one of several pathogens that can be involved in the bovine respiratory disease complex; others include bovine herpesvirus 1, bovine viral diarrhea virus, parainfluenza virus 3, bovine coronavirus, *Mannheimia haemolytica*, *Pasteurella multocida*, and *Hemophilus somnus*. Young calves are particularly susceptible to such respiratory infections. Vaccines against BRSV and the other pathogens have been routinely used in beef and dairy herds for decades, but large economic costs are still associated with respiratory diseases of calves. The study of BRSV has added significance because of the virus’ strong similarities with human respiratory syncytial virus (HRSV), a prevalent pathogen of infants. Immunization against HRSV presents significant problems because of disease-causing tendencies of early vaccines, and it has been proposed that BRSV infection in cattle can serve as a valuable research model for HRSV.

Immunological studies of bovine viruses and vaccines have traditionally concentrated on the humoral immune response. Assays for antibodies are relatively simple and cost-effective by nature, whereas analysis of adaptive cell-mediated immune responses is complicated by the biology of T cells and other leukocytes. New methods of testing cell-mediated responses have been adapted in recent years for use in veterinary species. As a result, there is now a greater appreciation for the importance of T cells in immunity against viral pathogens of cattle, such as BRSV. However, there is much room for improvement in
the availability of efficient, informative assays of T cell activity, both for basic research and for designing and evaluating vaccines.

**Aims of the Present Studies**

A broad goal of the work presented in this dissertation was to gain a fuller understanding of how different subsets of T cells can be primed against BRSV and a candidate vaccine vector for recombinant BRSV vaccines. An assay was previously developed in our laboratory that measured recall responses to viral antigens in vitro by using flow cytometry to detect CD25 upregulation on major T cell subsets. This technique was used and refined extensively during the studies described here. The first experiment described in this dissertation addressed whether or not T cell memory against BRSV can exist in calves that do not have detectable titers of antibody to the virus. The second experiment described in the dissertation tested the hypothesis that modified-live and inactivated BRSV vaccines from commercial sources differ in their ability to prime CD4, CD8, and γδ T cells against the virus. A third project was carried out to characterize the T cell stimulatory properties of modified vaccinia virus Ankara (MVA), a candidate vector for recombinant BRSV vaccines. In the fourth project, the hypothesis from experiment 2 was revisited in greater depth by further analyzing T cell responses to BRSV in seropositive calves receiving polyvalent modified-live virus or inactivated virus vaccines. This was facilitated by improvements to the flow cytometry assay that enabled more precise determination of cell phenotypes, and by using an enzyme-linked immunoassay (ELISA) for interferon-γ production.
Dissertation Organization

Chapter 2 of the dissertation is a literature review of prior BRSV research and of relevant T cell biology. Characteristics of the virus, pathogenesis of BRSV-induced disease, immune responses to infection, and immune responses to vaccines are discussed in the review. Chapter 3 is a paper submitted to the *Journal of the American Veterinary Medical Association*. It is an extension of the literature review, written for an audience of veterinary researchers and professionals. The Ph.D. candidate was primarily responsible for gathering references and writing the review paper, and the major professor was the author for correspondence. Chapter 4 is a paper that was published in *Veterinary Immunology and Immunopathology* (84: 111-123). The Ph.D. candidate was the primary researcher and author, and the major professor was the author for correspondence. Chapter 5 is a paper that has been accepted for publication in *Veterinary Immunology and Immunopathology*. The Ph.D. candidate was the primary researcher and author, and the major professor was the author for correspondence. Chapter 6 is a paper submitted to *Viral Immunology*. The Ph.D. candidate was primarily responsible for performing experiments and authoring the manuscript. The second author participated in the preparation of viruses and in the development of the flow cytometry assays that were utilized. The major professor was the author for correspondence. Chapter 7 is a paper that will be submitted to *Veterinary Immunology and Immunopathology*. The Ph.D. candidate was the primary researcher and author, and the major professor was the author for correspondence.
Chapter 2: Literature Review of Bovine Respiratory Syncytial Virus

Characteristics of the Virus and the Disease

Bovine respiratory syncytial virus (BRSV), a pneumovirus of the *Paramyxoviridae* family, is one etiologic agent of the bovine respiratory disease complex ¹. As a paramyxovirus, BRSV is enveloped and has a negative-sense RNA genome. Human respiratory syncytial virus (HRSV) is a closely related virus ², also classified in the *Pneumovirus* genus. Infections by BRSV and HRSV are similar with respect to epidemiology and disease ³.

Attachment of respiratory syncytial viruses to respiratory tract epithelial cells is thought to be mediated by the major glycoprotein (G), which is located in the viral envelope. Cellular heparin molecules were shown to be essential receptors for G-mediated attachment of HRSV ⁴. Additionally, Tripp et al reported that a cysteine signature motif (CX3C) in G facilitates virus binding through its interaction with chemokine receptor CX3CR1 ⁵. Since chemokine receptors are not normally expressed on epithelial cells, this interaction is more likely to be significant in leukocyte activity during the immune response than in the infection of respiratory tract epithelial cells. The fusion glycoprotein (F) facilitates fusion of an RSV envelope with the host cell and formation of syncytia among adjacent cells ⁶⁷. The small hydrophobic (SH) glycoprotein is another constituent of the envelope. Recent reports have added complexity to the known roles of the envelope glycoproteins in the infection process. An HRSV deletion mutant lacking G and SH replicated well in vitro, though it was attenuated in vivo according to human clinical experiments ⁸. BRSV mutants in which G or
SH were deleted by reverse genetics also proved competent to replicate in cell culture. It was further shown that F glycoprotein, in both HRSV and BRSV, binds to glycosaminoglycans, such as heparin, indicating that F participates in the attachment of virions to cells. For BRSV it was also reported that F-mediated cell fusion is optimized in the presence of G and SH.

BRSV infects respiratory tissues, and severe disease typically coincides with viral replication in the lower respiratory tract. In situ hybridization demonstrated BRSV replication in tracheal, bronchial, bronchiolar, and alveolar epithelial cells of naturally infected calves. The same study reported extensive syncytia in the bronchiolar epithelium and alveolar epithelium, but this contradicts a larger study of 32 naturally infected calves, in which syncytial cells were rare. New insight into RSV life cycle and tropism may be taken from an HRSV study that utilized a primary airway epithelial cell culture system that supports growth of a well-differentiated pseudostratified mucociliary epithelium. In this system, inoculation on the apical surface was essential for infection, and epithelial cells were susceptible only after having differentiated to a ciliated form. Virus was shed exclusively from the apical surface, then spread with the aid of ciliary beating.

Ten genes, each encoding a unique protein product, occur in the BRSV genome. The functions of these genes in the life cycle of BRSV are thought to contribute to a replication program that is characteristic of negative-sense RNA viruses in general. The genes encoding non-structural proteins 1 and 2 (NS1 and NS2) are unique to respiratory syncytial viruses, and may have special relevance to BRSV immunity. It was shown that the two gene products cooperatively suppress the antiviral effects of type I interferons, and the deletion of either gene causes attenuation in MDBK cell culture.
Genetic and antigenic diversity are important factors in understanding BRSV evolution and transmission, and in the effective design of immunization strategies. The subject has been explored in several studies. The attachment protein (G) displays more genetic variability than the other BRSV proteins. Its ectodomain contains two highly variable regions that flank a conserved, immunodominant sequence thought to be important for interaction with receptors. Other BRSV genes, such as F (fusion) and N (nucleoprotein), also accumulate sequence changes, but their evolution is more limited by structural constraints. Geographic clustering is a factor in sequence variability, and there can also be variability from one outbreak to the next in a closed herd. One study showed evidence that the evolution of G, F, and N sequences accelerates in countries where BRSV vaccination is widely adopted. Researchers have studied whether the global BRSV phylogeny splits into major subgroups, as is seen with HRSV subgroups A and B. Nucleotide sequences in BRSV isolates fall into distinct phylogenetic branches, distinguishable mostly by the divergent properties of their G proteins. The variation among these branches is similar to that within each HRSV subgroup, leading at least one research group to conclude that all BRSV strains belong to a single genetic and antigenic group. It remains to be determined how well divergent strains of BRSV can cross protect against one another. One BRSV strain primed partial protection against challenge by a strain with significant genetic and antigenic differences in G, and a secondary antibody response to the dissimilar region was observed.

BRSV can cause a broad range of disease severity. Cattle frequently undergo subclinical infection, but severe pneumonia also occurs, sometimes causing death. Characteristic signs of the acute disease include nasal and ocular discharge, fever, cough, and
shortness of breath. Young calves are particularly susceptible to BRSV outbreaks, which typically arise in the winter months. Cattle with past exposure to the virus gain a measure of resistance, but it is common for individuals to have subclinical or mild re-infections.

Primary BRSV lung lesions in calves are often secondarily infected with bacteria, such as *Hemophilus somnus* and *Mannheimia hemolytica*, which typically enhance clinical disease.

Gross examination of BRSV-infected lungs typically reveals interstitial pneumonia and widely distributed edema and emphysema. At the histologic level, the usual pattern involves bronchointerstitial pneumonia, bronchiolitis, syncytial cell formation, and viral antigen in the cranioventral region. Viral antigen can be detected by immunohistochemistry or immunofluorescence. Necrosis and hyperplasia of the airway epithelium are common. Alveolar edema and interstitial emphysema occur throughout the lung, and are most prominent in the dorsal lobes, where virus replication is not usually evident. This points to a significant role for inflammatory mediators in BRSV pathogenesis, and some studies have provided clues as to host factors that may be involved. There is evidence that complement is activated by BRSV-infected cells (independently of specific antibody), and that complement component C3 is deposited in infected portions of lungs from naturally infected calves. Signs of mast cell degranulation and of histamine release, extending outside the virus-positive areas, were associated with C3 detection in the same lungs. Another study revealed high levels of TNF-α in BAL fluids of experimentally infected calves at days 6 and 7 post-infection. Lesion severity and virus titers also peaked near that time, making it unclear whether the net effect of TNF-α was protective or pathogenic.
BRSV Immunity

Innate Immune Response

Surfactant proteins (SP) are early respiratory tract defenses against respiratory syncytial viruses. These molecules are produced by airway and alveolar epithelial cells. Recent genetic studies showed the importance of SP-A and SP-D in the outcome of human infection with HRSV \(^{25,26}\). In vitro experiments demonstrated that SP-A can neutralize HRSV by binding to F \(^{27}\) and opsonize HRSV uptake by phagocytes \(^{28}\).

Respiratory syncytial virus infection of epithelial cells induces expression of numerous genes that encode pro-inflammatory mediators. The finding that BRSV's NS1 and NS2 gene products cooperatively suppress IFN-\(\alpha\) and -\(\beta\) activity (mentioned above) \(^{14}\) indicates that type I interferons (IFN) are potent enough to exert evolutionary pressure on BRSV, and suggests that infected cells of the bovine respiratory tract cannot use the antiviral activity of type I IFN efficiently. However there is evidence from mouse experiments that type I IFN signaling through STAT-1 is vital to the formation of a protective, Th1-biased immune response to HRSV \(^{29}\). Also in mouse models, HRSV infection has been shown to induce the expression of IL-8 and RANTES by pulmonary epithelial cells \(^{30-34}\). These are turned on by signaling pathways that lead to nuclear factor-\(\kappa\)B (NF- \(\kappa\)B) and activator protein-1 (AP-1) translocation.

Non-adaptive recognition via CD14 and toll-like receptors (TLR) was reported with HRSV before any other virus \(^{35}\). Gene knockout experiments demonstrated that CD14 and TLR-4 were required for mouse macrophages to release IL-6 in response to HRSV F protein (as was also true for the response to lipopolysaccharide). Virus clearance in TLR-4-deficient mice was delayed significantly. In another study, TLR-4 function was associated with several
parameters of innate immunity in the murine response to HRSV. TLR-4-deficient mice had impaired natural killer (NK) cell and CD14+ cell trafficking to the lung, diminished NK cell cytotoxicity, and diminished IL-12 expression by BAL cells. None of these deficiencies were apparent in identical mice responding to influenza virus infection.

There is evidence that several types of innate immune cells are important in the response to respiratory syncytial viruses. Alveolar macrophages in lavage fluids from HRSV-infected children expressed interleukin-1β and tumor necrosis factor. TNF-α inhibits HRSV replication. These facts seem consistent with the restricted BRSV replication reported in bovine alveolar macrophages, but it has also been reported that BRSV inhibits alveolar macrophage functions. During the course of experimental BRSV infection in calves, there was evidence of neutrophils playing a major role in clearing virus from the lumen of the respiratory tract. As early as day 2 post-infection, neutrophils were seen migrating across the bronchial epithelium, into the lumen. Substantial amounts of BRSV antigen could be detected in neutrophils on several subsequent days. It has recently been theorized that eosinophils supply innate defenses against single-stranded RNA viruses. The proposed defense mechanism is attributed to ribonucleases like eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein, which have undergone evolutionary diversification that might correspond to the evolution of RNA viruses. Experiments with HRSV lent support to this theory. Human eosinophils, as well as recombinant EDN alone, reduced HRSV infectivity in vitro except when ribonuclease activity was inhibited.

NK cells play critical direct and indirect roles in protecting mice against HRSV. At an early stage in infection (day 4), NK cells were the most prominent IFN-γ-producing cell population in the lungs. Strong cytotoxicity toward infected cells was also observed in the
same cell population. NK cells probably exerted another direct effect on virus replication because IFN-γ mediates antiviral activity toward HRSV in human epithelial cells via the 2′-5′ oligoadenylate synthetase / RNase L pathway. Additionally, early NK cell accumulation and IFN-γ production in the lungs was correlated with subsequent accumulation of CD8⁺ T cells, suggesting that NK cells might modulate the adaptive immune response. The natural killer T cell population evidently has a similar role in mouse HRSV infection. Mice deficient in NK T cells had diminished CD8⁺ T cell recruitment to the lungs and delayed virus clearance.

Humoral Immune Response

Serological surveys have shown high percentages of BRSV-seropositive cattle in many areas of the globe. Most calves receive significant levels of BRSV-specific maternal antibody in colostrum. Passive antibody to BRSV does not prevent infection but can lessen the incidence and severity of disease. Titters of passive antibody to BRSV typically decline to the point of being undetectable by approximately six months of age, decaying at a half-life of approximately 27 days.

The active humoral immune response to BRSV infection involves the production of both mucosal and systemic antibody. IgA and IgM were demonstrated as the dominant isotypes of BRSV-specific antibody in the lung, nose, and eye of calves after primary or secondary infection. The same two isotypes were the first detected in serum after primary infection, followed by more stable titers of IgG1 and IgG2. Secondary infection led to increases in serum IgG1 and IgG2 and a new, more sustained presence of serum IgA. A correlation was observed between the speed of antibody appearance and virus clearance.
Epitope mapping has identified several antibody epitopes within the F protein, including some that have been characterized as neutralizing and/or targets of fusion-inhibition \textsuperscript{51,52}. The advantage of certain antibodies against F was demonstrated in gnotobiotic calves treated with one of three F-specific monoclonal antibodies prior to challenge \textsuperscript{53}. The two neutralizing, fusion-inhibiting monoclonal antibodies suppressed viral replication, syncytia formation, and pneumonic consolidation in lungs, whereas the non-neutralizing antibody provided no protection.

The fact that recovery from BRSV infection does not prevent recurrences suggests that adaptive immunity is induced inconsistently, immunologic memory declines over time, or cross-reactivity among strains is incomplete. One cause of weak active immune responses is the presence of passive maternal antibodies during infection at an early age. A study comparing antibody responses to experimental BRSV infection in colostrum-fed versus colostrum-deprived calves was reported \textsuperscript{50}. Calves that had passive, maternal IgG titers due to colostrum feeding showed little or no primary antibody response. They mounted significant systemic and mucosal responses to secondary infection but antibody titers were still lower than those in colostrum-deprived calves.

*Cell-Mediated Immune Response*

Numerous studies have indicated that cell-mediated immunity is critical to BRSV immunity, with a particularly important role evident for CD8\textsuperscript{+} T cells \textsuperscript{20}. Early evidence for a cell-mediated immune (CMI) response to BRSV came from delayed-type hypersensitivity, lymphocyte migration inhibition, and lymphocyte blastogenesis assays performed on cells from experimentally infected calves \textsuperscript{54,55}. Immunoperoxidase labeling was later used to
quantify lymphocyte subsets in the lungs of BRSV-infected calves. An influx of CD4⁺, CD8⁺, and gamma delta T cells was observed. T-lymphocyte subsets occurred in similar ratios throughout lung tissues, with CD8⁺ T cells typically making up a majority. In lung tissues of noninfected calves CD8⁺ T cells were a minority. Flow cytometric analysis in another study showed that the ratio of CD8⁺:CD4⁺ lymphocytes in calves undergoing infection shifted approximately from 1:1 to 3:1 in both the peripheral blood and the lung. The CD8⁺:CD4⁺ ratio shifted from 1:1 to 6:1 in tracheal tissues. While the pulmonary T cell population increased, the proportions of CD4⁺ and CD8⁺ cells in the bronchial lymph nodes were somewhat diluted by the immigration of B cells.

Expression of surface markers CD45R (high molecular weight CD45 isoform), CD45RO (low molecular weight isoform), and CD25 on CD8⁺ cells in these infected calves suggested a tendency for conversion to activation and memory. In bronchial lymph nodes and lungs the percentage expressing CD45RO increased modestly. By day 15 post-infection, lung CD8⁺ cells expressing CD45RO outnumbered those expressing CD45R, and in some cases the isoforms were coexpressed. CD8⁺ T-cell phenotyping in cattle has shown that CTL precursors fall into both the CD45RO⁺ and CD45RCT populations. Downregulation of L-selectin expression on CD8⁺ lymphocytes was not apparent during BRSV infection, which agrees with earlier research showing that bovine memory T cells are predominantly L-selectin⁺. The low proportion of blood, lung, and bronchial lymph node CD8⁺ cells expressing CD25 increased with BRSV infection, so that nearly half of lung CD8⁺ cells expressed CD25 on day 5.

An experiment was carried out in which monoclonal antibodies against individual subset markers were used to deplete calves of CD4⁺, CD8⁺, or WC1⁺ gamma delta T cells,
and results were reported in two publications. Depletion of CD8+ T cells led to more severe respiratory epithelial lesions upon infection. Lesions in the CD8+ -depleted individuals were consistent with persistent viral replication. Viral antigen was most extensive in these calves' lungs.

It was also reported that MHC class I-restricted CD8+ cytotoxic T lymphocytes (CTL) specific for BRSV appear in the peripheral blood and lungs of calves within 10 days of infection. Cross reactivity between BRSV strains was demonstrated in the CTLs. Detection of CTL occurred at about the same time post-infection that McInnes et al observed increased CD8+ cells in lungs, blood, and the trachea.

Helper T cell activation would be predicted to accompany CD8+ effector cell activation. The significance of the CD4+ T cell subset in immunity to BRSV was illustrated in the same lymphocyte depletion study that demonstrated the dominant role of CD8+ cells. Calves depleted of CD4+ T cells had suppressed antibody responses. The duration of infection in these calves was normal, but their lungs had more extensive pneumonic consolidation.

McInnes et al, the group that analyzed CD8+ T cell activation and migration to the airways after BRSV infection, also studied CD4+ lymphocytes during infection. These data showed more distinct modulation of activation and memory markers in CD4+ than in CD8+ cells, despite a reduced influx of CD4+ cells into lungs and trachea. Previous work in cattle showed that the CD45RO+ population is responsible for the CD4+ subset's memory response. Evidence for this included the detection of antigen-specific proliferation, IFN-γ and IL-4 transcription, and IFN-γ production solely in the CD45RO+ population. In lungs and bronchial lymph nodes following BRSV infection, the number of CD4+ cells expressing
CD45R decreased, while CD45RO expression increased significantly by day 10 post-infection. L-selectin expression was downregulated on CD4^+ cells in the BLN and lungs of infected calves, which is interesting since work by Howard et al cast doubt on L-selectin as a marker of bovine CD4^+ T cell memory. Expression of CD25 in CD4^+ cells, similar to that in CD8^+ cells, was upregulated in infected calves and peaked by day five post-infection in the lungs and BLN. Together, these findings illustrate the involvement of CD4^+ T cells in the CMI response to BRSV, and fit within the helper role.

CD4^+ T-cell epitopes were identified in the F and G proteins of BRSV, using an assay in which PBMC were stimulated with synthetic peptides. Lymphocyte proliferation was assayed in response to these stimuli. Multiple epitopes in the F1 region of F protein could be recognized in the context of diverse MHC class II haplotypes, and some of these corresponded with HRSV epitopes recognized by human T cells. Proliferative responses to epitopes from BRSV G protein were weak, with the only detectable responses directed toward sequences from the more conserved cytoplasmic region.

T helper responses influence important events such as isotype switching, macrophage activation, and the induction of CTL activity. These are critical not only in promoting clearance of a pathogen, but also in numerous pathogenic mechanisms such as IgE-mediated and delayed-type hypersensitivity. Immunity and immune-mediated disease are often sensitive to the differential activation of T helper subsets 1 and 2. Thus the activity of the CD4^+ T cell subset is probably an important determinant in the outcome of BRSV infection or vaccination. The following studies were undertaken to characterize cytokine patterns in cattle infected with the BRSV.
Several key cytokines (IFN-γ, IL-2, IL-4, and IL-10) were assayed by RT-PCR in mononuclear cells from BRSV-infected and control calves. This study was aimed at characterizing the helper T cell response to BRSV seven days after infection. Messenger RNA for IFN-γ was observed in pulmonary mononuclear cells from most animals, whether infected or not. In PBMC, infection was a distinct factor, i.e. all infected animals were positive, whereas most control animals were negative. IL-2, IL-4, and IL-10 messages were each detected in mononuclear cells from the blood and lungs of some infected calves. Cells from the control calves did not express any of these transcripts. IL-2 and IL-4 mRNA generally were not expressed together. Most interestingly, calves expressing IL-4 and IL-10 mRNA had more extensive pneumonic lesions at necropsy.

Gershwin et al examined IgE production and cytokine gene expression in pulmonary lymph from conventionally-reared calves infected with BRSV. These data also presumably reflect CD4+ helper T-cell activity. Lymph was sampled repeatedly from thoracic lymph fistulas. The concentration of BRSV-specific IgE in the lymph of infected calves increased significantly from baseline to day nine, and this factor correlated with clinical disease severity. Results of RT-PCR assays for IL-4 and IFN-γ messages in lymphocytes did not demonstrate a strong bias toward a Th1 or Th2 immune response. However, the IgE response is consistent with helper T cell activity that potentially drives class switching in this direction and contributes to BRSV pathogenesis.

Another study, in which calves were infected with non-cell-culture-passaged BRSV, demonstrated BRSV-specific IFN-γ synthesis in the blood of infected calves. Heparinized blood from infected and mock-infected calves was stimulated in vitro with BRSV, and IFN-γ
was measured by ELISA. At day seven most infected calves were producing IFN-γ in response to in vitro stimulus. By day 21 statistically significant IFN-γ synthesis was induced in five of the six infected animals, but none of the six mock-infected calves’ cells showed a significant gain in IFN-γ production. Similar data have been reported for naïve calves challenged with BRSV in subsequent studies. Collins et al characterized the relative capacity of bovine T-cell subsets to produce IFN-γ in response to in vitro restimulation with BRSV. Bovine peripheral blood mononuclear cells (PBMC) stimulated by BRSV and IL-12 produced IFN-γ. Flow cytometric analysis of intracellular cytokine expression indicated that IFN-γ synthesis is largely by the CD4⁺ population, in small part by CD8⁺ cells, and not by gamma delta T cells.

Experimental data on the role of bovine gamma delta T lymphocytes in response to BRSV and other viruses is limited. Depletion of γδ T cells did not appear to make calves more susceptible to lesions following BRSV challenge. However, the depletion of this cell subset was associated with higher titers of BRSV-specific IgM and IgA in postmortem lung washes. McInnes et al showed no significant change in γδ T cells’ phenotype during BRSV infection. Given the high number of γδ T lymphocytes in calves and their influx to the lungs after BRSV infection, they could be influential sometime in the course of infection.

T lymphocyte activity in response to HRSV infection has been the focus of numerous studies in humans and mice. It was demonstrated that infants and children acquire HRSV-specific CTL activity in the CD8⁺ T cell population. CTL activity declined from one HRSV season to the next, but the development of CTL responses in the first year was associated with a lower incidence of lower respiratory tract disease the following year. In the
same study, CTL activity was directly correlated with in vitro HRSV-specific IFN-γ production, but inversely proportional to IL-4 production. Another recent study examined the cytokine profiles of PBMC taken from HRSV patients and stimulated with virus. Compared with non-HRSV patients, the levels of both Th1 and Th2 cytokines were increased in cells from HRSV patients, and there was an increase in the ratio of IFN-γ to IL-4. However, an earlier report indicated that PBMC from infants with HRSV were biased toward a Th2 cytokine response when stimulated with mitogens. Ostler et al used the mouse infection model to investigate the activation and function of pulmonary CD8+ T cells during and after HRSV infection. They showed that a sizeable population of HRSV-specific CD8+ T cells persist in the lungs even fifty days after the influx brought on by acute infection. These cells retained a memory phenotype (CD69hi CD62Llo), but cytolytic activity was no longer detectable in them at fifty days. Reinfection elicited a fresh accumulation of pulmonary CD8+ T cells. These appeared to have been supplied from rapidly dividing memory cells in the mediastinal lymph node. This suggests that CD8+ T-cell memory in humans and cattle may involve cooperation between lung-resident cells and others induced in secondary lymphoid tissues.

Summary: Protective BRSV Immunity

Studies of BRSV immunity collectively indicate that a multifaceted response to this virus is ideal. The following points are important to consider:

- Neutralizing antibody can contribute a significant, though incomplete, level of protection when present at a sufficient levels.
The CD8$^+$ T-cell subset plays a critical role in mediating virus clearance from infected calves, which is consistent with findings in other RSV hosts.

BRSV-specific IFN-γ production is typically primed in PBMC during infection. This may be an important correlate of protection since there is evidence linking elevated IL-4 and IL-10 expression with increased lesion severity.

It is reasonable to hypothesize that BRSV-specific mucosal antibody and T cells in respiratory tract of previously infected cattle cooperate with systemic memory cells and IgG in the formation of a secondary response. Nonetheless, repeated infections are common.

**BRSV Vaccines**

The humoral response to BRSV immunization was analyzed in early work by Kimman et al\textsuperscript{74}. Parenteral administration of live virus to seronegative calves stimulated the production of BRSV-specific IgG in the serum. The same treatment primed the animals for mucosal IgM and IgA responses following a subsequent respiratory tract challenge. In that study, inactivated BRSV injection did not prime for detectable immune responses. In contrast, an early study by Howard et al showed significant humoral responses to an adjuvanted vaccine in which a BRSV-infected cell line was fixed with glutaraldehyde\textsuperscript{75}. Subsequently, there were a number of studies of antibody responses in cattle vaccinated with commercially available modified-live or inactivated BRSV. In two of these, Ellis et al compared antibody titers, specificities, and neutralization properties in serum from calves treated with commercial MLV or inactivated vaccines\textsuperscript{76,77}. Both classes of vaccine induced BRSV-specific antibodies, some of which bound the F protein. However, MLV vaccines
induced significantly stronger neutralizing antibody responses than inactivated viruses. An extensive set of serological assays was performed more recently with sera from treatment groups receiving commercial MLV or inactivated polyvalent vaccines. Again neutralizing antibody was generated more efficiently in response to MLV than to any of the inactivated vaccines, although one inactivated vaccine did induce a significant increase in neutralizing antibody titers. In addition, MLV-induced antibodies were superior in terms of fusion inhibition, further suggesting that commercial inactivation processes affect viral epitopes and weaken the function of resulting antibodies. Sera from all treatment groups had increases in antibody dependent complement mediated cytotoxicity following immunization.

Immunization with an experimental inactivated BRSV vaccine led to antibody responses biased toward different epitopes than in naturally infected cattle. Following natural infection, most F-specific antibody was directed against epitope A, and this was associated with virus neutralization. Animals that received inactivated vaccine made similar amounts of antibody to epitope A, but also made significantly more antibody to epitopes B and C than did the infected cattle. Nonetheless, virus neutralization titers were significantly lower in cattle that received the vaccine.

The mucosal antibody response to BRSV vaccination and challenge was revisited in a study by West et al. IgA and IgM titers in the bronchoalveolar lavage and nasal secretions were not significant following vaccination with MLV until after challenge with virulent BRSV. From that point mucosal antibody detection was accelerated in vaccinated animals, with a 1-3 day delay before the nonvaccinated calves began elaborating antigen-specific antibodies at those sites.
Cell-mediated responses to various BRSV vaccines have also been assessed in a number of studies. In one of these, commercial MLV and inactivated vaccines were compared by monitoring in vitro correlates of CMI, and responses were distinctly different. Antigen-specific lymphocyte proliferation was detected seven days after primary immunization with the adjuvanted, inactivated vaccine and seven days after secondary immunization with the MLV. Proliferation values of the MLV group surpassed the inactivated group following the third dosages. In vitro IL-2 production correlated with lymphocyte proliferative responses, and CD4 T cells were the predominant proliferating population. The strong cellular response in cattle given inactivated BRSV was consistent with previous lymphocyte proliferation data induced by glutaraldehyde-fixed, whole-cell vaccines formulated with Freund’s incomplete and Quil A adjuvants. In contrast, a later study of several commercial vaccines showed that MLV vaccines stimulated superior overall immunity. Lymphocyte proliferative responses were statistically significant only in the MLV treatment group.

Two more recent reports added insight into the practical efficacy of MLV and inactivated vaccines by including virus challenges. In one, the effects of glutaraldehyde-fixed, cell-associated BRSV and heat-inactivated, cell-free BRSV vaccines were tested in lambs. Both vaccines limited virus shedding and induced humoral and cellular immunity, as assessed by virus neutralization, virus-specific cytotoxicity, and lymphocyte blastogenesis. The glutaraldehyde-fixed vaccine gave superior protection and immune responses. A similar set of experiments in calves was performed with two commercial, polyvalent MLV vaccines. After challenge, clinical disease, virus shedding, and pulmonary lesions were reduced in calves receiving either vaccine. Protection was correlated with pre-challenge in vitro cellular
responses, as measured by antigen specific lymphocyte proliferation and interferon gamma (IFN-\(\gamma\)) production. Virus clearance in vaccinated and control calves coincided with the detection of BRSV-specific CTL in the lungs. Thus, while inactivated and modified-live BRSV vaccines both have the potential to stimulate the humoral and cellular immune responses of calves, the literature suggests that these responses differ to some degree. One study with particular relevance to this issue showed that incubation of bovine dendritic cells with live versus inactivated BRSV results in different transcription levels for three key cytokines \(^{82}\). According to real-time RT-PCR assays, live BRSV stimulated significantly more production of IL-10 mRNA, while inactivated virus stimulated significantly more IL-12p40 and IL-15 messages.

There is evidence that an inactivated BRSV vaccine primes CD4\(^+\)T cells to proliferate in response to numerous peptide targets derived from the F glycoprotein \(^{62}\). These targets did not differ greatly from those which were identified following experimental and natural BRSV infections.

Several recombinant BRSV vaccines have been constructed and tested experimentally. Glycoprotein F, vectored in baculovirus and adjuvanted with Quil-A, was effective in priming lambs for a response to BRSV \(^{83}\). This response entailed antigen-specific lymphocyte proliferation and cytotoxicity and virus neutralizing antibody. Reduced virus shedding following BRSV challenge was reported for vaccinated animals. Two studies examined the immunogenic properties of recombinant bovine herpesvirus-1 expressing BRSV's G glycoprotein \(^{84,85}\). This approach was shown to protect calves in terms of both virus shedding and pneumonic lesions, and this was associated with antibody and lymphocyte proliferative responses to immunization. However, the recombinant virus –
which was administered via the upper respiratory tract - was virulent. Limited protection was also seen following vaccination with DNA plasmids encoding G. In another study, vaccinia virus was used as a vector for F, G, N, and M2 genes from BRSV. Immunization with F, G, and N all conferred significant protection, but the immune responses differed among the four treatments. Calves primed with F developed higher titers of total antibody to BRSV than G or N, and no BRSV-specific antibody was detected in calves receiving M2. Only F induced detectable neutralizing antibody. Immunization with F favored IgG1 production over IgG2 in serum. BRSV-specific lymphocyte proliferation was observed in response to F and N.

Few field trials for BRSV vaccine efficacy have been published. Two commercial modified-live BRSV vaccines were tested for field efficacy in calves pre- or post-weaning or in cattle arriving at feedlot. In general, there was a trend toward reduced treatment for respiratory disease in immunized cattle. However, only two of eight vaccine treatment groups had statistically significant reductions in treatment rate compared with intermingled control animals, including a group that received vaccine upon arrival at feedlot and a group that were immunized before weaning. Other studies showed that an inactivated, whole-cell BRSV vaccine with Quil A adjuvant could provide significant protection from BRSV-associated respiratory disease in the field. In several published cases, commercial BRSV vaccines failed in field situations. Two large herds of beef calves experienced severe respiratory disease outbreaks weeks after having been immunized twice with a commercial inactivated BRSV vaccine. Antigen detection and serological results linked the outbreaks to BRSV. Maternal antibody blocking of the active immune response was suspected as the cause for this vaccine failure. In another outbreak, circumstantial evidence suggested that disease
severity was actually enhanced by the commercial inactivated BRSV vaccine that had been
given to part of the herd \(^90\). Pulmonary eosinophilia was associated with this severe form of
disease. MLV vaccine has also been implicated in the enhancement of a BRSV outbreak \(^91\).
Subclinical BRSV infection had apparently been spreading in this particular herd when a
portion of the calves were immunized, and severe disease arose in the vaccinated subset.

**Difficulties with Vaccines for BRSV and Other RSVs**

As mentioned previously, one difficulty in effective immunization against BRSV is
blockade by maternal antibody (IgG1) in young calves. One study examined several
components of the humoral response in calves primed with BRSV by different methods \(^74\).
Maternal antibody to BRSV severely suppressed the primary antibody response to live or
inactivated BRSV administered by either parenteral or mucosal routes. Passive antibody also
suppresses antibody responses to HRSV immunization in animal models \(^92\). Maternal
antibody blocking is generally thought to result from immunoglobulins masking the antigen
from the immune system. Another relevant mechanism may be the suppression of naïve B
cell activation that occurs when immune complexes cross-link surface antigen receptor with
Fc\(\gamma\)RIIb \(^93\). Work done in calves by Aldridge et al raises another possibility \(^94\). They
examined the expression of surface immunoglobulins on B cells in the lymph nodes of fetal
and neonatal calves. Surface IgG1 and IgG2 were readily detectable in cells from fetuses and
colostrum deprived calves, but expression in colostrum-fed calves was absent at 36 h of age.
This implied that a non-antigen-specific factor in colostrum could modulate calves' antibody
production.
There is evidence that exposure to viruses in the face of antigen-specific maternal antibody can prime some facets of immunity. Some studies have indicated that virus infection or vaccination in calves\textsuperscript{95,96}, infants\textsuperscript{97}, and mice\textsuperscript{98} with high maternal antibody titers may prime a T cell response despite the lack of an active antibody response. In addition, passively immunized calves that failed to mount primary antibody responses to bovine herpesvirus 1 (BHV-1)\textsuperscript{99} and BRSV\textsuperscript{74} did have memory-type antibody responses following secondary exposure. Thus, while maternal antibody-mediated inhibition of active immunity to BRSV is well documented, many of the details are unresolved. Some areas of ambiguity include the mechanisms of inhibition, how comprehensively a calf’s immune response is suppressed, and how the problem might be circumvented.

A vaccine trial with human infants demonstrated substantial risks associated with formalin-inactivated HRSV (FI-HRSV). Vaccinated children suffered more severe disease after natural infection with RSV than did their nonvaccinated counterparts, which led to longer hospital stays\textsuperscript{100}. This outcome has greatly slowed progress in developing HRSV vaccines for humans. It has also stimulated research in rodent, primate, and bovine disease models to discover the mechanisms underlying this disease enhancement.

Sera from children vaccinated with the FI-HRSV vaccine had low ratios of neutralizing to total antibody\textsuperscript{101}. Similarly, non-neutralizing antibodies to HRSV proteins F and G were prevalent in cotton rats that received formalin-inactivated HRSV (FI-HRSV) vaccines\textsuperscript{102}. Vaccinated rats developed much lower neutralizing antibody titers to surface glycoproteins F and G than rats which were infected with live virus. The predominance of nonneutralizing antibody in the rats was associated with enhanced pulmonary disease after challenge. It was theorized that the abundant nonneutralizing antibody is the basis for
enhanced disease upon infection of FI-HRSV vaccinated subjects. When rats were vaccinated with purified F protein, it induced antibodies capable of preventing syncytia. In contrast, FI-HRSV did not prevent syncytia or induce substantial levels of neutralizing antibody, suggesting that formalin treatment changed critical epitopes on the glycoproteins. It was proposed that nonneutralizing antibody forms immune complexes with the replicating virus and causes a type III hypersensitivity reaction. Neutrophilia in the initial lung infiltrate of vaccinated, infected rats was consistent with this notion. A recent study provided further validation for this concept. The absence of either mature B cells or complement component C3 abrogated a mouse model of FI-HRSV enhanced disease. In addition, the authors detected C4d deposition in lung sections from infant patients who died of FI-HRSV enhanced disease in the 1960's vaccine trial.

Most of the recent research on this subject has focused on another hypothesis, that RSV immunity in animals receiving formalin-inactivated vaccine is skewed toward a Th2 response. One study measured cytokine mRNA expression in groups of mice that were primed with inactivated HRSV, subunit F glycoprotein, or modified-live HRSV and challenged with live virus. Inactivated virus and F induced relative increases in IL-4 mRNA transcripts, whereas MLV led to an increased IFN-γ to IL-4 mRNA ratio. Other work showed that mouse RSV disease enhancement after challenge could be eliminated by depleting CD4$^+$ T cells at the time of challenge. A subsequent study tested the effects of depleting IFN-γ, IL-2, IL-4, and IL-10 from FI-vaccinated mice immediately before challenge. Mice depleted of IL-4 had partially reduced histologic lesions. Depletion of both IL-4 and IL-10 completely prevented lesions. Mice depleted of IFN-γ and/or IL-2 appeared to have the same enhanced pulmonary inflammation post-challenge as control
vaccinates. Another study examined cells from bronchoalveolar lavage after challenging mice that were vaccinated with formalin-inactivated HRSV \(^{107}\). Total cell, granulocyte, eosinophil, and CD4\(^+\) counts were increased in the BAL of FI-vaccinated mice, but the CD8\(^+\) count declined. Messenger RNA for several Th2-type cytokines also increased, but IFN-\(\gamma\) expression dropped. These results support the hypothesis that polarized immune regulation enhances RSV disease in mice immunized with FI-HRSV vaccine.

A study by de Swart et al \(^{108}\) provided evidence that similar Th2-driven pathogenic processes can take place in primates as well. Macaques that received FI-HRSV vaccine responded with a strong T cell response that included virus-specific proliferation and the production of IL-5 and IL-13. Following challenge with species-adapted HRSV there was significant eosinophil infiltration in the lungs of most vaccinated animals, in addition to the bronchointerstitial pneumonia that was also present in non-vaccinated animals.

It is not well established why FI-HRSV vaccine is linked to a Th2-polarized immune response. Several unique properties of G glycoprotein, e.g. chemokine mimicry and bias toward class II-restricted T cell priming, led researchers to investigate whether the glycoprotein inherently favors Th2 cytokine production and enhanced disease (reviewed by Graham et al \(^{109}\)). Vaccinia-vectored G can prime mice for enhanced disease after challenge, similarly to FI-RSV. However, the FI vaccine-induced version of disease enhancement requires a more complex Th2 cytokine milieu than the G-induced form. Therefore it is doubtful that G glycoprotein alone accounts for the disease complications. Other contributors to the association between FI-RSV and a Th2 response might include the obligatory use of endocytic antigen processing and RSV's antagonism toward type I interferon. As for mechanisms to link Th2 cytokines with enhanced disease, there are a few strong possibilities.
The recruitment of eosinophils by IL-5 from Th2 memory cells in the airways is thought to be a key step in immunopathogenesis\textsuperscript{109}. There is also evidence that IL-4 reduces the cytolytic activity of CD8\textsuperscript{+} T cells in ways that promote inflammation\textsuperscript{110,111}. Specifically, IL-4 increases the expression of Fas ligand (FasL) and TNF-\(\alpha\) in CD8\textsuperscript{+} T cells. Upregulation of FasL shifts the balance of cytolytic activity from the very targeted perforin-mediated mechanism to the more promiscuous Fas-mediated mechanism, which is prone to killing uninfected cells. By comparing HRSV infection in perforin knock-out and wild type mice, it was shown that HRSV clearance is delayed and illness is lengthened when perforin is disabled and other defense mechanisms (e.g. Fas-mediated killing) are used instead\textsuperscript{112}.

Some efforts have been made to determine whether inactivated BRSV vaccines cause enhanced subsequent disease in cattle. Gershwin et al performed an experiment in which calves were either vaccinated with FI-BRSV or mock-vaccinated, followed by challenge with BRSV\textsuperscript{113}. Virus clearance was less efficient and pulmonary disease was enhanced in FI-BRSV vaccinated calves. After challenge, IFN-\(\gamma\) production was significantly lower in PBMC from the vaccinated calves than in cells from control calves\textsuperscript{66}. These data appear to be consistent with results from HRSV studies in mice. Weak IFN-\(\gamma\) responses in FI-BRSV vaccinates may account for the inefficient clearance of virus and enhanced pulmonary disease. Another noteworthy finding from this work was that FI-BRSV induced only non-neutralizing antibodies, which also mirrors the data from HRSV studies in humans and rodents. It is noteworthy that another group tested a similar FI-BRSV vaccine in calves, with different results. They reported an overall reduction in clinical disease after challenge, except that disease onset was more rapid when calves had been vaccinated with FI-BRSV\textsuperscript{114}. It is not clear whether the licensed inactivated BRSV vaccines used routinely in the field can
drive a disease-enhancing response, but at least one field case was consistent with that scenario.\textsuperscript{90}

A consensus about the pathogenesis of vaccine-enhanced RSV disease has still not been reached. There is substantial evidence for both cytokine-mediated and antibody-mediated mechanisms, but no solid link between the two seems to have been demonstrated. There was a report that Th2-like responses are favored when specific antibody targets an antigen to APC via Fc receptors, even reversing the Th1 bias of activated macrophages.\textsuperscript{115} This could point to the high level of FI-RSV-induced nonneutralizing antibodies as the fundamental problem. Conversely, one could speculate that a Th2-like cytokine milieu diminishes neutralizing properties, perhaps by changing the balance of antibody isotypes. It is also possible that these two mechanisms are independently driven by exposure to FI-RSV, and perhaps their impact can be synergistic.

MLV and inactivated RSV vaccines each pose potential risks to bovine and human recipients. Both forms are also prone to blocking by passive maternal antibody. In order to provide safer and more reliable prophylaxis for bovine and human RSV, a more thorough understanding of host immune responses to vaccines is needed. It will be valuable to develop and test new generation RSV vaccines, such as subunit antigens vectored in avirulent viruses or encoded in DNA. These are likely to be useful tools for research, helping to identify critical targets for immunization, but such strategies also hold out promise to make immunization more effective.
Characteristics and functions of bovine T cells

Antigen receptors on classical T lymphocytes recognize viral peptides from proteins processed in the cell and presented on major histocompatibility complex (MHC). Classical CD8+ CTLs recognize peptides presented on MHC class I molecules. Class I presentation has generally been associated with antigen processing by the endogenous pathway, in which peptide loading takes place in the endoplasmic reticulum. CD4+ T cells ordinarily recognize viral peptides in the context of MHC II complexes on the surface of an antigen presenting cell (APC). Class II presentation has usually been associated with ingestion of antigen and antigen processing in the exogenous pathway. On a given αβ T cell, the TCR and the CD4 or CD8 co-receptor are matched for binding to the same class II or class I molecule. The cytoplasmic domains of CD4 or CD8 participate with the TCR-associated CD3 in extensive cell signaling. Co-stimulatory signals are also necessary for activation of a T cell. This interaction is classically carried out between CD28 from the T cell and B7 molecules from the APC. Antigen recognition in the absence of co-stimulation results in T-cell anergy. 116

Because the ability to drive a primary CD8+ T cell response is unique to professional APC, and because many intracellular pathogens do not infect professional APC, it is important that there be a special route for CD8+ T cell sensitization to these invaders. Professional APC can prime CD8+ T cells with exogenous antigens by a mechanism – or set of mechanisms – termed "cross presentation" 117. Dendritic cells (DC) were shown to be sufficient for this activity 118, and they are more efficient in cross presentation than macrophages 119. In this system, DC located throughout peripheral tissues can ingest various materials, such as debris from infected cells that may be undergoing apoptosis or necrosis 119.
The maturation of these DC likely depends on Toll-like receptor-mediated signaling triggered by heat shock proteins (especially in viral infection) or by numerous bacterial constituents, such as lipopolysaccharide. The DC can then migrate to secondary lymphoid tissues and present antigen to naïve CD8⁺ T cells. There are thought to be two routes for DC to present peptides from an exogenous antigen in the context of MHC class I. By one of these routes, peptide loading on MHC class I occurs in the endosomal-lysosomal system or directly on the cell surface. The other route uses a property unique to DC that allows antigens to pass from endosomes into the cytosol. From this point, the exogenous antigen can be processed and presented in the same manner as an endogenous antigen.

Gamma delta T cells express a set of antigen receptor genes distinct from those of the αβ T cells. This cell subset is a small portion of the circulating lymphocyte population in humans and rodent species 120, but large numbers of γδ TCR⁺ cells, such as intraepithelial lymphocytes (IEL), localize in various non-lymphoid tissues 121. In ruminants the γδ T cells are also a major lymphocyte subset in the circulation 122. Many aspects of γδ T cell biology are either poorly understood or difficult to generalize because there are major differences between species and there are heterogeneous subsets within species 123. Functional subsets have been distinguished using several parameters, including anatomical location, receptor specificity, and phase of immune response. The receptor diversity of γδ T cells is quite limited in humans and rodents 120,124. Many of the observed functions and activities of this population are consistent with a niche in the innate immune response. Endogenous molecules, such as heat-shock proteins, phosphoantigens, and non-classical class I molecules, can be upregulated on host cells (e.g. epithelial cells) in response to stresses like infection and transformation 123. Certain γδ T cells recognize these molecules, either through a
canonical TCR arrangement or a non-TCR receptor, and respond with a variety of activities. Gamma delta T cell functions have been shown to include cytotoxicity, cytokine production, and secretion of growth factors such as keratinocyte growth factor \(^{121}\). Through the production of cytokines like TNF-α and IFN-γ, these cells probably favor the activation of macrophages, natural killer cells, and αβ T cells early in an infection \(^{123}\). Later, during the resolution of some immune responses, certain γδ T cells release cytokines that dampen inflammation and growth factors to aid in healing lesions.

WC1, also known as T19, is a marker unique to ruminant γδ cells. It is a surface heterodimer with a large extracellular domain and a small intracellular tail, and can contribute to γδ T cell activation \(^{125}\). WC1 helps to differentiate two major γδ T cell subsets \(^{126}\). One subset is WC1\(^{-}\), CD2\(^{+}\), and CD8\(^{+}\) and the other is WC1\(^{+}\), CD2\(^{-}\), and CD8\(^{-}\). Many WC1\(^{-}\) γδ T cells are found in the intestinal tract and splenic red pulp. WC1\(^{+}\) cells can be detected in peripheral blood, the splenic marginal zone, skin, and gut lamina propria. Although the WC1\(^{-}\) population's high expression of CD8 suggests that they might bind MHC I, no such restriction requirement has been demonstrated. Analysis of cytokine production by WC1\(^{+}\) cells has indicated that they tend toward a Th1 profile \(^{127}\).

Ruminant species appear to have diverse gamma delta receptor genes and joining patterns, suggesting that they have sophisticated antigen recognition capabilities and a significant role in adaptive immunity \(^{124}\). Consistent with that idea, γδ T cells comprise a large fraction of the circulating bovine T cell population: sometimes nearly half of the PBMC in young calves \(^{69,128}\). However ruminant γδ T cells are quite rare in the conventional T cell zones of secondary lymphoid organs \(^{122}\). Studies in cattle have shown important roles for γδ
T cells in immune responses to a number of microorganisms. Gamma delta T cells from the
peripheral blood of *Mycobacterium bovis*-infected cattle give specific responses in vitro to
mycobacterial antigens. In one instance it was shown that the in vitro proliferative
response by αβ T cells was higher when γδ T cells were removed, suggesting an
immunomodulatory role for the latter. Gamma delta T cells were important for the
formation of granulomas in a SCID-bovine chimera model of *M. bovis* infection.
Vaccination of cattle against *Leptospira borgpetersenii* primed for antigen-specific in vitro
IFN-γ production by γδ T cells. Virus-specific in vitro γδ T-cell responses were also
acquired by cattle that were infected with bovine viral diarrhea virus or vaccinated for
bovine herpesvirus 1.

Some activities of bovine γδ T cells appear to fall in the category of innate immune
responses. Peripheral blood γδ T cells proliferate in response to autologous monocytes that
are metabolically inactivated or stimulated with granulocyte-macrophage colony-stimulating
factor (GM-CSF). They do not respond to normal monocytes, which secrete a specific
inhibitor of this activity. The proliferative response requires contact with the γδ TCR. It is
hypothesized that this mechanism directs a γδ T cell response specifically to altered
monocytes, which may have been transformed, infected, irradiated, or activated. In addition,
peripheral blood γδ T cells exhibited natural killer cell-like activity toward several viruses
after isolation from foot-and-mouth disease vaccinated cattle.

Bovine T lymphocyte populations undergo changes in distribution during the early
months of life. In a study of these dynamics, maximal concentrations of circulating CD4+ and
CD8+ T cells were observed in fetal calves (tested at 8 months gestation). Subsequently,
both subsets dropped below adult levels from parturition until after 120 days of age, with CD4$^+$ cell counts changing most significantly. Gamma delta T cell numbers peaked near the time of birth and then declined to adult level at around 120 days. The numbers of CD4$^+$ and CD8$^+$ T cells were significantly greater in the lymph nodes of fetal calves than in adults. A similar trend was seen for CD4$^+$ T cells in the spleen. These patterns of cellular traffic in fetal and neonatal calves suggest that a functional repertoire of T cells develops in prenatal calves. In a study of longer term lymphocyte population dynamics, the frequencies of CD4$^+$, CD8$^+$, and γδ T cells were lower in the peripheral blood and spleen of a group of cattle ≥five years of age than in a group of two-year-old animals. Fluctuations in the number of circulating WC1$^+$ γδ T cells have also been associated with a change in environment.

Mechanisms of T Cell Activation

Activation of T cells can be detected by analysis of several surface marker phenotypes, such as downregulation of CD45RA and L-selectin, and upregulation of CD45RO, MHC II, and the α-subunit of high-affinity IL-2 receptor. High-affinity IL-2 receptor (IL-2R) is a trimer composed of the α, β, and γc chains (CD25 is an alternate name for IL-2Rα). The critical function of IL-2 signaling in T cells is to induce the cell cycle transition from G1- to S-phase, thus driving proliferation during a response to antigen or mitogen. Resting T cells express undetectable or low levels of α (CD25), β, and γc chains. Signaling that is initiated by TCR engagement with peptide and MHC leads to transactivation of the IL-2 and CD25 genes. CD25 expression permits the assembly of high-affinity IL-2R, which tends to localize in membrane microdomains. This initiates a positive feedback loop in which IL-2 signal drives further expression of CD25 in a manner dependent on signal.
transducers and activators of transcription (STAT) 3 and STAT5a\(^{140}\). CD25 expression is more rapid on antigen-primed cells than on naïve cells\(^{141,142}\). In contrast, the ability of naïve T cells to engage TCR was shown to be equal to that of effector cells\(^{141}\). This suggests that efficient CD25 expression by effector T cells contributes to their superior responsiveness to antigen.

Recent studies in mice have identified a numerically small subpopulation of CD4\(^+\) regulatory T cells that constitutively express CD25 and demonstrate suppressive activity toward pathogenic, self-reactive T cells in vivo\(^{143,144}\). CD25\(^-\)CD4\(^+\) regulatory T cells are able to suppress the activation, IL-2 production, and proliferation of CD25\(^-\)CD4\(^+\) and CD25\(^-\)CD8\(^+\) T cells in response to antigen in vitro. It is thought that the regulatory T cells recognize and respond to self antigens, allowing them to suppress potentially pathogenic self-reactive T cells while both are interacting with antigen presenting cells. The mechanisms of this suppression are unclear. These regulatory T cells should not be confused with ordinary activated CD4\(^+\) T cells that upregulate CD25 in response to antigenic stimulation. No published studies concerning CD25\(^+\)CD4\(^+\) regulatory T cells in cattle are known to the author.

There are quantitative and qualitative reasons that effector/memory T cells respond more rapidly and efficiently than naïve cells of the same specificity. One key to the effectiveness of CD4\(^+\) and CD8\(^+\) memory T cells is numerical superiority over equivalent naïve cells in non-exposed individuals\(^{142,145}\). There are also critical qualitative differences in the antigen-driven responses of naïve and memory T cells, which is reflected in the above-mentioned fact that CD25 is more readily expressed on primed T cells. Qualitative factors that allow memory CD8\(^+\) T cells to respond more vigorously include a lower activation
threshold in terms of peptide concentration and co-stimulation, more direct migration to peripheral sites of inflammation, and more immediate induction of effector functions, such as cytotoxicity \(^{142,146}\). One reason for those qualitative differences is that memory cells are programmed for a different pattern of gene expression, due to their remodeled chromatin structure and the constitutive presence of mRNA transcripts that encode key effector molecules \(^{147}\).

Different subsets of naïve T cells behave in unique ways during antigen-driven responses, which is potentially significant in the development of vaccines. In studies of mice with deficiencies in costimulatory molecules, it was shown that CD4\(^+\) and CD8\(^+\) T cells have distinct requirements for costimulatory signals \(^{147}\). The kinetics of a proliferative response are also different between these two subsets. It was reported that CD4\(^+\) T cells stimulated with immobilized anti-TCR monoclonal antibody and B7-1 proliferated and expanded well beyond the 3-4 days at which CD8\(^+\) T cell activity (including lytic effector function) peaked and cell death began \(^{148}\). These data fit the current understanding that CD4\(^+\) T cells are slower to begin proliferating after engaging viral antigen \(^{147}\). For CD8\(^+\) T cells, the rapid proliferative burst is tightly correlated with the subsequent acquisition of effector functions \(^{147}\). In contrast, CD4\(^+\) T cells must engage antigen for a longer time, in the presence of proper cytokines, in order to be differentiated into effector cells. It would be valuable to learn more about the antigen-driven activation of γδ T cells, especially in ruminants, since they seem to span the innate and acquired branches of immunity. γδ T cells in sheep upregulate activation markers CD25 and MHC II in response to concanavalin A within just eight hours of stimulation, whereas CD4\(^+\) and CD8\(^+\) cells require 24 hours to do the same \(^{149}\).
There have been reports that a great majority of CD8$^+$ T cells proliferating during an acute anti-viral response are non-antigen-specific$^{150}$. Limiting dilution analysis was used in these studies to count antigen-specific CTLs. Those conclusions were contradicted by more recent data obtained with peptide-MHC I tetramer assays, intracellular IFN-γ staining, and single cell IFN-γ ELISPOT assays. These assays were used in one study to analyze responses to lymphocytic choriomeningitis virus (LCMV) and heterologous viruses in LCMV-immune mice$^{151}$. All three assays indicated that 50-70% of activated CD8$^+$ cells were specific for the infecting virus. Few cells from naïve mice were stained by LCM-derived tetramers or responded to its peptides; nor did lymphocytes from mice immunized with vaccinia. Another approach used to quantify specific versus "bystander" CD8$^+$ T cell activation involved adoptive transfer of polyclonal, virus-primed T cells$^{152}$. Tracer cells from Thy1.2$^+$ mice were transferred into Thy1.2$^+$Thy1.1$^+$ recipients. Donor cells taken from LCMV immune mice or vaccinia immune mice made up 60-80% of proliferating CD8$^+$ T cells upon infection of the recipients with the respective virus. Meanwhile, there was no evidence that donor cells from naïve mice or mice primed with the irrelevant virus contributed to expansion. Thus, several lines of evidence suggest that a primary anti-viral response stimulates mainly antigen-specific T cells. It has been suggested that activation-induced cell death caused underestimation of antigen-specific cells in limiting dilution assays$^{151}$.

The maintenance and re-stimulation of memory T cells are influenced by cytokine stimulation$^{153}$. In murine systems, type I interferon, IL-12, IL-15, IL-18, and IFN-γ have been shown to promote proliferation of memory CD8$^+$ T cells, even independently of TCR stimulation. IL-15 appears unique among these in its ability to directly stimulate memory CD8$^+$ T cell turnover. Evidence suggests that the other cytokines act indirectly by helping
drive IL-15 synthesis by cells such as macrophages. The proliferation of memory CD4\(^+\) T cells can be driven by IL-12, and to a lesser extent, IL-18\(^1\). One study examined the stimulation requirements for bovine CD8\(^+\) T cells responding to *Theileria parva* in vitro\(^2\). Naïve CD8\(^+\) cells required contact with antigen-stimulated CD4\(^+\) cells for development into parasite-specific CTL. Primed CD8\(^+\) cells could develop into CTL under less stringent conditions, such as the presence of T cell growth factors, recombinant bovine IL-2, or CD4\(^+\) T cells responding to another antigen. The authors reported no data concerning proliferation or activation marker expression. The fact that there are means of non-antigen-specific T-cell stimulation suggests that in vitro assays for antigen recall must be interpreted with some caution.

References


60. Howard CJ, Sopp P, Parsons KR. L-selectin expression differentiates T cells isolated from different lymphoid tissues in cattle but does not correlate with memory. *Immunology* 1992;77:228-234.


124. Hein WR, Dudler L. TCR gd+ cells are prominent in normal bovine skin and express a diverse repertoire of antigen receptors. Immunology 1997;91:58-64.


Chapter 3. Methods for Analysis of Cell-Mediated Immunity in Domestic Animal Species

A review paper submitted to the *Journal of the American Veterinary Medical Association*

Matthew R. Sandbulte and James A. Roth

**Introduction**

Past efforts to evaluate immune responses in domestic animals typically focused on humoral immunity. One reason for this is the relative ease with which serum antibody responses can be monitored, using assays such as virus neutralization, hemagglutination, and enzyme-linked immunosorbent assays (ELISA). The many facets of cell-mediated immunity (CMI) in response to infectious agents have been explored and appreciated more thoroughly in recent years. This has accentuated the importance of examining CMI in any thorough effort to characterize an immune response to infection or vaccine. When evaluating veterinary vaccines antibody titer is often cited as an important measure of vaccine efficacy. Yet, there are several realistic scenarios in which these data are insufficient to predict a positive or negative outcome.

- For many infectious agents, specific antibodies do not adequately protect the host.
- Antibodies to certain infectious agents enhance disease. One mechanism for this is antibody-dependent enhancement (ADE), where immunoglobulin-bound pathogens rely on Fc receptor-mediated phagocytosis for entry to host cells. Immune complexes can also contribute to the pathogenesis of infectious diseases by triggering complement activation and driving a type III hypersensitivity response.
Antibodies may not always be evident following an adaptive immune response. It has been documented with several viral agents that adaptive CMI responses can be mounted in the face of passive maternal antibody even while humoral responses are suppressed. This can result in animals with no detectable antibody titers being fully resistant to challenge.

CMI is a rather simple label for a broad and complex set of mechanisms that require T-cell activity. Cytotoxic T lymphocytes (CTL), which express CD8 on their surface, are important weapons against many intracellular pathogens. CTL are programmed to destroy infected cells when they recognize pathogen-derived peptides that have been processed and presented in the context of MHC class I via the endogenous pathway. Antigen-activated CD4+ helper T cells coordinate the overall adaptive immune response. They recognize peptides presented in the context of MHC class II by the exogenous pathway. Helper T cells influence the activities of many other immune cells through cytokine production and direct cell-cell contact (e.g. CD40-CD40 ligand signaling). For instance, both types of signal are used by T helper 2 (Th2) cells to induce antibody class switching and maturation of B cells. The T helper 1 (Th1) cells facilitate CTL activation and drive the inflammatory immune response. During an inflammatory response, signals from antigen-specific Th1 cells aid the rapid recruitment and activation of macrophages and neutrophils. Gamma delta (γδ) cells are the other major T-cell subset (reviewed by Hayday). The structure of γδ TCR is different from that of the αβ T cells (CD4 and CD8), and antigen recognition by γδ T cells is not usually MHC-restricted. Certain host cell markers, such as stress-induced heat-shock proteins, can activate γδ T-cell activity. The functions of γδ T cells in response to pathogens
are not well understood, but their prominent distribution in mucosal surfaces is consistent with a role in surveillance and rapid response to invaders. There is also a body of evidence that γδ T cells modulate inflammatory responses and promote tissue repair 4.

The balance of immune regulation has been one of the foremost topics in immunology in recent years. The Th1 versus Th2 paradigm has been used to explain striking divergences between protection and disease in several human and rodent infections, such as leprosy and leishmaniasis. There is some ambiguity as to the Th1/Th2 paradigm's relevance in other animal hosts, such as cattle 5, but there is evidence from several species to support it 6-8. The induction of cytokine activity promises to be a useful measure of vaccine efficacy for infectious agents of animals.

In this review, we briefly discuss strengths and drawbacks of the classical CMI assays. This is followed by a survey of contemporary methods for CMI assessment, with emphasis on those which are practical for studies in outbred animals. The assays monitor four categories of immune parameters: (1) antigen-specific in vitro activation and proliferation of distinct T-cell subsets, (2) antigen-specific cytokine production, (3) production of non-cytokine effector molecules, and (4) direct detection of peptide-specific receptors.

To set the context for this discussion, it is important to point out that there are two different reasons to assay for T cell activity. One is to examine basic immunologic mechanisms involved in an immune response, and the roles of these mechanisms in protection. This can be done with in-depth, molecular analysis on small numbers of specialized animals (such as gene-knockout mice or NIH mini-pigs). The second reason is to monitor T-cell responses to vaccines in populations of animals over time. This requires
assays that can be performed on large numbers of outbred animals. Some assays are very cumbersome to use for this purpose (e.g. CTL assays, discussed below). Other CMI assays are inherently more practical for this research aim (e.g. in vitro tests for antigen-specific lymphocyte activation, proliferation, or cytokine production, also discussed below). Ideally, some of these CMI methods will be enhanced to a point where they match the efficiency of serological assays for immunologic monitoring of domestic animals.

**Classical CMI methods**

The oldest technique for monitoring the cell-mediated response to an antigen is the delayed-type hypersensitivity (DTH) assay. This method involves observation of the swelling that results from leukocyte recruitment to a site of intradermal antigen injection in an immune animal. Therefore it permits *in vivo* detection of cell-mediated responses to a wide variety of infectious agents. Another advantage is that the test can be performed without expensive equipment. Among its drawbacks, the results are difficult to quantify and the test may only be valid the first time it is performed in an individual, if the test itself induces T cell responsiveness.

Antigen-specific lymphocyte proliferation has been used widely to assess antigen recall responses *in vitro*. High proliferation to an antigen reflects the expansion of antigen-specific lymphocytes in the course of immunization or infection, as well as the superior recall responses of memory cells in immune individuals. Lymphocyte proliferation has typically been assessed by cellular uptake of tritiated thymidine that is added during the final 24 hours of culture with antigen. This technique offers a quantitative read-out via scintillation counter, and it can be repeated routinely with peripheral blood mononuclear cells (PBMC) from an
individual animal. Data from the assay do not indicate which lymphocyte populations are responding to a given antigen. The assay only provides information about proliferation during the time period when tritiated thymidine is present. The hazards and restrictions of handling radioactive material are significant disadvantages.

Cytotoxic T lymphocytes (CTL), known to be a crucial component of many immune responses, have long been assayed by an in vitro method that measures the lysis of antigen-loaded autologous target cells. In the conventional chromium release format for this technique, $^{51}$Cr is loaded in the target cells, and its release is read as the indicator of target cell lysis. Several controls are necessary to demonstrate antigen-specificity and MHC restriction. One of the difficulties with this technique is handling radioactive material. Many related techniques that use non-radioactive labeling for target cells have been reported, but none of them seem to have been widely adopted. The major disadvantage when using this method for outbred animals is that autologous target cell lines susceptible to infection with the agent being studied must be maintained for each individual tested.

**Contemporary CMI methods**

*Monitoring the sensitivity of T lymphocytes to recall antigens in vitro*

Many of today's prevalent methods for CMI assessment are akin to the traditional lymphocyte proliferation assay, in that they are based on *in vitro* culture of PBMC with antigen. Lymphocyte proliferation is still a very widely used parameter for antigen-driven cellular activity in these systems, but significant efforts have been made to develop alternative proliferation assays that use non-radioactive signals and provide more information about the responding cell populations. Several colorimetric assays were evaluated as tests for
proliferation. Two indicator reagents which proved effective in domestic animal studies were alamar blue and the tetrazolium salt MTT (see Table 1). Both of these reagents undergo color changes under the metabolic conditions of proliferating lymphocytes, which can be quantified by standard spectrophotometry.

Flow cytometry-based proliferation assays have gained widespread acceptance in recent years. With these techniques, markers are incorporated into cells during culture, and precise measurements of antigen-driven proliferation can be derived from changes in marker intensity. Reagents that have been used for this purpose include 5-bromo-deoxyuridine (BrdU), the PKH series of dyes, and carboxyfluorescein diacetate succinimidyl ester (generally referred to as CFSE, though technically, CFSE is the fluorescent molecule derived when intracellular esterases remove two acetate side chains from the original molecule). All three of these have been used successfully in immunological studies of domestic animal species (see Table 1). BrdU is a nucleotide analog that is added to cells late in culture for incorporation into replicating DNA. Before flow cytometric analysis, BrdU-specific antibody must be applied for fluorescence labeling. Both PKH and CFSE are typically used to stain cells before culture with antigen, and no further reagents are required to achieve fluorescence. PKH dyes stain cells by integrating into the membrane, whereas CFSE is a membrane-permeant dye that binds covalently to cytoplasmic proteins. With either type of dye, each cell division results in two daughter cells with half the fluorescence intensity of the parent cell. This allows one to determine the proportion of cells that have undergone division and the number of generations that have occurred. Cells undergoing as many as eight rounds of division are routinely differentiated with these protocols. Another advantage of flow cytometric proliferation techniques is the capacity to phenotype the proliferating and non-
Table 1.

Contemporary methods for analysis of lymphocyte activation and proliferation utilized in domestic animal species

<table>
<thead>
<tr>
<th>Technique</th>
<th>Host Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD25 flow cytometry</td>
<td>Bovine(^9,10)</td>
</tr>
<tr>
<td></td>
<td>Goat(^11)</td>
</tr>
<tr>
<td></td>
<td>Pig(^12,13)</td>
</tr>
<tr>
<td>MHC II flow cytometry</td>
<td>Bovine(^9)</td>
</tr>
<tr>
<td></td>
<td>Pig(^12,14)</td>
</tr>
<tr>
<td>MTT proliferation</td>
<td>Dog(^15)</td>
</tr>
<tr>
<td></td>
<td>Chicken(^16)</td>
</tr>
<tr>
<td>Alamar blue proliferation</td>
<td>Cat(^17)</td>
</tr>
<tr>
<td></td>
<td>Chicken(^18)</td>
</tr>
<tr>
<td>BrdU flow cytometry</td>
<td>Bovine(^19)</td>
</tr>
<tr>
<td></td>
<td>Dog(^15)</td>
</tr>
<tr>
<td></td>
<td>Chicken(^20)</td>
</tr>
<tr>
<td>PKH flow cytometry</td>
<td>Pig(^21)</td>
</tr>
<tr>
<td></td>
<td>Bovine(^22)</td>
</tr>
<tr>
<td>CFSE flow cytometry</td>
<td>Bovine(^19)</td>
</tr>
</tbody>
</table>
proliferating cells. Fluorescent antibody conjugates are used directly or indirectly to label T lymphocyte subset markers, such as CD4, CD8, WC1, and γδ TCR.

There are potential weaknesses to the flow cytometry-based proliferation assays. As is the case in a traditional radioactivity-based lymphocyte proliferation assay, these techniques do not specify or prove effector functions of the responding cells. In addition, the dyes tend to be somewhat toxic to cells, and this complicates the interpretation of results, particularly negative data.

Along similar lines, lymphocyte responses to in vitro antigens can be detected by flow cytometric analysis of activation-induced cell surface markers. Fluorescent antibodies can be used to label these molecules. Activation markers that have been used for the purpose of detecting recall responses include CD25 (IL-2 receptor α subunit), CD69, and MHC class II11,23,24 (also see Table 1). The expression of such markers can be analyzed in parallel with T lymphocyte subset markers in order to assess the level of responses by distinct cell populations, such as CD4, CD8, and γδ T cells. Levels of activation marker expression in a cell population can be measured in terms of two statistics: the percentage of cells expressing the marker and the mean fluorescence intensity (MFI) of staining for the marker. Monoclonal antibodies to subset markers and activation markers are commercially available for a number of animal species, which makes this approach a practical tool for veterinary researchers. Under some circumstances, antigen-stimulated cells express activation markers but do not proliferate, suggesting that activation marker-based assays should detect some T cell responses that proliferation assays miss25. There are some important considerations regarding the interpretation of data from these assays.
As with proliferation, the demonstration of lymphocyte activation in response to antigen does not prove any particular effector function. If one subset of lymphocytes is better adapted to survive and proliferate in vitro, then the relative levels of antigen-driven activation across subsets may be skewed in favor of that subset. In addition, it is possible that antigen-specific cytokine production by one T-cell population in an in vitro system might drive nonspecific "bystander activation" of other T cells. Ordinarily, the activation of a T cell depends on antigen recognition by the TCR, and further stimulation by cytokines serves to amplify that antigen-specific response. Memory T cells, however, can be sensitive to stimulation by certain cytokines in the absence of antigen. Thus, it is plausible that an antigen-specific T-cell response in vitro might lead to the activation of accompanying memory T cells that do not share the same specificity. It has not been shown conclusively whether this causes measurements of antigen-specific T-cell activity to be inflated. Regardless, even bystander T-cell activation presumably depends on antigen-specific cells having made a recall response in the first place.

There are important choices to be made in terms of the population of leukocytes to test for antigen-specific priming. The most convenient source of cells for repeated assays of a systemic immune response is the peripheral blood, and PBMC are therefore used very frequently. Cells from lymphoid organs can arguably give a more direct indication of immune induction, especially in the early stages. Responses to systemic infections or parenteral vaccination are typically initiated in draining lymph nodes or the spleen. Infections of the respiratory tract or the gut (or intranasal immunization) will likely lead to immune responses in mucosal lymphoid tissues, e.g. tonsils or Peyer's patch. In some animal studies, draining lymph nodes can be biopsied, and in other instances cells are collected from
lymphoid tissues at necropsy. One technically demanding, but commendable approach used in bovine studies was to sample pulmonary lymph from cannulated lymphatics over an extended time. It is sometimes desirable to examine fractionated lymphocyte populations for antigen-specific responses. For instance, if a flow cytometry assay reveals CD8 T cell proliferation or activation in response to antigen, it can be useful to test a purified CD8 T cell population for dependence on CD4 T cell help. Separation of subsets before antigen stimulation also enables one to measure the contribution of each subset to cytokine production, using techniques like reverse transcriptase PCR (RT-PCR), ELISA, or ELISPOT (which are discussed in the next section). Several antibody-mediated techniques are available to either isolate one subset of T cells or deplete a given subset from the mixed population. These techniques include fluorescence activated cell sorting (FACS), magnetically activated cell sorting (MACS), and complement-mediated subset depletion. Homogenous lymphocyte subset populations may be inferior to mixed PBMCs in terms of creating an in vitro environment where antigen presentation, cognate T cell help, and the cytokine milieu can support T cell activation.

Researchers have utilized several approaches to culturing lymphocytes for antigen stimulation. In some studies antigens are simply added to a suspension of mononuclear cells in media. Alternatively, T-cell activation is often facilitated with differentiated antigen presenting cells (APC) prepared in advance. Mitomycin C-treated PBMC and adherent cells from peripheral blood monocyte culture have both been used as renewable sources of syngeneic APC. Recall antigens utilized in published studies include whole viruses (live and inactivated), recombinant viruses, protein extracts from bacterial culture, individual
proteins, and peptides. The more complex antigens may be better at provoking APC activity. They are also likely to contain more diverse epitopes for the stimulation of polyclonal T cell responses. According to dogma, live intracellular pathogens have optimal access to the endogenous antigen presentation pathway. This suggests that viable microorganisms may be ideal antigens for testing recall responses by CD8 T cells. Synthetic peptide libraries containing arrays of short sequences from within antigenic proteins are useful to screen for prominent T cell epitopes (reviewed by Rodda 30). Libraries tailored for analysis of CTL epitopes usually consist of peptides around 8-11 residues in length, whereas libraries for Th cell epitopes often have peptide lengths of 13-25 residues. A library of 16mer peptides representing two proteins from bovine respiratory syncytial virus was one example of a CD4+ T cell epitope library used in veterinary research 31.

*Monitoring antigen-driven cytokine production*

A variety of methods are used to analyze T cells' capacity for antigen-specific cytokine expression, and like the previously mentioned methods, most of these involve the incubation of cells with antigens. Some of the most frequently studied cytokines are Th1 mediators IFN-γ and TNF-α, and Th2 mediators IL-4, IL-5, and IL-10. One approach to analyzing cytokine production is to perform “bioassays.” In these assays, supernatants – such as from antigen-stimulated lymphocytes – are tested for biological effects upon a specific type of cell in culture. For instance, B cell proliferation can be associated with the presence of IL-4. Bioassays have been utilized for studies of domestic animals (see Table 2), especially in cases of limited reagent availability. One clear disadvantage to these methods is that a particular cytokine is typically not the only factor capable of causing a particular
biological effect. More confidence in the presence of a cytokine can be gained if the activity is blocked in the presence of cytokine-specific antibody. Because of this subjectivity in bioassays, direct detection of cytokines or the transcripts that encode them is generally considered to be more accurate and reliable.

Starting at the level of gene transcription, the first approach is to detect shifts in the level of cytokine mRNA after exposing cells to antigen. Methods of analyzing cytokine gene transcription include Northern hybridization, ribonuclease protection assays, and several variations of RT-PCR. Total RNA can be isolated for any of these methods with a number of commercially available kits. Because there are published genetic sequences for many of the key cytokines in domestic animal species, the development of probes or primers suited for these techniques is generally quite feasible. In their basic forms, the mRNA-based assays yield data that are qualitative or semi-quantitative, but adaptations with better quantitative readouts have been developed; some of these are mentioned below.

A ribonuclease protection assay for cytokine transcription relies on the construction of a radioactively labeled antisense RNA probe derived from the gene sequence. The probe is incubated with total RNA so that it may hybridize with target mRNA. Next the RNA mixture is treated with ribonuclease enzyme so that single-stranded RNA is digested, whereas the dsRNA complexes are protected. The sample is separated in a denaturing polyacrylamide gel. Radioactive signals are measured from the dried gel; intensity of signal is proportional to the amount of mRNA. RNA messages for multiple cytokines can be analyzed in a single assay, provided that probe sizes are differentiable on the gel $^{32,33}$.

RT-PCR offers the advantages of a partially automated assay that requires no radioactive isotopes. Total RNA is reverse transcribed into DNA by standard procedures that
Table 2.
Cytokine detection methods utilized in domestic animal species

<table>
<thead>
<tr>
<th>Technique</th>
<th>Host Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioassay</td>
<td>Bovine\textsuperscript{34}</td>
</tr>
<tr>
<td></td>
<td>Chicken\textsuperscript{35}</td>
</tr>
<tr>
<td></td>
<td>Pig\textsuperscript{36}</td>
</tr>
<tr>
<td>RNase protection</td>
<td>Bovine\textsuperscript{33}</td>
</tr>
<tr>
<td>Standard RT-PCR</td>
<td>Bovine\textsuperscript{37}</td>
</tr>
<tr>
<td></td>
<td>Chicken\textsuperscript{38}</td>
</tr>
<tr>
<td>Competitive RT-PCR</td>
<td>Bovine\textsuperscript{33,39}</td>
</tr>
<tr>
<td></td>
<td>Pig\textsuperscript{40}</td>
</tr>
<tr>
<td></td>
<td>Cat\textsuperscript{41}</td>
</tr>
<tr>
<td></td>
<td>Sheep\textsuperscript{42}</td>
</tr>
<tr>
<td>Real-time RT-PCR</td>
<td>Chicken\textsuperscript{43,44}</td>
</tr>
<tr>
<td></td>
<td>Bovine\textsuperscript{45}</td>
</tr>
<tr>
<td></td>
<td>Horse\textsuperscript{46}</td>
</tr>
<tr>
<td>Sandwich ELISA</td>
<td>Bovine\textsuperscript{8}</td>
</tr>
<tr>
<td></td>
<td>Chicken\textsuperscript{47}</td>
</tr>
<tr>
<td></td>
<td>Pig\textsuperscript{48}</td>
</tr>
<tr>
<td></td>
<td>Sheep\textsuperscript{49}</td>
</tr>
<tr>
<td>ELISPOT</td>
<td>Bovine\textsuperscript{45}</td>
</tr>
<tr>
<td></td>
<td>Pig\textsuperscript{50,51}</td>
</tr>
<tr>
<td>Intracellular staining</td>
<td>Bovine\textsuperscript{52,53}</td>
</tr>
</tbody>
</table>
utilize heat-stable reverse transcriptase enzyme. A pair of PCR primers derived from the cytokine sequence are used to amplify the specific signal of interest. In the conventional technique, product is electrophoresed on an agarose gel so that the target band can be visualized with ethidium bromide under UV light. Densitometry techniques can be used to compare band intensities.

Competitive RT-PCR is a modified version of the assay designed to make results somewhat more quantifiable, and it has been used often for the measurement of cytokines from domestic animals (see Table 2). This technique requires the construction of a competitor template which is recognized by the same two primers that target the cytokine mRNA. Amplification of the cDNA template and the competitor template yield products of distinct sizes. In a set of parallel reactions, the quantity of competitor template is reduced by serial dilutions. The key concept is that greater initial quantities of mRNA-derived template will outnumber the competitor template, and thus deliver signal, earlier in the series of competitor dilutions. PCR products are separated and visualized on agarose gels. The concentration of cDNA can be estimated by identifying the concentration of competitor template DNA in the particular lane where the two bands have equal intensity.

A major advance in this area is real-time RT-PCR, which enables the researcher to quantify cytokine transcripts with high sensitivity and accuracy. The read-out from this assay is derived from fluorescence signals that correspond with the synthesis of PCR product. The accumulation of signal is monitored in real-time from one PCR cycle to the next. One strategy is to use a dye in the reaction mixture that specifically labels double-stranded DNA. Greater specificity is attainable with fluorescently labeled DNA probes. These oligonucleotide probes, such as TaqMan® reagents, have a reporter dye at one end and a
quencher at the other. Fluorescence is quenched unless the probe is cleaved. The probe is specific to the DNA sequence of the target gene, and enough of it must be present to tag all amplified DNA strands. During the next primer elongation step, the 5' exonuclease activity of Taq polymerase cleaves the probe and unquenches the reporter fluorochrome. A fluorimeter built into the thermocycler measures this signal. The number of cycles required to achieve detectable signal and the kinetics of signal amplification are used to quantify the mRNA template, typically with the help of computer software.

Generally speaking, the key advantage of mRNA sequence-based tools for studying cytokine expression is their versatility in settings where monoclonal antibody reagents are not available, as is common in domestic animal research. Several disadvantages must be taken into account. First, the transcription of genes does not always correlate with synthesis of the respective proteins. Second, the expenses and labor required to perform RNA isolation, reverse transcription, and PCR, in addition to the initial cell culture, may be prohibitive in experiments where a large number of samples must be tested. Real-time PCR requires specialized equipment, which represents another significant expense.

Cytokines are often measured in supernatants from antigen-stimulated cells using ELISA analysis. The sandwich ELISA is preferred for this purpose due to its high specificity and sensitivity. This technique requires a pair of antibodies to a given cytokine, which ideally should bind to different epitopes. The detection antibody is frequently conjugated to biotin, so that an enzyme-streptavidin conjugate can be added in the next step, followed by addition of the enzyme substrate. Alternatively, an unlabeled detection antibody can be followed by indirect labeling with an isotype-specific antibody-enzyme conjugate. ELISA kits for many human and murine cytokines are marketed commercially, but there are only a few kits
available for domestic animal species. In some cases where kits do not exist, pairs of monoclonal antibodies can be purchased in order to develop ELISA assays in-house. One of the advantages to using ELISA in detection of cytokines is that many samples can be collected and frozen for storage, then tested efficiently at one time. Equipment suitable for this technique is available in most research laboratories. Quantitative results can be obtained using a standard curve based on purified or recombinant cytokine. Drawbacks to conventional ELISA assays are that they do not identify the cell populations producing a cytokine or indicate the frequency of cells that express it. Also, detection of a cytokine protein does not ensure that the protein is in its biologically active form. Cytokines that are characteristically produced at low protein concentrations, such as IL-4, can be difficult to detect by sandwich ELISA.

The frequency of cytokine-secreting cells can be determined with the ELISPOT assay, which is a variation on the sandwich ELISA. In this method, capture antibody is coated on the bottom of plastic wells before media, cells, and antigen are incubated in them. At each locus in the well where a cytokine-secreting cell lies, that cytokine will bind to capture antibodies within a small radius. At the completion of the culture period, approximately the same detection steps are carried out that would be employed in a conventional sandwich ELISA. The result is a pattern of "spots" that corresponds with the distribution of cytokine-secreting cells. These spots are examined by microscopy and quantified either manually or by computer, which leads to a straight-forward calculation of the frequency of cytokine secreting cells. This approach is often used to count effector cells after only one or two day of ex vivo incubation, though the detection of memory cells can require more time. Limitations to the ELISPOT method include the lack of information
about cytokine quantities and the identity of secretor cells. The latter can be dealt with if cells are sorted by phenotype before beginning the assay. Reagent availability for domestic animal species is likely to be the same as for conventional ELISA formats.

Cytokine-producing activity can be measured in different T-cell subsets using intracellular cytokine staining techniques and multi-parameter flow cytometry. Two key requirements for the success of this approach are that cells retain the cytokine molecules they produce and that cells be permeable to antibody during the staining steps. The first requirement is met by treating cells with an inhibitor of the golgi complex, such as brefeldin A, during culture. The second requirement is met by fixing cells and permeabilizing their membranes with a detergent solution before labeling. All of the reagents necessary for blocking secretion, fixing, and permeabilizing can be purchased together in a kit. Data are acquired and analyzed by much the same approach as the above-mentioned flow cytometric assays for T-cell activation. Antigen-specific cytokine-producing cells are often present at rather low frequencies, making assay sensitivity a potential problem. One approach to overcoming this problem is to amplify the responding cells by adding PMA-ionomycin to the cell culture near the end of incubation. Clearly, it is essential with this approach to run controls for non-specific T-cell activation. The main advantage of intracellular staining is its powerful capacity to trace cytokine production to specific populations of effector lymphocytes. However, the assay is complicated by the special requirements for inhibition of protein secretion and cell membrane permeabilization. Altered cellular activity may distort normal antigen-driven processes, and cell labeling procedures inevitably allows some leakage of cytokines. In addition, the technique is more time-consuming than flow cytometric assays for T-cell activation markers.
It was reported that highly sensitive labeling can detect certain cytokines (IL-10 and IFN-\(\gamma\), but not IL-2 or IL-4) on the membranes of lymphocytes that secrete them \(^{55}\). The key to this high assay sensitivity was a labeling reagent containing several thousand fluorochromes per particle, which was achieved with magnetofluorescent liposomes that incorporate carboxyfluorescein and specific antibody. Since the detection antibodies are able to block the biological activity of the cytokines they bind, it is unlikely that the staining technique labeled receptor-bound cytokines. The authors did not demonstrate a mechanism for the phenomenon of surface cytokine localization, but it was observed in human, murine, and hamster cell lines. This raises the possibility that the production of IL-10, IFN-\(\gamma\), and perhaps other cytokines could be assessed by surface labeling in conjunction with lymphocyte subset markers without having to block secretion.

**Assays for molecules involved in lytic function**

Effector lymphocytes, such as CD8+ CTL, express distinctive molecules. Some of these have been utilized as markers for immunity, and these methods are potentially very useful, though they have been little-used in veterinary research to this point. Effector molecules originating in the granules of CTL, most notably perforin, granzyme A, and granzyme B, are of particular interest. The synthesis of these proteins is induced upon activation of the naïve CTL precursor \(^{56}\). When an armed effector CTL recognizes an infected cell it releases perforin in a polarized fashion. Perforin facilitates the delivery of other granule contents, including the granzymes, which are serine proteases that trigger apoptosis pathways. An ELISPOT assay designed to directly measure granzyme B secretion was used to detect peptide-specific CTL in PBMC from human patients \(^{57}\). An alternative
approach, which probably requires less investment in the development of reagents, is to measure granzyme activity with serine protease substrates as indicators. Strong correlations have been observed between enzymatic activity and target cell killing\textsuperscript{58,59}. Influenza-specific CMI responses were analyzed in humans using parallel assays for \textsuperscript{51}Cr release and granzyme activity\textsuperscript{60}. Granzyme protease activity was detected in PBMC by measuring digestion of a substrate specific to granzyme B. Data obtained by the two methods were well-correlated. Techniques aimed at monitoring granzyme production or activity would appear to offer better efficiency and fewer difficulties with radioactivity and target cell preparation than conventional \textsuperscript{51}Cr release assays.

\textit{Tetramer labeling of T cell receptors}

A major breakthrough in immunology in the past five years has been the development of reagents and techniques that allow the direct identification of peptide-specific CD8 T cells. The key concept is that peptide-specific CD8 T cells can be identified from within a polyclonal T cell population by staining the cells with a fluorescently labeled complex of peptide and autologous MHC class I. This is a powerful tool for identifying critical epitopes and measuring the dynamics of a complex immune response. In order to achieve high enough avidity to label T cells, multimers of MHC-peptide must be constructed. Typically, four MHC-peptide units are combined in a tetramer. “Tetramer” has become shorthand for this class of labeling reagents. Since there are typically many peptide targets in a given protein, a large set of tetramers is often necessary to characterize the CD8 T cell response to an antigen. It is much easier to prepare the necessary tetramers when working with a strain of inbred mice than with outbred groups of individuals because the MHC genotype is uniform, well-
defined, and shared amongst mice in many different laboratories. This technology has been used to analyze murine immune responses to many pathogens and tumor antigens. There are a significant number of human studies utilizing tetramers that are matched to the HLA genotypes of test subjects. A helpful factor for some of this work is the high prevalence of HLA-A*0201 in humans (>95% of US population)\(^\text{13}\). It is difficult to predict if or when sufficient resources might become available for the practical use of tetramer technology in veterinary research. Even if it eventually proves feasible, there are limitations to the technique. It would be rather cumbersome in studies aimed at assessing the overall T cell response to a pathogen because of the many peptides that would have to be tested in each of the six MHC I or II types in each individual. Also, tetramer labeling by itself does not characterize any functions of cells that it identifies, so other assays would often need to be performed with tetramer-positive cells.

**Considerations for experimental design**

CMI assays are inherently more complex than serological assays because they measure the in vitro functions and activities of live cells. Therefore, considerable day to day variability is typical in experiments that incorporate these assays. This makes it essential that statistical analysis be designed to test for differences between a control group and treatment groups that are vaccinated or infected, as opposed to differences across time. Valid statistical comparisons are only possible if cells from control and subject animals are tested in parallel on the same day.
Conclusion

Many of today's experimental vaccines for veterinary pathogens are designed to target critical epitopes by utilizing recombinant gene technology. In these sophisticated approaches, genes are more and more routinely being deleted from wild-type microorganisms, vectored in heterologous viruses or bacteria, or inserted into plasmids for DNA vaccination. In order for these cutting edge vaccines to be thoroughly evaluated and optimized for practical use, it is important that researchers and regulatory professionals utilize effective and comprehensive tests of immunity. There is also a strong trend toward methods for evaluating vaccines and therapeutics that minimize the use of challenge infections in animal subjects. Modern assays for cell-mediated immunity will very likely be essential for accomplishing all of these goals.

References

7. Fischer T, Buttner M, Rziha HJ. T helper 1-type cytokine transcription in peripheral blood mononuclear cells of pseudorabies virus (Suid herpesvirus 1) primed swine indicates efficient immunization. Immunology 2000;101:378-387.


Chapter 4. T Cell Populations Responsive to Bovine Respiratory Syncytial Virus in Seronegative Calves

A paper published in *Veterinary Immunology and Immunopathology* *

Matthew R. Sandbulte and James A. Roth

Abstract

Calves lacking detectable serum antibodies against bovine respiratory syncytial virus (BRSV) were screened for virus-specific T cell memory. Peripheral blood mononuclear cells were cultured in vitro with live BRSV and analyzed by dual-color flow cytometry for surface expression of CD25 on CD4\(^+\), CD8\(^+\), and \(\gamma\delta\) T cells. Significant recall responses were detected in some of the seronegative calves. Modified-live BRSV vaccine was administered to these and to a group of non-responding calves. Following vaccination, virus-specific IgG, virus neutralizing antibody, and T cell recall responses were all elevated more rapidly in the group with BRSV-sensitive T cells than in the T cell-negative group, which suggested that calves in the first group were previously exposed to BRSV. This demonstrates that exposure to BRSV can induce T and B cell memory in young calves without causing seroconversion. The calves were presumably exposed to BRSV while they had maternal antibody, which inhibited the calves from developing an antibody response.

Keywords: Bovine respiratory syncytial virus; maternal antibody inhibition; T cells; modified-live virus; anamnestic immune response

* Reprinted from volume 84, pages 111-123, © 2002, with permission from Elsevier.
Abbreviations: BHV-1, bovine herpesvirus 1; BRSV, bovine respiratory syncytial virus; BVDV, bovine viral diarrhea virus; E.I., expression index; HBSS, Hanks' balanced salt solution; IFN-γ, interferon gamma; LSD, least significant difference; PBS++, phosphate-buffered saline with 0.5% bovine serum albumin and 0.1% sodium azide; PE, phycoerythrin; PI3, parainfluenza 3; SVN, serum virus neutralization

1. Introduction

Various characteristics of bovine immune response to infection and vaccination with bovine respiratory syncytial virus (BRSV) have been reported. Seronegative calves produce systemic (Kimman et al., 1987a; West and Ellis, 1997; Uttenthal et al., 2000) and mucosal (Kimman et al., 1987b; Kimman et al., 1989) antibodies to the virus following primary exposure. Cattle in the United States and many other countries are predominantly seropositive for BRSV (Ames, 1993; Uttenthal et al., 2000). Correlations between humoral responses and protection from secondary challenge have been inconsistent (Kimman et al., 1989; West et al., 1999a; West et al., 1999b). However, the capacity for a secondary mucosal IgA response has been noted as a predictor of protection (Kimman et al., 1987b; West et al., 1999b). Vaccines vary in their potential to induce neutralizing and fusion-inhibiting antibodies to the virus; higher ratios of both parameters (compared to ELISA titers) are induced by modified-live viruses than by inactivated viruses (Ellis et al., 1995; West and Ellis, 1997). Substantial BRSV-specific maternal antibody titers can partially protect young calves (Kimman et al., 1988; Belknap et al., 1991). Maternal antibody decays at a half-life estimated between 23 and 27 days (Kimman et al., 1987a; Uttenthal et al., 2000).
Results of several studies have demonstrated the importance of a cell-mediated immune response to BRSV. In one study, lymphocytes from the respiratory tract, bronchial lymph nodes, and peripheral blood all progressed toward an activation phenotype (as measured by CD45R, CD45RO, L-selectin, and CD25 expression) following infection with BRSV (McInnes et al., 1999). CD8$^+$ T cell recruitment to the respiratory tract has been observed in the same time frame (Thomas et al., 1996; McInnes et al., 1999). In one set of experiments, monoclonal antibodies were used to deplete T cell subsets from cattle before and during infection with BRSV (Taylor et al., 1995). Virus shedding was prolonged and pathologic lesions were most severe in the group lacking CD8$^+$ T cells. Antigen-specific lymphocyte proliferation, interferon gamma (IFN-γ) production, and chromium-release cytotoxicity assays have been used to demonstrate T cell-mediated immune responses to various whole BRSV vaccines (Ellis et al., 1992; Ellis et al., 1995; Ellis et al., 1996; Keles et al., 1998; West et al., 1999b). In calves immunized with commercial modified-live vaccines and later challenged, virus specific proliferation and IFN-γ responses before challenge correlated better with rapid virus clearance than did serum antibody titers (West et al., 1999b).

Passive antibody has been shown to suppress the active humoral response to infection or vaccination (Kimman et al., 1987a; Kimman et al., 1987b; Kimman et al., 1989). In the latter two studies, live virus administered to maternal antibody-positive calves via the respiratory tract did, however, appear to prime for memory antibody responses upon secondary challenge after maternal antibody was lost. This suggests that the immune system did respond to BRSV in some fashion in the face of maternal antibody. The effect of maternal antibody on T lymphocyte priming is uncertain, but there is evidence that viruses can prime T cells of passively immune cattle. For example, lymphocyte proliferative responses to
BRSV were detected in seropositive neonatal calves following a single vaccine dose (Ellis et al., 1996).

In preparation for another study, we tested sixteen beef calves that lacked BRSV-specific IgG and serum virus neutralizing (SVN) antibody for T-cell mediated immunity to the virus. A flow cytometric assay measuring in vitro virus-specific upregulation of surface CD25 (the alpha chain of the interleukin-2 receptor) was used to analyze the sensitivity of CD4⁺, CD8⁺, and γδ T cells to live BRSV. These animals were assumed to be immunologically naïve to BRSV, but for three calves the assay repeatedly detected T cells responding to BRSV. This suggested that the animals had previously been infected with BRSV. In response to the conflicting serological and cellular data, and given the evidence cited above that a cell-mediated immune response may be activated in calves with antigen-specific maternal antibody, we hypothesized that these calves had been infected while maternal antibody was still present. The antibody-negative, T-cell-positive animals and a group of the naïve calves were immunized with a commercial modified-live vaccine in order to compare their functional immune responses and to clarify the unconventional immune status of the first group. If the hypothesis was correct, this would be a secondary exposure for the T-cell positive calves, and the subsequent humoral and cell-mediated responses would be expected to reflect immunologic memory.

2. Materials and methods

2.1. Animals

Sixteen mixed breed beef calves, which had tested negative for serum virus neutralizing (SVN) antibody to BRSV, were purchased. All calves were from the same herd and had an
average body weight of approximately 180 kg. Additional serum samples were collected (weeks -4 and 0) to test for SVN activity and ELISA antibody against BRSV. Peripheral blood mononuclear cells were isolated three times (weeks –4, –2, and 0) and cultured in vitro to test for recall responses to BRSV using flow cytometry and CD25 as an activation marker. Upon finding that all calves were seronegative by virus neutralization, and that three of them consistently had BRSV-responsive T cells, treatment groups were formed. Eight of the naïve calves with negative T cell responses and all three of those with positive T cell responses were vaccinated (on day 0) intramuscularly with 2 ml of modified-live vaccine containing BHV1, types I and II bovine viral diarrhea virus (BVDV), parainfluenza virus 3 (PI3), and BRSV (Titanium™ 5, Agri Laboratories, Ltd., St. Joseph, MO). At week four post-vaccination, these calves received a second, identical dose of Titanium™ 5. The other five naïve calves were not vaccinated, and composed a negative control group. In one of these individuals, the T cell assay was unreliable, due to high background expression of CD25. The data from this animal was not included.

2.2. Viruses

Modified-live BRSV from Bovi-Shield™ BRSV vaccine (Pfizer Animal Health, Exton, PA) was used for in vitro stimulation of peripheral blood mononuclear cells (PBMC). The vaccine was reconstituted with the manufacturer’s diluent, aliquotted into small volumes, and stored at –70°C. The optimal dilution of virus for stimulation of T cells was determined by titrating with PBMC from a separate group of positive control animals.
2.3. Antibodies

Primary and secondary antibodies used to label PBMC for flow cytometry were the same as listed previously (Quade and Roth, 1999), except that two fluorochrome-conjugated secondary antibodies were purchased from Southern Biotechnology Associates (Birmingham, AL). These were goat anti-mouse IgG1-fluorescein isothiocyanate (FITC) and goat anti-mouse IgG2a-phycoerythrin (PE). A peroxidase-labeled goat anti-bovine IgG (with specificity for heavy and light chains) was purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD) for detection of BRSV-specific IgG in an ELISA assay.

2.4. Isolation and culture of PBMC

Blood samples were collected from calves in tubes containing 60 U.S.P. units heparin per ml blood at weeks -4, -2, 0, 2, 4, and 6. PBMC were isolated for in vitro culture. The samples were kept on ice until being centrifuged at 800g for 20 minutes at 4°C. The buffy coat was collected and diluted at least 2-fold in Hanks' balanced salt solution without Ca\(^{2+}\) and Mg\(^{2+}\) (HBSS), and layered onto an equal volume of Histopaque 1077 (Sigma, St. Louis, MO). This was centrifuged as before, and the mononuclear cell layer was washed in HBSS. Contaminating erythrocytes were lysed by hypotonic flash lysis. PBMC were counted and resuspended to 1.6 X 10^7/ml in HBSS. Then the cells were cultured with or without virus in 96-well, round-bottomed plates. Wells received 200 μl of growth medium (RPMI\(^{+}\)), consisting of RPMI 1640 with 25mM HEPES and L-glutamine (Life Technologies, Gaithersburg, MD), plus 10% fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA) and 0.1% tissue culture penicillin/ streptomycin (Sigma, St. Louis, MO). The FBS tested
negative for BVDV by five blind passages in cell culture, followed by immunocytochemistry using bovine antiserum to BVDV. Each well received $4 \times 10^5$ PBMC in 25μl volume and 25μl of either BRSV suspended in RPMI$^{++}$ ($10^4$ TCID$_{50}$/ml) or control RPMI$^{++}$ alone. Cells with each stimulus (BRSV and control RPMI$^{++}$) were dispensed in triplicate wells. Plates were incubated in a humidified 5% CO$_2$ atmosphere at 39°C for 5 days.

2.5. Fluorescent labeling and flow cytometry

Following culture, PBMC underwent washing and antibody labeling. This was carried out as follows: Plates of PBMC were initially chilled on ice, then washed four times, twice with cold phosphate-buffered saline (PBS) and twice with cold PBS with 0.5% BSA and 0.1% sodium azide (PBS$^{-}$). Cells were incubated with two primary monoclonal antibodies: one against CD25 and another against CD4, CD8, or γδ TCR. Each well received a 50-μl PBS$^{++}$ suspension of its two antibodies. In these mixtures the concentrations of anti-CD4 and -CD8 were 3.3 μg/ml and the concentrations of anti-γδ TCR and anti-CD25 were 1.67 μg/ml. In isotype control wells, mouse IgG2A, IgG1, and IgG2b were used in place of anti-CD25, anti-CD4 and anti-CD8, and anti-γδ TCR, respectively. Cells were incubated with the primary antibodies at 4°C for 30 minutes, followed by four washes with cold PBS$^{++}$. Appropriate secondary antibodies were added to all wells. Each one received anti-IgG$_{2a}$-PE conjugate (0.17 μg/ml) and either anti-IgG$_{1}$-FITC (1.0 μg/ml) or anti-IgG$_{2b}$-FITC (0.33 μg/ml) conjugate. Four washes with PBS followed. Cells were fixed with 1% formaldehyde in HBSS, and the contents of triplicate wells were pooled in tubes. A FACScan flow cytometer (Becton-Dickinson) was used to acquire raw data from samples. Excitation was by a 488 nm laser. FITC fluorescence was measured in the FL1 channel and PE fluorescence was
measured in the FL2 channel. Forward and side scatter parameters were also acquired, and an acquisition gate was set around the lymphocyte populations. Enough cells were analyzed to accumulate 5000 gated events for each sample.

2.6. Serology

At weekly intervals, blood samples were collected in evacuated serum separator tubes. After centrifuging tubes for 30 minutes at 1200g, aliquots of serum were removed and saved at −20°C until submission for testing. Samples were submitted to the Iowa State University Veterinary Diagnostic Laboratory for measurement of BRSV serum neutralizing antibody titers. Neutralization titers were defined as the reciprocal of the highest serum dilution that inhibited 100% of BRSV cytopathic effects in cell culture.

An indirect ELISA protocol was used to detect BRSV-specific IgG in frozen serum samples, using a protocol adapted from Bruckova et al. (1981). Briefly, BRSV antigen was partially purified from BRSV-infected bovine turbinate cell monolayers, and negative control antigen was prepared from noninfected cells. Lysates from freeze-thawed and sonicated cells were ultracentrifuged, resuspended in PBSS (pH 8.5), and coated onto ELISA plates. Serum samples from weeks -4 and 0-6 were diluted 1:5 and dispensed into duplicate BRSV antigen-coated and control antigen-coated wells. Plates were incubated at room temperature, washed, and treated with peroxidase-labeled goat anti-bovine IgG, diluted 1:500. After application of substrate, absorbance was read at 405 nm. Final optical density (OD) values were tabulated for BRSV-coated wells receiving serum by subtracting the background OD level in wells without serum added.
2.7. Data analysis

Analysis of CD25 expression index data was performed as described previously (Quade & Roth 1999). Gates were set on the basis of side-scatter versus forward-scatter to include most lymphocytes. Quadrant markers were selected by comparing staining with specific antibodies versus isotype controls. For each combination of T-cell subset and stimulant, the percentage of cells expressing CD25 and the geometric mean fluorescence intensity of CD25-specific staining among these cells were tabulated. CD25 expression indices (E.I.) were calculated by the following formula for each combination of animal, stimulant, and subset:

\[
\text{E.I.} = \frac{\% \text{ of subset CD25}^+ \times \text{geometric mean intensity of CD25 staining}}{\% \text{ of subset CD25}^+ \times \text{geometric mean intensity of CD25 staining}}
\]

For statistical tests, a log(2) transformation was performed on all CD25 expression index values and SVN titers. All SVN titers of <2 were defined to be 1 prior to log(2) transformation. Differences in mean log₂(CD25 E.I.) and mean log₂(SVN titer) among groups at each time point were determined by Analysis of Variance in the SAS program (SAS Institute Inc., Cary, NC) with P < 0.05. Pairwise comparisons between treatment effects in the three groups were made by Least Significant Difference tests (LSD).

3. Results

3.1. Baseline immunity

T cells from group 3 cattle responded to BRSV in vitro at all three pre-vaccination time points. At two of these samplings, the log₂(CD25 EI) for CD4⁺, CD8⁺, and γδ T cells in group 3 were all significantly greater than corresponding values in groups 1 and 2 (P < 0.05)
(Figures 1-3). SVN titers were also assayed three times prior to vaccination. Negative titers were recorded consistently for most calves in every group (Figure 4). The exceptions were titers of 2 for three calves (one in group 1 and two in group 3) and 4 for another (in group 2) at week 0. One week later, negative titers were recorded again for one of the calves that had registered at 2 (in group 1) and for the calf that had registered at 4 the week before. Archived serum samples were later assayed by ELISA for BRSV-specific IgG in order to test whether T-cell positive group 3 calves had in fact seroconverted with non-neutralizing antibody against the virus. Among groups 1, 2, and 3 there were no significant differences in OD values at week -4 or week 0, the only pre-vaccination time points tested. The week 0 OD values (± standard deviation) for groups 1, 2, and 3 were 0.109 ± 0.041, 0.114 ± 0.029, and 0.107 ± 0.015 respectively. Thus, cattle in groups 1 and 2 were naïve to BRSV with respect to T cells and antibody titers. The animals in group 3 had been primed for T cell responses to BRSV, but were antibody negative.

3.2. Virus-specific T cell sensitivity

Groups 2 and 3 were vaccinated with a commercial modified-live vaccine against BRSV, BVD types I and II, BHV1, and PI3 at week 0 and boosted with the same vaccine at week 4. BRSV-specific CD4⁺ T cells were detected in initially naïve calves (group 2) by week 4, when the geometric mean log(2) CD25 E.I. was significantly greater than that of control group 1 (P < 0.05) (Figure 1C). This was observed again at week 6. At weeks 2, 4, and 6 CD4⁺ T cells from group 3 calves maintained a significantly higher E.I. over their counterparts in the other two groups.
Figure 1.
BRSV-specific CD25 upregulation by CD4\(^+\) T cells from BRSV-seronegative calves vaccinated with modified-live vaccine. Vaccine was given to group 2 (T-cell negative, n=8) and group 3 (T-cell positive, n=3) at weeks 0 and 4, while group 1 (n=4) served as negative controls. At each sampling, PBMC were cultured with and without live BRSV for 5 days and analyzed for CD25 expression by two-color flow cytometry. Shown are the percentage of CD4\(^+\) T cells expressing surface CD25 (A), the mean fluorescence intensity of CD25 expression among CD4\(^+\)CD25\(^+\) T cells (B), and the CD25 expression index (E.I.) of CD4\(^+\) T cells (C). Error bars represent standard errors of the means. * Group 2 E.I. significantly greater than group 1, \(P < 0.05\). † Group 3 E.I. significantly greater than group 1, \(P < 0.05\). ‡ Group 3 E.I. significantly greater than group 2, \(P < 0.05\).
% of CD4+ T Cells Expressing CD25

CD4+ T Cell Mean Fluorescence Intensity of CD25 Expression

CD4+ T Cell CD25 Expression Indices
Figure 2.

BRSV-specific CD25 upregulation by CD8\(^+\) T cells from BRSV-seronegative calves vaccinated with modified-live vaccine. Vaccine was given to group 2 (T-cell negative, n=8) and group 3 (T-cell positive, n=3) at weeks 0 and 4, while group 1 (n=4) served as negative controls. At each sampling, PBMC were cultured with and without live BRSV for 5 days and analyzed for CD25 expression by two-color flow cytometry. Shown are the percentage of CD8\(^+\) T cells expressing surface CD25 (A), the mean fluorescence intensity of CD25 expression among CD8\(^+\)CD25\(^+\) T cells (B), and the CD25 expression index (E.I.) of CD8\(^+\) T cells (C). Error bars represent standard errors of the means. * Group 2 E.I. significantly greater than group 1, \(P < 0.05\). † Group 3 E.I. significantly greater than group 1, \(P < 0.05\). ‡ Group 3 E.I. significantly greater than group 2, \(P < 0.05\).
% of CD8+ T Cells Expressing CD25

A.

CD8+ T Cell Mean Fluorescence Intensity of CD25 Expression

B.

CD8+ T Cell CD25 Expression Indices

C.
Figure 3.

BRSV-specific CD25 upregulation by γδ T cells from BRSV-seronegative calves vaccinated with modified-live vaccine. Vaccine was given to group 2 (T-cell negative, n=8) and group 3 (T-cell positive, n=3) at weeks 0 and 4, while group 1 (n=4) served as negative controls. At each sampling, PBMC were cultured with and without live BRSV for 5 days and analyzed for CD25 expression by two-color flow cytometry. Shown are the percentage of γδ T cells expressing surface CD25 (A), the mean fluorescence intensity of CD25 expression among CD25⁺ γδ T cells (B), and the CD25 expression index (E.I.) of γδ T cells (C). Error bars represent standard errors of the means. * Group 2 E.I. significantly greater than group 1, $P < 0.05$. † Group 3 E.I. significantly greater than group 1, $P < 0.05$. ‡ Group 3 E.I. significantly greater than group 2, $P < 0.05$. 
Primary vaccination did not have a large effect on the magnitude of CD8$^+$ T cell responses (Figure 2C). The mean CD25 E.I. in CD8$^+$ T cells from group 2 was elevated at week 4 to the point that it was not significantly different from group 3. In the only assay following secondary immunization (week 6), the mean CD25 E.I. in CD8$^+$ T cells from group 2 had become significantly different from that of control group 1 ($P < 0.05$). Also at week 6, the mean log(2) CD25 E.I. for CD8$^+$ T cells in group 3 was once again significantly greater than those in groups 1 and 2.

γδ T cell responses were also observed after vaccination (Figure 3C). At weeks 4 and 6, the mean log(2) CD25 expression indices for γδ T cells in group 2 were significantly greater than in control group 1 ($P < 0.05$). At approximately the same time, an elevation in the mean CD25 E.I. of group 3 was evident. At weeks 4 and 6, γδ T cells from group 3 once again showed significantly greater responses than either other group.

Levels of CD25 expression on the different subsets of T cells appear to fall into somewhat different ranges following culture with and without the presence of BRSV. Variation in the percentages of subsets expressing CD25 probably reflects differences in their viability in culture and their rate of clonal expansion upon antigen recognition.

### 3.3. Humoral responses to BRSV vaccine

Serological data also suggested that group 3 calves had been primed for specific immunity to BRSV (Figure 4). Following immunization calves that already had BRSV-specific T cells (group 3) produced a strong, rapid SVN response to BRSV. In contrast with the weak primary antibody response in fully naïve calves (group 2), this appeared to be a memory response. One week after vaccines were given, the mean log(2) SVN titer of group 3 was
Figure 4.

Serum virus neutralizing antibody responses of seronegative calves immunized with modified-live BRSV. Vaccine was administered to group 2 (T-cell negative) and group 3 (T-cell positive) at weeks 0 and 4, while group 1 served as negative controls. Values given are log₂(SVN titer). * Group 2 significantly greater than group 1, $P < 0.05$. † Group 3 significantly greater than group 1, $P < 0.05$. ‡ Group 3 significantly greater than group 2, $P < 0.05$. 
statistically greater than those in groups 1 (naïve, non-vaccinated) and 2. This trend continued until the week after booster vaccination (week 5). At this point a statistically significant difference was detected in the SVN titers of control group 1 and group 2. The anamnestic antibody response in group 2 after secondary immunization produced titers very similar to those observed in group 3 after a single vaccine dose.

4. Discussion

A group of calves that had T cells primed to respond to BRSV, but lacked detectable titers of serum virus neutralizing antibody against BRSV was identified. These calves responded to vaccination by producing a greater antibody and T cell response than did animals without BRSV-specific T cells. This suggests that the animals were previously exposed to the virus, which enabled priming of T and B lymphocytes, but they were prevented from mounting an antibody response. The data are consistent with the hypothesis that these calves were infected with BRSV while still possessing virus-specific maternal antibody in their serum. Some studies have shown that virus infection or vaccination in calves (Bradshaw and Edwards, 1996; Lemaire et al., 2000), infants (Gans et al., 1999), and mice (Siegrist et al., 1998) with high maternal antibody titers may induce a T cell response despite the absence of an active antibody response. In other studies, passively immunized calves that failed to mount primary antibody responses to BHV-1 (Brar et al., 1978) and BRSV (Kimman et al., 1989) did have memory-type antibody responses following secondary exposure. In the latter case virus shedding was monitored, revealing that intranasal priming conferred protection to colostrum fed calves. Our laboratory recently evaluated T-cell and neutralizing antibody responses in groups of colostrum fed and colostrum deprived calves that were challenged and later re-
challenged with bovine viral diarrhea virus (BVDV) types I and II (Endsley et al, submitted for publication). Colostrum fed calves, with very high passive antibody titers to BVDV I and II, did not produce neutralizing antibody following initial exposure. However, T cell priming was evident in this group of animals. Upon challenge, they mounted anamnestic antibody responses and were protected from disease. Other researchers vaccinated neonatal calves with BRSV in the face of maternal antibody and subsequently detected lymphocyte proliferative responses to BRSV, which demonstrated some degree of lymphocyte priming (Ellis et al., 1996). Therefore, our observation that T cell-mediated immunity to BRSV can occur without seroconversion is consistent with the reported immune responses of cattle, humans, and mice possessing maternal antibody to several viruses.

The mechanisms by which maternal antibody could block antibody production by B cells are not clear. It is generally thought that immunosuppression by passive antibody results from antigen being bound and, in the case of some viruses, neutralized. It seems plausible that the resulting immune complexes would be readily taken up and processed by antigen presenting cells. This would enable naïve, antigen-specific T cells in secondary lymphoid tissue to recognize immunodominant epitopes and proliferate. Another relevant mechanism is the suppression of naïve B cell activation that occurs when immune complexes crosslink surface antigen receptor with FcγRIIb (Fridman, 1993). Work done in calves by Aldridge et al poses another possible explanation (Aldridge et al., 1998). They examined the expression of surface immunoglobulins on B cells in the lymph nodes of fetal and neonatal calves. Surface IgG1 and IgG2 expression in colostrum fed calves was absent at 36 h of age, but readily detectable in fetuses and colostrum deprived calves. This implied that a non-antigen-specific factor in colostrum could have a part in modulating calves’ antibody production. It is
questionable whether any of these mechanisms would allow for B cell priming while suppressing antibody production. Since we did not measure B cell activation in this experiment, we cannot rule out the possibility that T cells were the only lymphocyte population to initially develop memory. In this scenario, one could speculate that the presence of many antigen-specific helper T cells in calves from group 3 accelerated the priming of B cells after vaccination.

In conclusion, the data presented here demonstrate that cattle can acquire T cell responsiveness to BRSV while remaining seronegative to the virus. This agrees with findings in some other host-virus systems. It demonstrates that BRSV seronegative cattle should not be presumed to be immunologically naïve to BRSV, because the IgG ELISA and SVN assays are not sufficient to identify all calves primed for a response to the virus. Seronegative calves with T cells primed against BRSV respond more rapidly and vigorously to vaccination than naïve animals.

Acknowledgements

The authors thank Dagmar Frank and Thomas Skadow for excellent technical assistance. This research was partially supported by the John G. Salsbury Endowed Chair in Veterinary Medicine at Iowa State University.

References


2000. Effects of bovine herpesvirus type 1 infection in calves with maternal antibodies
responses in calves infected with bovine respiratory syncytial virus. Immunology 96,
396-403.
of cattle immunized with a modified-live bovine herpesvirus 1 vaccine. Viral Immunol.
12, 9-21.
Siegrist, C., Barrios, C., Marinez, X., Brandt, C., Berney, M., Cordova, M., Kovarik, J.,
antibody but not T cell responses allows successful early prime-boost strategies in mice.
lymphocyte subsets in recovery from respiratory syncytial virus infection in calves. J.
Virol. 69, 6658-6664.
Thomas, L. H., Cook, R. S., Howard, C. J., Gaddum, R. M., Taylor, G., 1996. Influence of
selective T-lymphocyte depletion on the lung pathology of gnotobiotic calves and the
distribution of different T-lymphocyte subsets following challenge with bovine
Uttenthal, A., Larsen, L. E., Philipson, J. S., Tjornehoj, K., Viuff, B., Nielsen, K. H., Nielson,
T. K., 2000. Antibody dynamics in BRSV-infected Danish dairy herds as determined by
West, K., Ellis, J., 1997. Functional analysis of antibody responses of feedlot cattle to bovine
61, 28-33.
The effect of formalin-inactivated vaccine on respiratory disease associated with bovine
respiratory syncytial virus infection in calves. Vaccine 17, 809-820.
West, K., Petrie, L., Konoby, C., Haines, D. M., Cortese, V., Ellis, J. A., 1999b. The efficacy
of modified-live bovine respiratory syncytial virus vaccines in experimentally infected
calves. Vaccine 18, 907-919.
Chapter 5. Priming of Multiple T Cell Subsets by Modified-Live and Inactivated Bovine Respiratory Syncytial Virus Vaccines

A paper accepted for publication in Veterinary Immunology and Immunopathology

Matthew R. Sandbulte and James A. Roth

Abstract

T cell activity is a critical component of immunity to bovine respiratory syncytial virus (BRSV). We tested the effects of immunization by modified-live and inactivated BRSV vaccines on cell-mediated and humoral immunity in young calves. The two forms of vaccine stimulated similar serum neutralizing antibody production, although the early kinetics of those responses differed. CD4⁺, CD8⁺, and γδ T cells were analyzed before and after immunization for BRSV-specific in vitro recall responses, as evaluated by CD25 upregulation measured by flow cytometry. Modified-live virus (MLV) primed each of the three subsets for statistically significant in vitro responses to antigen. Inactivated vaccine also primed each T cell population for significant antigen-driven CD25 upregulation, including responses by CD4⁺ and γδ T cells that were stronger and longer-lasting than those primed by MLV. Monoclonal antibody was used in additional assays to block MHC class I during incubation of BRSV antigen with peripheral blood mononuclear cells from an animal in the inactivated vaccine group. The recall response by CD8⁺ T cells was more inhibited by this treatment than the other subsets, further suggesting that the inactivated vaccine had primed antigen-specific CD8⁺ T cells. In summary, the data indicate that balanced BRSV-specific T cell responses can be induced by inactivated, as well as modified-live, conventional vaccines, which may implicate an alternative pathway of MHC class I antigen presentation.
Keywords: Bovine respiratory syncytial virus, vaccination, T cells, CD25

Abbreviations: BRSV, bovine respiratory syncytial virus; IFN-γ, interferon gamma; HBSS, Hanks' balanced salt solution without Ca\(^{2+}\) and Mg\(^{2+}\); HRSV, human respiratory syncytial virus; MLV, modified-live virus; PBS\(^{++}\), phosphate-buffered saline plus BSA and sodium azide; PE, phycoerythrin; RPMI\(^{++}\), RPMI 1640 media plus fetal bovine serum and antibiotics

1. Introduction

Bovine respiratory syncytial virus (BRSV), of the Paramyxoviridae family, is an important etiologic agent of respiratory disease in dairy and beef cattle (Larsen, 2000). Calves less than one year of age are particularly susceptible to outbreaks of the virus (Van der Poel et al., 1994; Larsen, 2000). BRSV infects bronchiolar and alveolar epithelial tissues, and infected epithelial cells may fuse into syncytia (Viuff et al., 1996). Bronchiolitis and interstitial pneumonia in the cranioventral lung region, and alveolar edema and interstitial emphysema throughout the lung are typical (Kimman et al., 1989a; Baker et al., 1997). Human respiratory syncytial virus (HRSV) is a closely related virus that causes similar lesions and clinical symptoms in infants (Duncan and Potgieter, 1993; Van der Poel et al., 1994).

Cattle in many countries are predominantly seropositive for BRSV (Baker et al., 1997; Uttenthal et al., 2000). Infection with the virus does not generally induce sufficient immunity to prevent subsequent infection. Seroprevalence is high even in young calves, due to colostrum-borne maternal antibodies (Kimman and Westenbrink, 1990). Passive antibody
may provide partial protection from disease when titers are high (Duncan and Potgieter, 1993), but it inhibits the humoral response to vaccine or infection, even after titers decline below protective levels (Kimman et al., 1989b). Another complicating factor in the development of RSV vaccines is the potential for immunopathologic effects. Immunization of infants with a formalin-inactivated HRSV vaccine enhanced subsequent disease (Kim et al., 1969). Therefore the quality of immunization attained in other RSV hosts, such as cattle, is of special interest. Calves that received an experimental formalin-inactivated BRSV vaccine also suffered enhanced disease following challenge (Gershwin et al., 1998). In addition to that work, several studies have examined the efficacy of inactivated and modified-live BRSV vaccines in stimulating different facets of the immune response and protecting against challenge.

In one study, commercial MLV and inactivated vaccines were compared by monitoring in vitro parameters of cell-mediated immunity, and responses were distinctly different (Ellis et al., 1992a). Antigen-specific lymphocyte proliferation was detectable after one dose of adjuvanted, inactivated vaccine, but only after secondary immunization with MLV. The strong cellular response to inactivated BRSV was consistent with previous lymphocyte proliferation data induced by glutaraldehyde-fixed, whole-cell vaccines formulated with Freund's incomplete (Taylor et al., 1987) or Quil A adjuvants (Howard et al., 1987). In contrast, another study of several commercial vaccines showed that MLV vaccines stimulated superior immune responses in terms of both neutralizing antibody and lymphocyte proliferation (Ellis et al., 1995).

Additional studies tested the protective effects of MLV and inactivated vaccines against viral challenge. Glutaraldehyde-fixed, cell-associated BRSV and heat-inactivated, cell-free
BRSV vaccines were tested in lambs (Keles et al., 1998). Both vaccines limited virus shedding and induced humoral and cellular immunity, as assessed by virus neutralization, virus-specific cytotoxicity, and lymphocyte blastogenesis. The glutaraldehyde-fixed vaccine gave superior protection and immune responses. A similar set of experiments in calves was performed with two commercial, polyvalent MLV vaccines (West et al., 1999). After challenge, clinical disease, virus shedding, and pulmonary lesions were reduced in calves receiving either vaccine. Protection was correlated with in vitro cellular responses before challenge, that is, antigen specific lymphocyte proliferation and interferon gamma (IFN-γ) production. Virus clearance in vaccinated and control calves coincided with the detection of BRSV-specific CTL in the lungs. Thus, while inactivated and modified-live BRSV vaccines can both stimulate humoral and cellular immune responses, the literature suggests considerable variation in these responses. It is not expected that an inactivated virus would prime CD8⁺ cytotoxic T lymphocytes because these cells recognize peptides presented on MHC class I, and this mode of presentation is generally thought to require virus replication in the cytoplasm.

CD8⁺ T lymphocytes and associated virus-specific cytotoxicity are important for a protective immune response to BRSV (Taylor et al., 1995; Thomas et al., 1996; West et al., 1999). CD4⁺ T cell responses to BRSV are of special interest due to their complex roles as helper cells, i.e. production of type 1 and/or type 2 cytokines. The γδ T cells are a prominent cell population in cattle, and have at least an immunomodulatory role in the responses to numerous pathogens (Hein and Mackay, 1991; Howard et al., 1999; Rhodes et al., 2001; Naiman et al., 2001), possibly including BRSV (Taylor et al., 1995). Conventional lymphocyte proliferation assays do not distinguish activation of the CD4⁺, CD8⁺, γδ T cell,
and B-cell populations. For an improved comparison of how different BRSV vaccines prime the cell-mediated immune response it is important to measure the stimulation of each population of T cells. Dual-color flow cytometry has been used to measure in vitro activation of antigen-specific ruminant T cells by monitoring expression of CD25, which is the inducible alpha chain of IL-2 receptor (Quade and Roth, 1999; Liebana et al., 1999; Whist et al., 2000; Sandbulte and Roth, 2002). In the present study a similar technique was used to monitor T cell priming in cattle after immunization with inactivated or modified-live commercial BRSV vaccines.

2. Materials and Methods

2.1. Animals

Holstein steers, weighing approximately 225-275 kg, were randomly divided into two treatment groups. Before treatment, peripheral blood mononuclear cells were isolated twice to assay baseline in vitro responses to BRSV and ConA (Sigma, St. Louis, MO). All calves were negative for BRSV serum neutralizing antibodies and negative for BRSV-specific T cell responses before vaccination. A group of four calves were vaccinated intramuscularly with 2 ml of monovalent modified-live BRSV (Bovi-Shield BRSV, Pfizer Animal Health, Exton, PA). The other four animals were vaccinated intramuscularly with 2 ml of monovalent killed BRSV with Xtend III adjuvant (Syn Shield, Grand Laboratories, Inc., Larchwood, IA). In accordance with both vaccine labels, vaccinated cattle were re-vaccinated after four weeks with doses identical to their first ones. Both vaccines were produced from virus grown in bovine cell lines, so it is unlikely that the recipient cattle would have developed immunity to cell culture components. Two BRSV-negative Holstein
steers served as negative control animals and two that had been immunized multiple times
with polyvalent modified live vaccine (BRSV Vac 4, Miles, Inc., Shawnee Mission, KS),
were positive controls.

2.2. Isolation and culture of PBMC

Calves were bled at biweekly intervals from week two to week ten. For this set of assays,
PBMC were isolated and cultured with virus in vitro as previously described (Sandbulte and
Roth, 2002). To summarize, heparinized blood was centrifuged, and the buffy coat was
collected, diluted in Hanks’ balanced salt solution without Ca$^{2+}$ and Mg$^{2+}$ (HBSS), and
separated on Histopaque 1077 (Sigma, St. Louis, MO). Mononuclear cells were washed and
contaminating erythrocytes were lysed by hypotonic flash lysis. Then the cells were cultured
with and without virus in 96-well, round-bottomed plates. Wells received 200µl of growth
media (RPMI$^{16}$), consisting of RPMI 1640 with HEPES and L-glutamine, 10% fetal bovine
serum FBS, and 0.1% tissue culture penicillin / streptomycin. The FBS tested negative for
BVDV by five blind passages in cell culture, followed by immunocytochemistry. Each well
received $4 \times 10^5$ PBMC and either $2.5 \times 10^3$ TCID$_{50}$ of BRSV diluted in RPMI$^{16}$ or an equal
volume of RPMI$^{16}$ alone, bringing the final volume to 250 µl. Cells were cultured in
triplicate wells for each treatment. The in vitro viral antigen was modified-live BRSV from
the Bovi-Shield BRSV product (Pfizer Animal Health, Exton, PA), which was constituted
with the manufacturer’s diluent, aliquotted into small volumes, and stored at $-70^\circ$C. The
optimal virus titer for in vitro stimulation of T cells was determined by titrating the antigen
with PBMC from the positive control animals. Plates were incubated in a humidified 5% CO$_2$
atmosphere at 37°C for 5 days.
For a subsequent experiment it was necessary to prepare PBMC suspensions in which two T cell subsets were depleted, in order to study the third subset of T cells in isolation. The cell isolation protocol described above was extended by using negative magnetic sorting. After the standard preparation of PBMC, the cells were resuspended in a coating buffer consisting of HBSS with 10% FBS and 20 mM Hepes, to $2 \times 10^7$ per ml. For samples being depleted of CD4$^+$ T cells, at least 40 $\mu$g anti-CD4 were added per ml. Samples being depleted of CD8$^+$ T cells received 25 $\mu$g and those being depleted of $\gamma\delta$ TCR$^+$ T cells received 33 $\mu$g of the corresponding antibodies. Samples were incubated 30 minutes with rocking motion while on ice. Two washes with coating buffer followed, and then 50 $\mu$l of Dynabeads® were added per ml of sample. Samples were incubated 60 minutes at 4°C with gentle tumbling motion.

Sample tubes were placed in a magnetic particle concentrator (Dynal A.S, Oslo, Norway) for three minutes before transferring the unbound contents to a new tube. An additional round of depletion was performed by adding fresh Dynabeads® and repeating the incubation and separation steps. Remaining cells were cultured exactly as described for nondepleted PBMC. Depletion efficiencies of CD4$^+$, CD8$^+$, and $\gamma\delta$ TCR$^+$ T cells were 94-99%, 88-99%, and 90-95%, respectively.

Another experiment was designed to test T cell responses to BRSV while inhibiting MHC class I antigen presentation. PBMC used in this experiment did not undergo T cell subset depletion. Monoclonal antibody to MHC I and isotype control antibody were dispensed into selected wells at the beginning of culture, using antibody concentrations of 100, 200, and 400 ng/ml.
2.3. Antibodies

Specific details concerning the antibodies used to label T cells for flow cytometry in this study were provided previously (Sandbulte and Roth, 2002). Briefly, primary monoclonal antibodies used to label cell surface markers CD4, CD8α, delta chain of γδ TCR, and CD25 were purchased from VMRD (Pullman, WA). Isotype control antibodies and affinity-purified, fluorochrome-conjugated secondary antibodies were purchased from Caltag Laboratories (Burlingame, CA).

The same primary antibodies to CD4, CD8α, and delta chain were used in an additional experiment as primary reagents for the depletion of specific T cell subsets by magnetic sorting. The secondary reagent for T cell depletion was a pan mouse IgG-specific monoclonal antibody attached to superparamagnetic beads (Dynabeads®, product number 110.22; Dynal A.S, Oslo, Norway). For the MHC class I blocking experiment performed in this study, a monoclonal antibody to MHC I was purchased from VMRD (cell line H58A); an IgG2a isotype control antibody was purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL). Both antibodies were dialyzed extensively in HBSS. The isotype of the blocking antibody (IgG2a) required that CD25 be labeled with a directly conjugated primary antibody. The PhycoLink® R-phycoerythrin (PE) conjugation kit from ProZyme, Inc. (San Leandro, CA) was used to label anti-CD25 from the original source.

2.4. Fluorescence labeling and flow cytometry

PBMC were prepared for flow cytometric analysis by a previously described protocol (Sandbulte and Roth, 2002), described here in brief. The PBMC were chilled on ice at the conclusion of the culture period and washed twice with cold PBS. Cold PBS with 0.5% BSA
and 0.1% sodium azide (PBS++) was used to wash cells two more times. Cell samples were incubated with two primary monoclonal antibodies: one against CD25 and another against CD4, CD8α, or γδ TCR (or corresponding isotype control antibodies). Cells were incubated with the primary antibodies at 4°C for 30 minutes. Four washes with PBS++ followed. Secondary antibodies were used to stain the T cell subset markers CD4, CD8, and γδ with FITC and CD25 with PE. Cells were incubated with the secondary antibodies in the dark at 4°C for 30 minutes, followed by four washes with PBS. Cells were fixed with 1% formaldehyde in PBS. A FACScan flow cytometer (Becton-Dickinson) was used to acquire raw data. An acquisition gate was set around the lymphocyte population, based on forward-scatter and side-scatter parameters. Each sample was analyzed until five thousand gated events were collected. Flow cytometric data was analyzed similarly to that in prior studies (Quade and Roth, 1999). Briefly, gates were set on the basis of side-scatter versus forward-scatter to include most lymphocytes. Quadrants were defined by comparing cell staining with specific antibodies versus isotype controls. For each combination of T cell subset and stimulant, the percentage of cells expressing CD25 was calculated.

2.5. Virus neutralization assays

At the same biweekly intervals, blood samples were collected in evacuated serum separator tubes. After centrifuging tubes for 30 minutes at 1200 x g, aliquots of serum were removed and saved at −20°C until submission for testing. Samples were submitted to the Iowa State University Veterinary Diagnostic Laboratory for measurement of BRSV serum neutralizing antibody titers. Neutralization titers were defined as the reciprocal of the highest serum dilution that inhibited 100% of BRSV cytopathic effects in cell culture.
2.6. Statistical Analysis

The T cell CD25 expression data were analyzed by week and by subset. BRSV-specific responses were determined by calculating the difference between percent CD25 expression in samples receiving in vitro antigen versus control samples cultured without antigen. Within a treatment group, the mean of this CD25 differential (Δ%CD25) was determined to be significant if its 95% confidence interval excluded zero. Student's T test was used to test for significant differences between the two vaccine treatment groups at each time point, in terms of Δ%CD25. Serum virus neutralization values were also analyzed by week, using Wilcoxon's ranked sum test to determine significant differences between the two groups. Statistical significance for each test was defined as p < 0.05.

3. Results

3.1. Virus Neutralization

Vaccination with MLV and inactivated BRSV vaccines stimulated serum virus neutralizing antibody titers that peaked at about the same level (Figure 1). However, these two responses developed somewhat differently. Significant neutralizing antibody was detected in calves from the MLV group four weeks post-vaccination (p < 0.05). Booster doses of both vaccines were administered at week four. There were no detectable neutralizing antibody titers in calves from the inactivated vaccine group until week six (p < 0.05). From week six until the final assay, at week ten, titers in the killed and MLV groups were very similar, but these appeared to be diminishing substantially by week ten.
Figure 1.

Vaccine-induced BRSV neutralizing antibody levels. Serum samples collected the day of immunization and at biweekly intervals were tested for virus neutralizing activity in cell culture. Serum virus neutralization (SVN) activity was analyzed in two-fold serial dilutions. Titers were defined as the reciprocal of the highest serum dilution that inhibited 100% of BRSV cytopathic effects. Data is presented as the log₂ of SVN titer. Error bars represent standard error of the mean (n=4). * $P < 0.05$; level of statistical significance of the difference between the two groups at the time point indicated.
3.2. T cell priming

Both the MLV and inactivated vaccines primed CD4⁺ T cells to respond to BRSV in vitro (Figure 2A). Beginning at week two, the difference in CD25 expression between cells cultured with and without antigen was statistically significant in the inactivated vaccine group (p < 0.05). This was continued through the tenth week. For the MLV group, significant antigen-driven CD25 expression was observed at weeks 6 and 8, although the mean response was also higher (p > 0.05) by week 2. At weeks 8 and 10, CD4⁺ T cells from the inactivated vaccine group expressed significantly more CD25 in response to BRSV than their counterparts in the MLV group (p < 0.05).

Immunization induced elevated responsiveness to BRSV by CD8⁺ T cells from both vaccine groups (Figure 2B). During the pre-immunization baseline assays there were low, but sometimes statistically significant, levels of CD25 expression by CD8⁺ T cells cultured with BRSV (p < 0.05). For six weeks following immunization there were steady increases in sensitivity to the virus by CD8⁺ T cells from both treatment groups. In the later weeks of the study, responses by the MLV group declined to nonsignificant levels, while responses by the inactivated vaccine group were quite steady. As a result, the in vitro CD8 response by cattle in the inactivated vaccine group was significantly greater than that of MLV vaccinates at week 10 (p < 0.05).

γδ T cell responses to in vitro BRSV antigen followed a similar kinetic pattern. For both groups, the difference between CD25 expression in γδ T cells cultured with and without antigen was significant from week 2 through at least week 8 (p < 0.05) (Figure 2C). The responsiveness of γδ T cells from the MLV group declined after week 6 to statistically nonsignificant levels at week 10. Responses in the inactivated vaccine group were stable.
CD25 expression on CD4+ (A), CD8+ (B), and γδ TCR+ (C) T cells in response to culture with BRSV in vitro. PBMC were isolated from cattle in the inactivated virus and MLV vaccine treatment groups. Cell samples were incubated with and without the presence of BRSV for five days, and the level of CD25 expression was determined for both conditions. Data presented are the percent CD25-positive for BRSV-stimulated samples minus the percent CD25-positive for samples without BRSV (Δ%CD25). Error bars represent standard error of the mean (n=4). * Denotes a Δ%CD25 that is statistically significant, i.e. its 95% confidence interval excludes zero. † Denotes a significant difference in BRSV-driven CD25 expression between cells of the two vaccine treatment groups at a given time point, as determined by Student's t-test (P < 0.05).
A. CD4+ T Cells
- Inactivated UMLV
- MLV

B. CD8+ T Cells
- Inactivated UMLV
- MLV

C. γδ T Cells
- Inactivated UMLV
Consequently, there were statistically significant differences between vaccine treatment
groups at weeks 8 and 10, regarding γδ T cell sensitivity to BRSV (p < 0.05).

Cumulative data from positive control samples during the experiment (n=13) showed
differential CD25 expression values for CD4⁺, CD8⁺ and γδ T cells of 39.1±3.0, 27.7±2.1,
and 60.6±3.9, respectively. Equivalent data from negative control samples (n=12) showed
differential CD25 values for CD4⁺, CD8⁺ and γδ T cells of -1.6±3.1, 0.2±2.2, and 0.0±4.1.

3.3. Roles of accompanying T cell subsets and MHC class I in in vitro activation

Additional experiments were carried out to better understand the T cell responses to
inactivated vaccine, with particular attention to CD8⁺ T cells. In the first approach, each
subset of T cells was tested for in vitro responsiveness to BRSV after having been isolated
from the other two subsets. Before culturing PBMC with antigen, magnetic sorting was used
to deplete two T cell phenotypes and, in effect, enrich the third. On two occasions the three T
cell subsets from one calf were tested under both protocols: subset isolation and ordinary
PBMC preparation (Table 1). For CD4⁺ cells, isolation from the other T cells did not have a
large influence upon the percentage expressing CD25 after culture with BRSV. In contrast,
BRSV did not stimulate a perceptible response by isolated CD8⁺ T cells; these cells had
significantly less CD25 upregulation during culture than non-isolated CD8⁺ T cells (p =
0.04). The antigen-specific CD25 response by γδ T cells was also reduced when CD4⁺ and
CD8⁺ T cells were depleted, but the difference was not statistically significant.

This raised the question of why the CD8⁺ T cells required the presence of other T cells to
respond to BRSV in vitro. To test the hypothesis that the vaccine had primed antigen-specific
CD8⁺ T cells that required some form of T cell help, anti-MHC class I antibody was used to
Table 1.

**BRSV Responses by Fractionated and Nonfractionated T Cells**

<table>
<thead>
<tr>
<th>Cell Phenotype</th>
<th>Cell Preparation</th>
<th>BRSV-Driven CD25 Expression (%\text{CD25 differential} \pm \text{SEM})</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4⁺</td>
<td>PBMC</td>
<td>53.3 ± 7.9</td>
</tr>
<tr>
<td></td>
<td>CD8 &amp; (\gamma\delta)-depleted (^b)</td>
<td>43.8 ± 7.9</td>
</tr>
<tr>
<td>CD8⁺</td>
<td>PBMC</td>
<td>36.0 ± 5.3 (^d)</td>
</tr>
<tr>
<td></td>
<td>CD4 &amp; (\gamma\delta)-depleted (^b)</td>
<td>-0.1 ± 5.3</td>
</tr>
<tr>
<td>(\gamma\delta) TCR⁺</td>
<td>PBMC</td>
<td>70.5 ± 19.1</td>
</tr>
<tr>
<td></td>
<td>CD4 &amp; CD8-depleted (^b)</td>
<td>29.7 ± 19.1</td>
</tr>
</tbody>
</table>

\(^a\) CD25 expression was measured on CD4⁺, CD8⁺, and \(\gamma\delta\) TCR⁺ T cells cultured for five days with or without BRSV. Initial preparation of cells was performed in two ways: simple isolation of PBMC and PBMC isolation plus magnetic depletion of two T cell subsets to isolate the third subset.

\(^b\) Depletion efficiencies of CD4⁺, CD8⁺, and \(\gamma\delta\) TCR⁺ T cells were 94-99%, 88-99%, and 90-95%, respectively.

\(^c\) Data represent the percent CD25-positive in BRSV-stimulated cells of a subset minus the percent CD25-positive in corresponding nonstimulated control cells (%CD25 differential). Values in the table represent the mean ± SEM of two independent assays. SEM uses a pooled estimate of error variance for each subset.

\(^d\) Denotes a statistically significant difference in \(\Delta \%\text{CD25}\) between non-fractionated and fractionated T cells of a given subset (p < 0.05).
Figure 3.

Influence of blocking antibody against MHC class I upon BRSV-driven CD25 expression in vitro. PBMC incubated with and without BRSV antigen were treated with either a monoclonal antibody to MHC class I or an isotype control antibody (IgG2a) and analyzed for CD25 expression after five days. The difference in CD25 expression by virus-stimulated CD4⁺, CD8⁺ and γδ TCR⁺ T cells versus non-stimulated controls was calculated. Antigen-driven CD25 upregulation during treatment with anti-MHC I is expressed as a percentage of the corresponding value for cells treated with isotype control antibody. The data represent mean values from two assays and error bars represent the standard error of the mean.
inhibit or block antigen presentation to CD8+ T cells. Again, the full assay was performed twice with cells from one animal. In Figure 3, antigen-driven CD25 upregulation during treatment with monoclonal antibody to bovine MHC I is expressed as a percentage of the corresponding value for cells treated with isotype control antibody. In the presence of increasing anti-MHC I concentrations there were decreasing proportions of CD8+ T cells that upregulated CD25 in response to antigen. It should be noted that anti-MHC I antibody also diminished the responses of CD4+ and γδ T cells to some extent.

4. Discussion

Neutralizing antibody responses to MLV and inactivated BRSV vaccines were similar, with the exception that titers in the inactivated vaccine group were not detectable until after secondary exposure. The SVN antibody response to two doses of MLV was lower than in some reports (Ellis et al., 1992b; Ellis et al., 1995; West and Ellis, 1997), but similar to that reported in another study where cattle were seronegative at vaccination (West et al., 1999). In the latter case, B lymphocytes were primed sufficiently to mount a strong anamnestic SVN antibody response following challenge. Researchers have reported widely varying SVN responses to inactivated BRSV vaccines. During studies by Ellis and co-workers, eight commercial inactivated BRSV vaccines were tested for antibody induction (Ellis et al., 1992b; Ellis et al., 1995; West and Ellis, 1997; Ellis et al., 2001). Each vaccine induced BRSV-specific antibody, detectable by ELISA, but the levels of neutralizing antibody were generally quite low, with a few exceptions. In calves immunized with inactivated BRSV in the present study, SVN titers were detected after booster dosage at 28 days, and from that point mirrored the response to MLV. This indicates that chemical inactivation of this virus
did not denature neutralizing epitopes. Interestingly, the BRSV strain in this vaccine was also in one of the polyvalent, inactivated vaccines previously reported to induce significant BRSV neutralizing antibody (Ellis et al., 1995), whereas most induced ELISA antibody but low neutralizing activity.

Both vaccines primed cellular immune responses, and the initiation of T cell priming was almost concurrent in the two groups. For individual T cell subsets, significant CD25 responses to BRSV were observed in both groups within no more than one sampling interval of each other. Nonetheless, there were notable differences during the latter stages of the experiment. The inactivated vaccine appeared to stimulate a more durable T cell response, including elevated CD25 responses after secondary immunization at week four. The T cell responses to MLV dropped to statistically insignificant levels by the tenth week. These data are consistent with the results of other studies, in which MLV and inactivated, adjuvanted BRSV vaccines primed for virus-specific lymphocyte proliferation (Taylor et al., 1987; Ellis et al., 1992a; Ellis et al., 1995; Keles et al., 1998; West et al., 1999) and IFN-γ production (Ellis et al., 2001). In some instances, inactivated vaccines were more effective in priming for proliferation than MLV (Taylor et al., 1987; Ellis et al., 1992a).

In an early study that used light microscopy to analyze CD4 and CD8 expression on lymphoblasts from a proliferation assay, CD4+ cells were reported to be the main population responding to BRSV after vaccination with MLV or inactivated vaccines (Ellis et al., 1992a). Our flow cytometric data indicates that both forms of vaccine primed significant proportions of CD4+, CD8+, and γδ T cells to respond to BRSV, rather than one dominant subset. CD4+ T cell responses were lower in the MLV group than in the inactivated vaccine group at the latter time points. This may have been due to the fact that inactivated vaccine was formulated
with an adjuvant, a difference which could have been accentuated if the MLV replicated poorly. Yet, CD8+ T cell priming was quite similar in the two treatment groups.

γδ T cell responses developed against BRSV were quite evident in both treatment groups. We are not aware of other studies that have examined γδ T cell priming by BRSV vaccines. The large percentages of the γδ T cells that upregulated CD25 during culture with virus suggest that they either proliferated very rapidly upon recognition of antigen or were stimulated via nonadaptive receptors, such as NKG2D, a receptor for stress-inducible MICA (Bauer et al., 1999). The first possibility is more likely because the capacity to respond to BRSV was only evident after immunization (Fig. 2C). It is interesting that γδ T cells from vaccinated animals were activated in the presence of BRSV because lymphocyte studies in gnotobiotic calves undergoing mild BRSV infection did not indicate a direct role for γδ T cells in protective immunity or pathogenesis (Taylor et al., 1995; Thomas et al., 1996; McInnes et al., 1999).

Antigen-driven CD25 upregulation was detected on the surface of CD8+ T cells from cattle immunized with either the inactivated or MLV vaccines. This phenotypic change is consistent with the expected response of memory or effector CD8+ T cells since CTL precursors in TCR-transgenic mice respond to antigen in vitro with high CD25 expression compared to naïve cells (Cho et al., 1999). Because the classical CD8+ T cell response is initiated with endogenous antigen processing and presentation, priming of this subset is generally attributed to intracellular replication of a pathogen. Therefore, it was somewhat unexpected that the inactivated vaccine would prime CD8+ T cells for a strong in vitro response to BRSV. To address the nature of this response in recipients of the inactivated BRSV vaccine, two further experiments were carried out. First, the antigen was added to
negatively selected PBMC samples such that CD4\(^+\), CD8\(^+\), and \(\gamma\delta\) T cell responses could each be evaluated in the near absence of other T cells. While the CD4 response was not changed significantly, responses by the other two subsets were inhibited, particularly that of the CD8\(^+\) T cells. Thus, the antigen-driven in vitro CD8 response was dependent on help from other T cells, presumably CD4\(^+\) cells. One possible explanation is that cytokines produced by class II-restricted T cells might activate CD8\(^+\) T cells without any TCR engagement (bystander activation). Another possibility is that help from CD4\(^+\) T cells was an essential co-factor with peptide-class I ligand for the activation of CD8\(^+\) T cells. This requirement has been reported previously in cattle (Taracha et al., 1997; Walravens et al., 2002). In order to distinguish between these possibilities, another method was used to determine if MHC class I recognition was required for the recall CD8 response. Anti-MHC class I monoclonal antibody added to PBMC at the beginning of culture with BRSV inhibited the upregulation of CD25 on CD8\(^+\) T cells in a dose-dependent manner. The meaning of this result is somewhat obscured because anti-MHC I also inhibited the in vitro activation of CD4\(^+\) T cells, to a lesser extent, for unknown reasons. Perhaps impeding the CD8 response dampens the responses of CD4\(^+\) T cells, or the IgG may have nonspecific inhibitory effects on cells. Nonetheless, anti-MHC I was more suppressive toward the CD8 recall response than toward the CD4 recall response. This suggests that the inactivated vaccine did prime MHC class I-restricted, BRSV-specific CD8\(^+\) T cells. BRSV-induced stimulation of \(\gamma\delta\) T cells was inhibited by anti-MHC I at a level intermediate between the other two subsets. This observation is difficult to interpret, but the possibility that some bovine \(\gamma\delta\) T cells might be class I-restricted is noteworthy. A minor proportion of bovine \(\gamma\delta\) T cells express CD8,
which is also consistent with the possibility that some γδ T cells may recognize antigen in the context of MHC I (Howard et al., 1999).

CD8+ T cell priming by this inactivated BRSV vaccine is consistent with some previous reports. Keles et al detected BRSV-specific CTL in the peripheral blood of lambs that had been immunized with a glutaraldehyde-fixed, whole-cell vaccine with adjuvant (Keles et al., 1998). Heat-inactivated Sendai virus (another paramyxovirus) primed for an MHC class I-restricted CTL response in mice (Liu et al., 1995). Further studies in cell lines (Liu et al., 1997b) and splenic antigen presenting cells (Liu et al., 1997a) showed that inactivated Sendai virus antigen was processed independently of LMP2/7 proteosome subunits and TAP1/2 peptide transporters, which are elements of the classical MHC I antigen presentation pathway.

There are several potential reasons for CD8+ T cell priming in response to inactivated virus. Antigen cross-presentation is a special activity of professional APCs that allows them to present epitopes from microbes that do not infect APCs (Kurts, 2000). By this mechanism, an APC ingests exogenous antigens in peripheral tissues and presents them on MHC class I to naïve CD8+ T cells in secondary lymphoid tissues. In our scenario, the inactivated BRSV might have been taken up by APC and processed into CD8+ T cell ligands in this manner. The Sendai virus studies in mice pointed instead to an endosomal pathway in which peptides were loaded onto class I molecules recycling from the cell surface (Liu et al., 1997a, 1997b). Alternatively, it is plausible that unique characteristics of paramyxoviruses make them accessible to the endogenous antigen presentation pathway. If functional attachment (G) and fusion (F) proteins remain intact after virus inactivation, they might mediate virion entry into the cytoplasm of host cells. Finally, the inactivated vaccine tested in
our study contained a proprietary adjuvant that also could have mediated direct entry of antigen into the endogenous antigen presenting pathway, a role which some adjuvants are designed to play (Vogel, 2000).

In conclusion, the MLV and inactivated vaccines examined in this study appear to have distinct stimulatory effects upon the humoral and cell-mediated immune response to BRSV. With respect to cell-mediated immunity, the inactivated vaccine was more effective in priming CD4+ and γδ T cell subsets to respond in vitro to BRSV antigen. Most notably, CD8+ T cells from cattle in the inactivated vaccine group responded to BRSV as well as those from MLV vaccinates. These findings provide new insight into the cell-mediated immune responses stimulated by conventional BRSV vaccines and support the idea that inactivated BRSV vaccines can induce effective cell-mediated immunity in cattle.

Acknowledgements

This work was partially supported by the John G. Salsbury Endowed Chair in Veterinary Medicine at Iowa State University.

References


Keles, I., Woldehiwet, Z., Murray, R.D. 1998. Vaccination with glutaraldehyde-fixed bovine respiratory syncytial virus (BRSV)-infected cells stimulates a better immune response in lambs than vaccination with heat-inactivated cell-free BRSV. Vaccine 16 (11-12), 1172-1178.


Chapter 6. T Cells from a High Proportion of Apparently Naïve Cattle Can Be Activated by Modified Vaccinia Virus Ankara (MVA)

A paper submitted to *Viral Immunology*

Matthew R. Sandbulte, Ratree Platt, and James A. Roth

**ABSTRACT**

Modified vaccinia virus Ankara (MVA) was used as a vector to express genes from bovine respiratory syncytial virus (BRSV). Using these recombinant viruses as recall antigens for cells from BRSV-immune cattle proved to be problematic because non-recombinant MVA itself frequently stimulated high levels of T lymphocyte activation. This phenomenon was observed in a high percentage of cattle from multiple herds. Gamma delta T cells were more sensitive to activation by MVA than other classes of T cells. A serological assay for MVA neutralization detected low, fluctuating titers of serum virus neutralizing (SVN) activity toward MVA in some cattle, but these were lower titers than those observed in cattle that underwent MVA vaccination. T cell reactivity in non-vaccinated cattle did not correlate significantly (p > 0.05) with SVN activity, undermining the notion that any adaptive immune response was responsible for the observed T cell sensitivity. More probable explanations are that MVA has mitogenic or superantigenic properties, or that the virus induces γδ T cell activation through interactions with innate pattern recognition receptors.
INTRODUCTION

Modified vaccinia virus Ankara (MVA), a highly attenuated vaccinia virus strain, is one of several poxviruses that have been used as vaccine vectors for pathogen-derived genes. Recombinant MVA constructs have been used experimentally to immunize against a number of important pathogens, including Dengue virus (13), human immunodeficiency virus (7), measles virus (21), Mycobacterium tuberculosis (5), and Plasmodium falciparum (18). The original vaccinia virus isolate from Ankara, Turkey, was attenuated by over 570 passages in chick embryo fibroblast culture (15). The genome of MVA has six major deletions, which correspond with at least 31 kb of wild type vaccinia virus DNA (14). In mammalian cells the MVA replication cycle is blocked at a late stage, but viral genes can be expressed, as can heterologous genes placed under MVA promoters (22).

In at least two studies, recombinant vaccinia viruses have been used in vitro to deliver recall antigens for human peripheral blood lymphocytes (10, 20). In one of these studies (20), non-recombinant MVA stimulated lymphocytes from some individual donors to release varying amounts of interferon gamma (IFN-γ). It has also been reported that MVA and other poxviruses could stimulate lymphocytes from domestic animals that presumably had no previous exposure to such agents (2, 4). For instance, inactivated parapox ovis virus (PPOV), fowlpox virus, vaccinia virus Lister, and MVA all induced responses by porcine lymphocytes (4). These responses included cell proliferation and the production of cytokines. Activities like these might explain the known immunostimulatory properties of inactivated PPOV in vivo. PPOV is the main component in a commercial therapeutic product (Baypamun, Bayer AG, Leverkusen, Germany) that reportedly enhances resistance to a number of animal infections (3, 9, 24).
We pursued the possibility of using MVA as a vector for genes of bovine respiratory syncytial virus (BRSV). One of the aims was to use several recombinant MVAs as \textit{in vitro} recall antigens to determine the dominant target antigens through the course of an immune response to BRSV. During these experiments, we observed that non-recombinant MVA has stimulatory properties toward T cells from some cattle, despite the animals’ lack of known exposure to poxviruses. This posed a major problem for using recombinant MVAs to express \textit{in vitro} recall antigens, because background responses to MVA alone were often nearly as high as responses to recombinant MVAs encoding BRSV genes. We performed more in-depth studies of the interactions between MVA and the immune system of immunized and non-immunized cattle.

\textbf{MATERIALS AND METHODS}

\textbf{Animals and experimental design.} Cattle used in this study originated from several Iowa dairy and beef herds. Most were calves less than one year of age. Groups of cattle are named in this section in the order that they were obtained. Fifteen Holstein calves (Group A) were housed in a biosafety level 2 animal facility. Twelve of them were inoculated with live recombinant MVAs that encoded BRSV genes F, G, or N. MVA-F, MVA-G, MVA-N, and non-recombinant MVA were each used to immunize three calves. Three control calves were not inoculated with MVA or recombinant MVA. Two doses of $5 \times 10^8 \text{ TCID}_{50}$ were administered subcutaneously, seven weeks apart. Blood was collected at several time points prior to and after inoculation so that serological and cellular responses to MVA and BRSV could be analyzed. Blood samples were also obtained from a group of 34 Holstein calves (Group B), a group of 18 Holstein calves (Group C), and a group of 24 Angus calves (Group
D) during different phases of the work. Calves in groups B, C, and D were kept in conventional housing at Iowa State University, and were not inoculated with MVA or recombinant MVA.

**Media and reagents.** DMEM-F12++ growth medium was prepared from DMEM-F12 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. RPMI++ growth medium consisted of RPMI 1640 with 2 mM L-glutamine and 25 mM HEPES buffer (Mediatech, Inc., Herndon, VA); 10% fetal bovine serum (bovine viral diarrhea virus-free, Atlanta Biologicals, Norcross, GA); and 1% penicillin/streptomycin solution (Mediatech), providing final concentrations of 100 IU penicillin/ml and 100 μg streptomycin/ml.

Primary antibodies to bovine lymphocyte markers were purchased from VMRD (Pullman, WA). These murine immunoglobulins included anti-CD4 IgG1 (CACT138A), anti-CD8α IgG1 (CACT80C), anti-CD8α IgM (BAQ111A), anti-γδ TCR IgG2b (GB21A), and anti-CD25 IgG2a (CACT108A). Anti-mouse IgG1-FITC and anti-mouse IgG2a-PE secondary antibodies were purchased from Southern Biotechnology Associates (Birmingham, AL). Anti-mouse IgG2b-Tri-Color® and anti-mouse IgM-AlexaFluor® 647 secondary antibodies were purchased from Caltag Laboratories (Burlingame, CA) and Molecular Probes (Eugene, OR), respectively.

**Viruses.** MVA was provided by Dr. Bernard Moss (Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, MD). The virus was amplified by infecting a chicken embryo fibroblast (CEF) cell line (ATCC CRL-1590). CEF were grown in D-MEM/F-12++. Virus was isolated after extensive cytopathic effects were visible. This was accomplished by freezing and thawing the washed, infected cells;
resuspending the contents in a small volume of PBS or DMEM-F12++; and sonicating the suspension to liberate virus. After the suspension was centrifuged 10 minutes at 400 x g, the clarified supernatant was aliquotted and stored at -70°C. Mock antigen was prepared by identical treatment of non-infected CEF cultures. High-titer virus suspensions intended for the inoculation of cattle or for density gradient purification were prepared by lysing infected cell layers in the medium in which they were grown, which already contained free virus. Partially purified virus was obtained by density gradient separation. MVA was isolated by the initial steps listed above, then concentrated by ultracentrifugation of the material onto a cushion of 50% OptiPrep (Sigma, St. Louis, MO). This spin was performed in a SW 28 rotor (Beckman Coulter, Fullerton, CA) for 3 hours at 27,000 rpm (average RCF = 96000 x g).

Bands collected from these tubes were further concentrated by spinning in a Beckman MLS 50 rotor (Beckman Coulter) for 2 hours at 40,000 rpm (average RCF = 128000 x g), with the same type of cushion as before. The resulting virus suspension was layered onto a discontinuous OptiPrep gradient containing adjacent layers of 22% and 32% OptiPrep in 10mM Tris-Cl, pH 9.0. This design was adapted from another study in which vaccinia virus was purified over a continuous gradient with the same minimum and maximum concentrations of OptiPrep (11). The tubes were ultracentrifuged at 25,000 rpm for 3 hours (average RCF = 50000 x g), and the band found between the two gradient layers was collected and stored at -70°C. The titer of virus infectivity was 3x10^7 TCID_{50}/ml.

**T cell stimulation.** Cattle were bled by jugular venipuncture, and blood was collected in acid citrate dextrose or sodium heparin solutions. Samples were centrifuged at 800 x g for 25 minutes. Buffy coat layers were aspirated, and the remaining erythrocytes were treated with two rounds of hypotonic lysis by adding two volumes of buffered water for 1 minute and
then restoring the suspension to normal osmolarity with one volume of triple strength PBS (16). Two different systems were used for culturing peripheral blood mononuclear cells (PBMC) with MVA in vitro. For cells analyzed by two-color flow cytometry, each well of a round-bottom 96-well microtiter plate received 200 μl RPMI++ growth media, 4 x 10^5 PBMC, and 2.5 x 10^2 TCID₅₀ of MVA. Plates were incubated for five days at 37°C in a humidified 5% CO₂ atmosphere. For cells analyzed by three- and four-color flow cytometry, each well of a flat-bottom 96-well microtiter plate received 1 x 10⁶ PBMC in 200 μl RPMI++. Plates were incubated for four days at 39°C in a humidified 5% CO₂ atmosphere. The quantity of MVA added was 2.5 x 10³ TCID₅₀ per well, except in titration experiments where dose effects were tested. In the first such experiment, cells from 34 cattle (Group B) were stimulated by the original MVA preparation at three five-fold dilutions: 5.0 x 10² TCID₅₀ / well, 2.5 x 10³ TCID₅₀ / well, and 1.25 x 10⁴ TCID₅₀ / well. In the second titration experiment, PBMC from five cattle (Group C) were stimulated by the original MVA preparation and by semi-purified MVA at three ten-fold dilutions: 2.5 x 10² TCID₅₀ / well, 2.5 x 10³ TCID₅₀ / well, and 2.5 x 10⁴ TCID₅₀ / well. Control wells received plain media or mock-infected CEF lysate / sonicate instead of virus suspension.

**Flow cytometry.** Following the incubation period, flow cytometry was used to analyze the sensitivity of CD4, CD8, and γδ T cells to MVA antigen. T cell activation was assessed by measuring CD25 upregulation upon in vitro exposure to the virus. Because we developed improvements to the flow cytometry protocol during the course of these studies, three versions of the CD25 assay were used at different stages. In the initial two-color flow cytometry assay, triplicate samples were used to analyze the CD4⁺, CD8⁺, and γδ TCR⁺ subsets in parallel, as described previously (17). Briefly, all three received anti-CD25
primary antibody (IgG2a) and individual samples received either anti-CD4 (IgG1), anti-CD8α (IgG1), or anti-γδ TCR (IgG2b). Then FITC- and PE-labeled secondary antibodies were used to detect subset markers and CD25, respectively. In the three-color technique, which used the same primary antibodies, anti-CD8α, anti-γδ TCR, and anti-CD25 were combined to label one sample. Secondary antibody conjugates used in this format were anti-IgG1-FITC, anti-IgG2a-PE, and anti-IgG2b-Tri-Color. This allowed CD8+ γδ TCR− cells, CD8+ γδ TCR+ cells, and CD8− γδ TCR+ cells to be distinguished from one another. CD4 T cell analysis in the other sample was unchanged from the two-color assay. Finally, the four-color assay allowed CD25 expression to be analyzed on all three T-cell subsets in a single test sample. In this technique, CD8 was labeled with anti-CD8α IgM primary antibody and anti-IgM-AlexaFluor 647 secondary antibody.

Two- and three-color flow cytometric analysis of FITC, PE, and TriColor fluorescence signals were performed on a FACScan cytometer (Becton-Dickinson). Four-color analysis was performed on an Epics Altra cytometer (Beckman-Coulter). FITC, PE, and TriColor dyes were excited by a 488 nm krypton laser, and AlexaFluor 647 was excited by a spatially separated 633 nm helium-neon laser. A gated amplifier was used to differentiate TriColor and AlexaFluor 647 signals, which were detected in the same PMT. Analysis of data from both flow cytometry methods was performed using CellQuest or FlowJo software. Live lymphocytes were gated according to forward- and side-scatter distribution. Data from CD4+ T cells was plotted directly from this gate. Plots of CD8 versus γδ TCR were used to draw gates that delineated three separate T-cell subsets: CD8 single-positive, γδ TCR single-positive, and CD8 / γδ TCR double-positive. For each of the four subsets, two CD25 parameters were tabulated: the percentage of cells expressing CD25 (%CD25+) and the
geometric mean fluorescence intensity (MFI) of CD25 signal among positive cells. The CD25 expression index (EI) was calculated according to the following formula: 

\[
\frac{(%CD25^+ \times (CD25 \text{ MFI}) \text{ of MVA-stimulated cells})}{(%CD25^+ \times (CD25 \text{ MFI}) \text{ of non-stimulated cells of the same animal})}
\]

**Serum virus neutralization (SVN) assay.** Serum samples collected from cattle were stored at -20°C. Before performing the assay, samples were inactivated by heating in a 56°C water bath for 30 minutes. For each sample, two-fold serial dilutions were made from 1/4 to 1/4096 in duplicate rows of a microtiter plate. DMEM-F12++ culture media was used as diluent, and the volume of diluted serum was 100 µl per well. In addition, each well received \(1.0 \times 10^1\) TCID\(_{50}\) of MVA suspended in 100 µl of DMEM-F12++. Serum and virus were allowed to incubate for 1 hour at 37°C. Then each well received 100 µl of cells freshly subcultured from 3-5 day-old CEF culture. This combination of serum, virus, and cells was incubated in a humidified 5% CO\(_2\) atmosphere for 6 days at 37°C. Plates were read for the presence of cytopathic effects (CPE) by observation under an inverted microscope. CPE was present in 100% of control wells that were set up in each plate with MVA but no serum. The SVN titer was defined as the inverse of the highest serum dilution that neutralized MVA in at least 1 of 2 wells. Serum samples that did not neutralize MVA at the lowest dilution (1:4) were assigned a titer of 2. When non-immunized cattle were tested for SVN activity, two serum samples were collected within two months of each other, and the mean of the two results was the final titer.

**Statistics.** Statistical analyses were performed with JMP 5 software (SAS Institute Inc., Cary, NC). Student’s T test was used to test for differences in group means. Bivariate analysis of SVN titer versus CD25 expression index was performed for each T cell subset.
Coefficient of determination ($R^2$) values were computed for these relationships, and the statistical significance of each $R^2$ value was determined by analysis of variance. For multiple comparisons among three treatments, the Tukey-Kramer HSD test was used. In the experiment where calves received MVA inoculations, SVN and CD25 data points collected at weeks 0, 7, and 11 were handled as distinct from one another. In the experiment where non-immunized calves were tested, mean SVN values from two bleedings were plotted versus mean CD25 expression index values from three bleedings; all samples were taken within a two-month period.

**RESULTS**

MVA-induced activation was frequently observed in T cells from conventionally reared Iowa cattle. This effect was quantified in a large number of animals when we carried out repeated four-color flow cytometry assays on MVA-stimulated PBMC from groups of 18 Holstein calves (Group C) and 24 Angus calves (Group D). Data from Group C, which is representative of both groups, is displayed in Figure 1. Activation of CD4$^+$ T cells in response to the virus was rare, and the level of CD25 expression induced in those cases was modest. Activation of CD8$^+$ $\gamma\delta$ TCR$^-$ T cells by MVA was observed somewhat more frequently, and in a few instances the magnitude of their CD25 expression was high. Both subsets of $\gamma\delta$ T cells (CD8$^+$ and CD8$^-$) were more responsive than CD4$^+$ T cells or CD8$^+$ $\gamma\delta$ TCR$^+$ T cells. In particular, there was a high frequency of responses among the CD8$^-$ $\gamma\delta$ TCR$^+$ T cell subset, with varying levels of CD25 expression. Responses by individual animals were rather variable among the four repeated assays, but there was a trend that
FIGURE 1. T cell activation in response to MVA. PBMC from non-immunized cattle in Group C were incubated with MVA for four days. CD25 expression was measured in the major T cell subsets: CD4+ (A), CD8+ γδ TCR− (B), CD8+ γδ TCR+ (C), and CD8− γδ TCR+ (D), by four-color flow cytometry. CD25 expression index values > 30 are plotted as 30 in the graphs.
responders could be distinguished from non-responders.

MVA concentrations were titrated to examine dose dependency in PBMC from 34 calves. Three five-fold dilutions of MVA were tested: $5 \times 10^2$, $2.5 \times 10^3$, and $1.25 \times 10^4$ TCID$_{50}$/well. In all four T cell subsets, which were analyzed by three-color flow cytometry, CD25 expression was elevated at the highest concentration of viral stimulus (Figure 2). In the CD8$^-$γδ TCR$^+$ subset, which make up most of the γδ T cells, this increase was particularly large. Apparently there is a rather narrow threshold of MVA concentration for the activation of γδ T cells. There was variation among individuals with respect to reactivity, as some responded readily to virus at the two lower concentrations, whereas others only responded to the highest concentration.

MVA viral antigen had been prepared from infected CEF. To help assess the virus specificity of T cell responses, stimulation by MVA was compared with stimulation by mock MVA prepared from uninfected CEF. PBMC from 27 calves (three from Group C and 24 from Group D) were incubated with equal dilutions of the two stimuli and analyzed by four-color flow cytometry (Table 1). The mean CD25 expression indices in response to mock antigen were less than 2.0 for all four subsets. Cells belonging to the CD4$^+$, CD8$^+$γδ TCR$^+$, and CD8$^+$γδ TCR$^+$ subsets had significantly higher mean CD25 expression in response to MVA stimulation ($p < 0.01$). The CD8$^-$γδ TCR$^+$ T cells had a mean CD25 expression index greater than 10 in response to MVA, the highest among the four subsets. To further test for virus specificity, we compared the stimulatory effects of original MVA and semi-purified MVA (Figure 3). PBMC from five cattle were used in the assay, and both virus preparations were dosed at three ten-fold dilutions. Results from four-color flow cytometry showed that the two preparations had similar dose-dependent effects toward each
FIGURE 2. Dose response of bovine T cells to MVA. PBMC were isolated from 34 calves and incubated with three five-fold dilutions of MVA for four days. CD25 expression in each of the T cell subsets (CD4<sup>+</sup>, CD8<sup>+</sup>γδ TCR<sup>-</sup>, CD8<sup>+</sup>γδ TCR<sup>+</sup>, and CD8<sup>-</sup>γδ TCR<sup>+</sup>) was then analyzed by three-color flow cytometry. Error bars represent the standard error of the mean. Statistical differences were tested by Tukey-Kramer HSD test: <sup>a</sup> CD25 expression induced by virus at high titer (1.25 x 10<sup>4</sup> TCID<sub>50</sub>/ well) is significantly greater than by virus at low titer (5 x 10<sup>2</sup> TCID<sub>50</sub>/ well), p < 0.05. <sup>b</sup> CD25 expression induced by virus at high titer is significantly greater than by virus at medium titer (2.5 x 10<sup>3</sup> TCID<sub>50</sub>/ ml), p < 0.05.
TABLE 1. Virus specificity of T cell responses to MVA grown in CEF.

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>CD4⁺</th>
<th>CD8⁺ γδ TCR⁺</th>
<th>CD8⁺ γδ TCR⁺</th>
<th>CD8⁺ γδ TCR⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>MVA</td>
<td>1.71 ± 0.22</td>
<td>2.40 ± 0.40</td>
<td>4.80 ± 1.09</td>
<td>10.41 ± 2.04</td>
</tr>
<tr>
<td>Mock MVA</td>
<td>0.93 ± 0.09</td>
<td>1.18 ± 0.14</td>
<td>1.45 ± 0.26</td>
<td>1.57 ± 0.32</td>
</tr>
</tbody>
</table>

¹ MVA and mock MVA were used to stimulate PBMC isolated from 27 cattle. The two stimuli were added to PBMC cultures at equal dilutions.

² PBMC were analyzed by flow cytometry for CD25 expression in the four main T-cell subsets after four days of incubation. In each of the four subsets there was a statistically significant difference between stimulation with MVA virus and stimulation with mock MVA (Student's t-test, p < 0.01).
FIGURE 3. Effects of MVA virus purity on T cell stimulation. Original MVA lysate / sonicate was compared with MVA that was partially purified by ultacentrifugation on a density gradient. PBMC from five cattle were incubated with each virus preparation, or with no antigen, for four days. Error bars represent the standard error of the mean.
subset of T cells, and the semi-purified MVA appeared somewhat more stimulatory. This suggests that the reactivity is not due to factors released by infected tissue culture cells during propagation of the virus.

A serum virus neutralization (SVN) assay was designed to measure serological reactivity with MVA. MVA-positive sera were archived from the experiment in which calves (Group A) were inoculated with live MVA. Those calves were also tested for MVA-specific CD4⁺, CD8⁺, and γδ TCR⁺ T cell activation, using two-color flow cytometric analysis of CD25 expression. Sera from all twelve inoculated calves, as well as three control calves, were tested one week before immunization, seven weeks after primary immunization (the day of secondary immunization), and eleven weeks after primary immunization. The humoral response to MVA inoculation resulted in geometric mean SVN titers of approximately 27 and 256 at weeks 7 and 11, respectively (Figure 4). In control calves, SVN titers against MVA remained low or undetectable (data not shown). The seroconversion of the vaccinated animals corresponded with CD4⁺, CD8⁺, and γδ TCR⁺ T cell responses to MVA, which also increased substantially after immunization with the virus (Figure 4). CD4⁺ T cells showed evidence of a secondary response at week 11, which fits well with the apparent secondary SVN antibody response. The range of SVN titers measured by the assay was quite broad, with readings extending from 4 to 1024, which suggested high assay sensitivity. Bivariate analysis was performed to model linear fit between the log₂ transformed CD25 expression indices for CD4⁺, CD8⁺, and γδ TCR⁺ T cells versus the corresponding log₂ transformed SVN titers. R² values obtained for each of these models were modest: 0.25, 0.19, and 0.31, respectively. However, these correlations were statistically significant (p < 0.01).

Next, the SVN assay was used to test 42 normal (non-MVA-inoculated) calves (Group C
FIGURE 4. Immune responses induced in calves following subcutaneous inoculation with MVA. Twelve calves each received $5 \times 10^8$ TCID$_{50}$ MVA. Booster doses of vaccine were administered at week 7. PBMC were incubated with MVA in vitro for five days and analyzed for CD25 upregulation on CD4$^+$, CD8$,^+$, and $\gamma\delta$ TCR$^+$ T cells by two-color flow cytometry. Serum samples were frozen and later tested for MVA neutralizing activity in CEF culture. Error bars represent the standard error of the mean.
and Group D) for serological reactivity with MVA. T cells from the same calves were also
tested by four-color flow cytometry for responses to MVA (Figure 1). Therefore, any
potential correlations between serological reactivity and T cell reactivity could be identified.
The two sets of normal sera were collected two weeks apart, and in that low range of SVN
titers there was not great consistency for individual animals from the first assay to the
second. Most of the normal cattle (41/42) had a detectable level of serological reactivity with
MVA in at least one of two serum samples. However, 17 of the normal cattle had
undetectable reactivity in one of two samples. The mean \( \log_2 \) SVN titer for normal calves
overall was 3.05, which is less than the titer present in calves after primary vaccination with
MVA (Figure 4). With regard to normal cattle, no statistically significant correlation was
found between SVN activity and CD25 responses by any subset of T cells.

**DISCUSSION**

It has been hypothesized that the complexity and large size of poxviruses tends to make
them highly immunogenic or mitogenic, but that the wild-type poxviruses have adapted by
encoding genes to interfere with the host immune response (4). These suppressive factors
include soluble receptors for tumor necrosis factor-\( \alpha \), interleukin-1, and interferon-\( \gamma \),
chemokine binding proteins, and vaccinia virus complement control protein (19). The
attenuation of MVA is partly attributed to the loss of several soluble cytokine receptor genes
(1). It is possible that some intrinsic immunostimulatory properties of pox viruses are
"unveiled" in attenuated strains like MVA or in inactivated poxviruses. The results of a
study examining the immunostimulatory properties of several viable and inactivated
poxviruses were consistent with this notion (2). There it was shown that viable MVA
induced significant IFN-α production by PBMC from humans, sheep, and swine. In contrast, no detectable IFN-α was induced by equivalent doses of the less attenuated Elstree, Bern, and Copenhagen vaccinia strains. However, when Elstree strain vaccinia was inactivated with β-propiolactone it became a strong inducer of IFN-α. It appears likely that a non-structural product (or set of products) from replicating, wild type poxviruses is able to suppress that host response.

Fachinger et al. tested several inactivated poxvirus strains for a wider range of immunostimulatory properties toward porcine lymphocytes (4). They showed that chemically inactivated fowl poxvirus, parapox ovis virus, and MVA could all induce lymphocyte proliferation and the secretion of IL-2, IFN-α, and IFN-γ. Flow cytometric analysis of lymphocytes demonstrated virus-induced CD25 and MHC class II upregulation, predominantly in the CD4+ subset of T cells.

The results of our experiments with lymphocytes from young cattle indicate that bovine T cells are also responsive to stimulation by MVA. Responses by γδ T cells were the greatest and most frequent. Most individuals upregulated CD25 expression on the surface of γδ T cells in an MVA dose-dependent manner. Gamma delta T cells of cattle reportedly fall into two main phenotypes: a CD8+WC1− subset and a CD8−WC1+ subset that predominate in the periphery (12). We also observed CD8+ and CD8− subpopulations of γδ T cells after incubation with or without MVA. The CD8−γδ TCR+ subpopulation was much greater in number and generally more responsive to MVA. Gamma delta T cells make up a large proportion of the lymphocyte population in the blood in calves and other young ruminants (8, 23). It is important to understand the interactions of γδ T cells with MVA if the virus is to be used as a vector for bovine vaccines. The MVA dose-response curve of T cells from
presumably naïve cattle was interesting for at least two reasons. First, T cells belonging to the CD4\(^+\) and CD8\(^+\) \(\gamma\delta\) TCR\(^-\) subsets generally did not respond in parallel with the responses by \(\gamma\delta\) TCR\(^+\) T cells. In certain animals, CD4\(^+\) and CD8\(^+\) \(\gamma\delta\) TCR\(^-\) T cells were activated upon incubation with MVA, but this was a fairly rare occurrence. Second, the multiplicity of infection (MOI) required to optimize T cell reactivity for these non-immunized animals (1.25\(\times\)10\(^{-2}\)) was more than an order of magnitude higher than the MOI that activated T cells from vaccinated calves (6.25\(\times\)10\(^{-4}\)).

It was unclear whether the reactivity of bovine T cells with MVA was a result of adaptive immunity to some cross-reactive antigen or an innate response to the virus. An adaptive response seemed questionable because there are no recognized poxviruses circulating among North American cattle. We reasoned that serological reactivity with MVA would correlate with T cell reactivity if cattle had acquired antigen-specific immunity. Using a SVN assay that was developed and validated with sera from MVA-vaccinated cattle, we tested two sets of serum samples from 42 calves that had no known previous exposure to poxviruses. High sensitivity in the assay was achieved partly by using 10 TCID\(_{50}\) per well rather than the standard 100 TCID\(_{50}\). SVN titers were relatively low in these cattle, and they appeared somewhat unstable from the first assay to the second. These data might reflect fluctuations in non-antigen-specific serum contents, such as type 1 interferons or tumor necrosis factor-\(\alpha\). If the MVA-responsive T cells had been primed by exposure to a poxvirus or other cross-reactive antigen, greater corresponding SVN titers would have been likely.

Cross-reactivity with another virus (or viruses) is a potential explanation for the MVA-reactive T cells and SVN titers observed in a high proportion of non-vaccinated cattle. It is possible that such cross-reactivity arises from exposure to papular stomatitis virus or
pseudocowpoxvirus. These are parapoxviruses with minor clinical significance that occur commonly in cattle worldwide (6). However, our observations of low serological reactivity with MVA and a general lack of reactivity by CD4$^+$ and CD8$^+$ γδ TCR$^-$ T cells suggest that the MVA-responsiveness of γδ T cells is innate rather than acquired. There are several plausible mechanisms for this. One possibility is that the virus has mitogenic or superantigenic properties, causing polyclonal activation of lymphocytes. Another possibility is that pattern recognition receptors on lymphocytes bind to conserved poxvirus motifs and mediate activation. Alternatively, the virus could cause stress-induced molecules, such as heat-shock proteins, to be expressed on some subset of the PBMC. In this case, lymphocyte activation may result from pattern recognition receptors binding to those stress-induced molecules. Cells from some individual calves appeared more MVA-sensitive than others. Similarly, it was shown previously that inactivated parapox ovis virus stimulated varying degrees of proliferation by PBMC from different pigs (4). These results could be due to differences in the genetic makeup of individual animals, such as heterogeneity in major histocompatibility complex (MHC) types. For instance, if MVA has superantigenic activity, it is logical that cattle with different MHC types could have different levels of sensitivity to the virus. In conclusion, the use of MVA as a vector for genes from bovine pathogens is complicated by that virus’ unexplained reactivity with host lymphocytes. While this reactivity hampers the use of recombinant MVA viruses as in vitro recall antigens, it is possible that it can provide a useful adjuvant effect in vivo.
ACKNOWLEDGEMENTS

This work was supported by the John G. Salsbury Endowed Chair in Veterinary Medicine at Iowa State University. We gratefully acknowledge Tom Skadow for his animal handling and technical assistance, and Shawn Rigby for flow cytometry expertise and operation of the Epics Altra cytometer in the ISU Flow Cytometry Facility.

REFERENCES


Chapter 7. In Vitro Analysis of BRSV-Specific T Cell Priming in Vaccinated Cattle by a Multi-Parameter Flow Cytometry Assay for Activation and by ELISA for Interferon-γ Production

A paper to be submitted to *Veterinary Immunology and Immunopathology*

Matthew R. Sandbulte and James A. Roth

Abstract

Immune responses to bovine respiratory syncytial virus (BRSV) were analyzed in calves that were inoculated with polyvalent modified-live and inactivated virus vaccines from commercial sources. In addition to standard serological assays for virus neutralization, antigen-specific T cell responses were monitored before, during, and after the vaccine regimen. The priming of T cells against BRSV was tested by a flow cytometry-based assay for CD25 upregulation in response to viral antigens in vitro. In this assay, the expression of CD4, CD8, γδ TCR, and CD25 were analyzed simultaneously on T cells from antigen-stimulated peripheral blood mononuclear cell suspensions. Four phenotypes of T cells were analyzed: CD4+, CD8+ γδ TCR−, CD8+ γδ TCR+, and CD8− γδ TCR+. In addition, an ELISA was used to quantify the production of interferon-γ (IFN-γ) in response to antigen. One treatment group received two doses of inactivated virus vaccine with adjuvant (n=6). A second treatment group received two doses of modified-live virus (MLV) vaccine. A control group was inoculated with saline solution (n=6). At the time of vaccination, calves were all seropositive to BRSV by virus neutralization, but naïve according to T cell responses, suggesting that the BRSV-specific antibody was of maternal origin. Both the inactivated virus and MLV vaccines primed T cells of all four subsets for BRSV-specific activation, and
inactivated vaccine induced a stronger T cell activation response and IFN-γ production than the MLV vaccine after the booster dose was administered.

1. Introduction

BRSV is a Paramyxovirus that causes mild to severe respiratory disease in cattle. It is one of several infectious agents of the bovine respiratory disease complex in young calves, which causes substantial economic losses. Replication of the virus is largely restricted to respiratory epithelial cells (Viuuff et al., 1996). The cell-mediated branch of the bovine immune system plays a large part in recovery and protection from BRSV infection. During experimental infection of young calves, there was an influx of CD8^+ T cells into the lungs, and pulmonary CD4^+ and CD8^+ T cells had increased expression of activation markers (McInnes et al., 1999). Under similar experimental conditions, MHC-restricted, BRSV-specific cytotoxic T lymphocytes (CTL) were observed in the peripheral blood and lungs of infected calves (Gaddum et al., 1996). The depletion of T cell subsets from gnotobiotic calves prior to experimental BRSV challenge indicated that CD8^+ T cells have an important role in the clearance of virus (Taylor et al., 1995). Pneumonic lesions were more extensive in individuals lacking CD4^+ or CD8^+ T cells. With regard to humoral immunity, there are data indicating that passive maternal antibody to BRSV can lessen the severity of infection (Kimman et al., 1988), as can BRSV-neutralizing monoclonal antibodies (Thomas et al., 1998).

Numerous efforts have been made to characterize the ability of MLV and inactivated vaccines to elicit antibody, cell-mediated immunity (CMI), and protection against BRSV challenge. Vaccines representing both forms have been shown to induce neutralizing
antibody to BRSV, but inactivated viruses often result in lower ratios of neutralizing antibody titer to ELISA-detectable IgG titer (Ellis et al., 1992b; Ellis et al., 1995; West and Ellis, 1997; Ellis et al., 2001). It has been known for some time that both categories of vaccine can prime lymphocytes for proliferative responses to BRSV (Ellis et al., 1992a; Ellis et al., 1995). A study that monitored in vitro correlates of CMI after vaccination with modified-live BRSV and after subsequent challenge indicated that protection was correlated with enhanced antigen-specific lymphocyte proliferation and IFN-γ production (West et al., 1999). One inactivated BRSV vaccine conferred significant clinical protection that corresponded with the priming of antigen-specific IFN-γ production (Ellis et al., 2001).

In a previous study, we partially characterized the in vitro T cell responses to BRSV that were primed by commercially available MLV and inactivated vaccines given to seronegative calves (Sandbulte and Roth, in press). Following vaccination, CD4⁺, CD8⁺, and γδ TCR⁺ lymphocytes all upregulated activation marker CD25 during in vitro incubation with the virus. Results from an MHC class I blocking experiment suggested that the CD8⁺ T cell response was class I-restricted. There was no attempt to differentiate CD8⁺ γδ TCR⁻ T cells from CD8⁺ γδ TCR⁺ T cells. The latter phenotype occurs in a small proportion of bovine PBMC (MacHugh et al., 1997). Because responses by γδ TCR⁺ T cells tended to be high, it was uncertain whether any BRSV-responsive CD8⁺ T cells belonged to the αβ T cell category.

In this study, we again analyzed cell-mediated and humoral responses to BRSV following immunization with two polyvalent vaccines. These were a MLV vaccine and an inactivated virus vaccine with adjuvant. One aim of the present study was to evaluate a different pair of commercial MLV and inactivated vaccines for efficacy in priming antibody and T cell
responses to BRSV in calves with pre-existing antibody titers. Other aims were to characterize the phenotypes of responding T cells more precisely and to monitor antigen-specific IFN-γ production as a parallel measure of T cell function.

2. Materials and Methods

2.1. Animals and Experimental Design

Eighteen beef calves weighing approximately 300 kg were included in the study. At the time of vaccination they were approximately ten months of age. Calves were assigned randomly to three treatment groups (n=6). On day zero, commercial polyvalent vaccines were administered to two treatment groups. One group was inoculated with Triangle 4 + Type II BVD, an inactivated virus vaccine containing BHV-1, BVDV type 1 and 2 strains, bovine respiratory syncytial virus (BRSV), parainfluenza virus 3, and a proprietary adjuvant (Fort Dodge Animal Health, Overland Park, KS). Another group was inoculated with Titanium™ 5, which is a MLV vaccine also consisting of BHV-1, BVDV types 1 and 2, BRSV, and PI-3 (Agri Laboratories, St. Joseph, MO). The control group received inoculations of saline solution. All inoculations were 2 ml given subcutaneously, in accordance with manufacturers’ instructions. Three weeks later, booster doses of both vaccines were given to the respective treatment groups, and the control group received second doses of saline solution. All cattle were bled on six occasions, which included two times prior to primary vaccination (week -1 and week 0), the day of secondary vaccination (week 3), and three times after secondary vaccination (week 5, week 7, and week 9). Cattle were bled by jugular venipuncture, and blood for cell isolation was collected in acid citrate dextrose, while an additional sample was collected in serum separation tubes. Three adult
Holstein cattle that had received multiple vaccinations with Titanium 5 and demonstrated strong T cell responses to BRSV were used as positive control animals.

2.2. Media and reagents.

RPMI++ growth medium consisted of RPMI 1640 with 2 mM L-glutamine and 25 mM HEPES buffer (Mediatech, Inc., Herndon, VA); 10% fetal bovine serum (bovine viral diarrhea virus-free, Atlanta Biologicals, Norcross, GA); and 1% penicillin / streptomycin solution (Mediatech), providing final concentrations of 100 IU penicillin/ml and 100 μg streptomycin/ml. PBS++ solution consisted of phosphate buffered saline (PBS) plus 0.5% bovine serum albumin and 0.1% sodium azide. Primary antibodies to bovine lymphocyte markers were purchased from VMRD (Pullman, WA). These murine immunoglobulins included anti-CD4 IgG1 (CACT138A), anti-CD8α IgM (BAQ111A), anti-γδ TCR IgG2b (GB21A), and anti-CD25 IgG2a (CACT108A). Goat anti-mouse IgG1-FITC and goat anti-mouse IgG2a-PE secondary antibodies were purchased from Southern Biotechnology Associates (Birmingham, AL). Goat anti-mouse IgG2b-Tri-Color® and goat anti-mouse IgM-AlexaFluor® 647 secondary antibodies were purchased from Caltag Laboratories (Burlingame, CA) and Molecular Probes (Eugene, OR), respectively.

2.3. Virus

Live BRSV was used as the recall antigen for in vitro T cell response assays. It was obtained in the form of a monovalent modified-live BRSV vaccine (Bovi-Shield™ BRSV, Pfizer Animal Health, Exton, PA). Virus was reconstituted with manufacturer's diluent,
aliquotted, and stored at -70°C. The virus was titrated prior to the experiment to determine the optimal dilution for in vitro activation of T cells from BRSV-immune cattle.

2.4. *In vitro* T cell stimulation

Peripheral blood samples were centrifuged at 800 x g for 20 minutes. Buffy coat layers were collected, and remaining erythrocytes were lysed by adding two volumes of buffered water for 1 minute and then restoring the suspension to normal osmolarity with one volume of triple strength PBS (Roth et al., 1982). The resulting suspension of peripheral blood mononuclear cells (PBMC) was pelleted by slow speed centrifugation (250 x g for 10 minutes), resuspended in PBS, and treated with a second round of lysis. Then the PBMC were resuspended to 5 x 10^6 cells/ml in RPMI++ culture medium. In each well of a 96-well, flat-bottomed microtiter plate, 10^6 PBMC (200 µl of suspension) were combined with either 50 µl of BRSV antigen diluted in Hanks balanced salt solution, 50 µl concanavalin A (ConA) solution (0.6 µg/well), or 50 µl of plain diluent. Plates were incubated for four days at 39°C in a humidified 5% CO₂ atmosphere. After incubation, cells were transferred to round-bottomed microtiter plates and centrifuged at 200 x g for 1 minute, allowing supernatants to be collected and stored at -20°C.

2.5. Flow cytometry

Following the incubation period, four-color flow cytometry was used to analyze the reactivity of T cells with BRSV antigen or ConA. CD4, CD8α, and γδTCR surface markers were detected simultaneously with CD25, a marker of activation. First, plates were chilled on ice 15 minutes. Then cells were washed three times with PBS++ before being labeled via
an indirect antibody protocol. The primary antibodies were diluted together in PBS++, with anti-CD4 and -CD8α diluted to 10 μg/ml, and anti-γδ TCR and -CD25 diluted to 1.3 μg/ml. Each well of a microtiter plate received 50 μl primary antibody mixture, and plates were incubated 30 minutes on ice, followed by three washes with PBS++. In the secondary antibody mixture, anti-IgG1, anti-IgG2a, anti-IgG2b, and anti-IgM were diluted to 2.5 μg/ml, 0.6 μg/ml, 0.5 μg/ml, and 10 μg/ml, respectively, in PBS++. Each well received 50 μl secondary antibody mixture, and plates were incubated 30 minutes on ice, followed by three washes with plain PBS. Cells were fixed in a PBS solution with 1% formaldehyde. Four-color analysis was performed using an Epics Altra cytometer (Beckman-Coulter). FITC, PE, and TriColor dyes were excited by a 488 nm krypton laser, and AlexaFluor 647 was excited by a spatially separated 633 nm helium-neon laser. A gated amplifier was used to differentiate TriColor and AlexaFluor 647 signals, which were detected in the same photo multiplier tube. Approximately 10,000 live lymphocyte events were analyzed per sample. Analysis of flow cytometry data was performed using FlowJo software. The first step in analysis was gating out doublet events (clumps) according to their distribution in plots of peak forward scatter versus linear forward scatter. Then live lymphocytes were gated according to linear forward scatter and side scatter properties. Histograms for CD4 expression were plotted directly from this gate. Plots of CD8 versus γδ TCR were used to draw gates that delineated three separate T-cell subsets: CD8 single-positive, γδ TCR single-positive, and CD8 / γδ TCR double-positive. Histograms of CD25 expression were drawn for each of the four subsets, and the percentage of cells expressing CD25 (%CD25+) was determined. Antigen-driven CD25 upregulation was calculated for each of the T-cell subsets.
using the Δ%CD25⁺ formula, which equals [(%CD25⁺ for cells incubated in the presence of stimulant) – (%CD25⁺ for control cells incubated in the absence of stimulant)].

2.6. Measurement of in vitro IFN-γ production

Supernatant samples collected from PBMC incubated with and without BRSV were thawed and mixed well. IFN-γ concentrations in the supernatants were quantified with the Bovigam™ ELISA test kit (Biocor Animal Health, Omaha, NE). The manufacturer’s recommended protocol was followed, except that sample volumes were 25 µl instead of 50 µl. A Vmax Microplate Reader (Molecular Devices, Sunnyvale, CA) was used to analyze optical density at a wavelength of 450 nm. A two-fold serial dilution of recombinant bovine IFN-γ (Serotec, Oxford, UK) was used to calculate a linear standard curve for each ELISA plate. Data reported here are IFN-γ protein concentrations derived from those curves.

2.7. Serology

Blood samples in serum separation tubes were centrifuged for 30 minutes at 1200 x g. Serum samples were removed and submitted to the Iowa State University Veterinary Diagnostic Laboratory where a routine serum virus neutralization (SVN) assay against BRSV was conducted. Each SVN titer was the reciprocal of the highest dilution that inhibited 100% of cytopathic effects in cell culture.

2.8. Statistical analysis

CD25 expression data from the six tests were expressed as Δ%CD25⁺ and grouped into three periods during the vaccination regimen. Period 1 included both pre-vaccination assays
(week -1 and week 0). Period 2 included only the week 3 assay, which was representative of responses to one dose of vaccine. Period 3 included all three assays performed after secondary vaccinations were administered: week 5, week 7, and week 9. Analysis of variance was performed using the general linear models (glm) procedure in SAS software (SAS Institute Inc., Cary, NC). For the first and third period, which included multiple assays, repeated measures analysis was performed by assigning “animal within treatment group” as the error term. Tests for differences in CD25 expression index among treatment groups were performed by the least squares means method. SVN data was log2-transformed and analyzed by week, using standard ANOVA. In all statistical tests, significant differences between means were declared when $P < 0.05$.

3. Results

3.1. Serology

Serum virus neutralization (SVN) titers were thus far obtained for three post-vaccination time points, at weeks 3, 5, and 7. Across the three assays, all calves in the non-vaccinated control group had antibody to BRSV (Figure 1). Since the calves were randomly distributed into treatment groups, it may be concluded that all of them were seropositive to BRSV prior to vaccination. Data from week 3 suggest that primary immunization with MLV versus inactivated virus vaccines caused similar increases in SVN titers. Data from weeks 5 and 7 indicate that there was no increase in antibody titer after secondary vaccination.
Figure 1. Serum virus neutralization titers against BRSV. Control calves were inoculated with saline solution, whereas experimental groups were inoculated with two doses (weeks 0 and 3) of inactivated virus vaccine or two doses of MLV vaccine. Error bars represent the standard error of the mean log$_2$ SVN titer within a treatment group (n=6). Statistically significant differences between groups ($P < 0.05$) are denoted as follows: † MLV > Control; * Inactivated virus > Control. Double symbols denote $P < 0.01$. 
3.2. T Cell Responses

The four main subsets of T cells identified within cultured PBMC have the following phenotypes: CD4$^+$, CD8$^+$ γδ TCR$^-$, CD8$^+$ γδ TCR$^+$, and CD8$^-$ γδ TCR$^+$. In PBMC cultures that were incubated in the absence of virus, the mean proportions of these subsets were 27.8%, 11.9%, 1.9%, and 38.2%, respectively, during a representative assay of all 18 cattle. PBMC from positive control cattle were sampled and tested throughout the study for recall responses to live BRSV. CD25 expression was upregulated in all four subsets, as CD4$^+$, CD8$^+$ γδ TCR$^-$, CD8$^+$ γδ TCR$^+$, and CD8$^-$ γδ TCR$^+$ T cells had A%CD25$^+$ values across the study of $16.6 \pm 3.0$, $7.8 \pm 1.6$, $34.5 \pm 5.3$, and $35.0 \pm 5.3$ (mean ± standard error, n = 18), respectively. Each T cell subset, in each treatment group, had strong CD25 responses to ConA (A%CD25$^+ > 40$) during each period (data not shown).

T cell responsiveness to BRSV before, during, and after the vaccination regimens are illustrated by the CD25 expression data in Figure 2. Despite the fact that all calves were seropositive to BRSV, no significant T cell responses against the virus were detectable in any of the treatment groups before vaccination. After primary vaccination (Period 2), there were no significant differences between the MLV and inactivated virus vaccine groups in terms of T cell responses to BRSV, but one or both vaccine groups had higher responses than controls in the CD4$^+$, CD8$^+$ γδ TCR$^-$, and CD8$^+$ γδ TCR$^+$ subsets ($P < 0.05$). There was an apparent trend for T cell responses to increase in the inactivated virus vaccine group after secondary vaccination, but not in the MLV vaccine group. This led to statistically significant differences between the two vaccine groups in all subsets except CD8$^+$ γδ TCR$^-$ in Period 3 ($P < 0.05$). Data on the capacity of PBMC for in vitro IFN-γ production in response to BRSV is presented in Figure 3. Increased IFN-γ production was apparent in BRSV-
Figure 2.

T cell responsiveness to BRSV before, during, and after vaccination regimens. PBMC from control calves, calves inoculated with MLV vaccine, and calves inoculated with inactivated virus vaccine were incubated with and without BRSV antigen. After four days, CD25 expression on the major T cell subsets was measured by four-color flow cytometry. A%CD25 was the difference in frequency of CD25 expression between BRSV-stimulated and non-stimulated samples. Period 1 of the experiment consisted of the week -1 and week 0 timepoints. Period 2 consisted of the week 3 timepoint, representing the primary response to vaccines. Period 3 consisted of weeks 5, 7, and 9 – all timepoints after secondary immunization. Error bars represent the standard error of the mean within a treatment group. Statistically significant differences in CD25 expression between groups (P < 0.05, n = 6) are denoted as follows: † MLV > Control; * Inactivated virus > Control; ‡ Inactivated virus > MLV. Double symbols denote P < 0.01.
A. CD4+ T Cells

B. CD8+ γδ TCR- T Cells

C. CD8+ γδ TCR+ T Cells

D. CD8+ γδ TCR+ T Cells
**Figure 3.**

IFN-γ production by BRSV-stimulated PBMC sampled before, during, and after vaccination regimens. Supernatants collected from PBMC samples that were incubated with and without BRSV were later assayed by ELISA for levels of IFN-γ. The background IFN-γ level observed in non-virus-stimulated samples was subtracted from the level observed in corresponding samples that were incubated with BRSV. Error bars represent the standard error of the mean within a treatment group. Statistically significant differences between groups ($P < 0.05$, $n = 6$) are denoted as follows: † MLV > Control; * Inactivated virus > Control; ‡ Inactivated virus > MLV. Double symbols denote $P < 0.01$. 
stimulated cultures from the MLV and inactivated virus vaccine groups after primary vaccination, though not to levels significantly different from the controls ($P > 0.05$). After secondary vaccination, there was no indication of a further increase for the MLV group. In contrast, elevated IFN-$\gamma$ was measured for the inactivated virus group, and this level was significantly greater than in either the MLV group or the controls ($P < 0.05$).

4. Discussion

The calves in this experiment had neutralizing antibodies to BRSV before vaccines were given. Since the calves lacked detectable BRSV-specific T cell immunity before immunization, it is likely that the antibodies were of maternal origin. The calves were approximately ten months of age at the time of vaccination. It is generally thought that maternal antibodies wane by the time calves reach that age, but there is little data in the literature addressing this issue for cattle. Interfering levels of maternal antibody to measles virus have been reported in human infants at nine months of age (Gans et al., 2001). An alternative explanation is that previous exposure to BRSV primed an active antibody response without leaving evidence of a CMI response. This scenario would complicate the interpretation of subsequent T cell responses, but mechanistically, it seems unlikely. We reported evidence of T cell priming against BRSV despite the lack of an antibody response in calves that were apparently exposed to BRSV in the face of maternal antibody (Sandbulte and Roth, 2002).

For immunization against BRSV, both the MLV and inactivated virus vaccines are recommended by their manufacturers to be administered in two doses, two to four weeks apart. These are typical guidelines for BRSV immunization using commercial MLV and
inactivated virus vaccines. In contrast, only one dose of MLV is typically recommended for immunization against bovine viral diarrhea virus, bovine herpesvirus 1, and parainfluenza virus 3. Since the calves in this study were seropositive to BRSV, pre-existing antibodies might have inhibited the replication of modified-live BRSV.

After primary immunization, increased responsiveness to BRSV was observed in T cells from calves that received either of the vaccines. Both vaccine treatments led to statistically significant responses by CD8⁺ γδ TCR⁻ T cells, and significant CD4⁺ and CD8⁺ γδ TCR⁺ T cell responses were also induced by the inactivated virus vaccine ($P < 0.05$). In Period 3, which encompassed the three timepoints after secondary immunization, CD25 expression levels were higher in the inactivated virus vaccine group than the MLV vaccine group for all four T cell subsets. Secondary immunization with inactivated virus vaccine led to elevated BRSV-responsiveness in all T cell subsets except CD8⁺ γδ TCR⁻ T cells. In contrast, there was no evidence of a booster effect by the second dose of MLV. As a result, there were statistically significant differences between the inactivated virus and MLV treatments groups in all T cell subsets except CD8⁺ γδ TCR⁻ T cells during Period 3. The second dose of MLV also failed to increase the capacity of PBMC for IFN-γ production. The lack of a booster effect by MLV contradicts results from another study, in which two doses of modified-live BRSV induced higher levels of virus specific lymphocyte proliferation and IFN-γ production than one dose (West et al., 1999), though statistical significance was not indicated in those comparisons.

The trend for inactivated BRSV to induce stronger cellular immunity than MLV supports findings from our earlier vaccine study with BRSV-naïve calves (Sandbulte and Roth, in press), even though different adjuvanted, inactivated vaccines were used in the two studies.
In the earlier study, CD8\(^+\) T cell responses to BRSV were among the T cell responses detected, and there was evidence that responding CD8\(^+\) T cells were MHC I-restricted. This went against the concept that MHC I-restricted T cells are only primed by endogenous antigens. One drawback with our earlier findings was that CD8\(^+\) T cells were not differentiated from CD8\(^+\) \(\gamma\delta\) T cells, leaving doubt as to whether the responding CD8\(^+\) T cells belonged to the subset normally associated with CTL activity. In the present study, activation assays were performed by four-color flow cytometry, which allowed CD8\(^+\) \(\gamma\delta\) TCR\(^-\) cells to be distinguished from CD8\(^+\) \(\gamma\delta\) TCR\(^+\) cells. Results from this assay confirmed significant recall responses by CD8\(^+\) \(\gamma\delta\) TCR\(^-\) (presumably \(\alpha\beta\) TCR\(^+\)) cells after immunization with inactivated BRSV.

Also in this study, we examined IFN-\(\gamma\) production as an in vitro indicator of T cell priming. This property was shown to correlate statistically with clinical protection from BRSV challenge in another vaccine study (West et al., 1999). In the present experiment, BRSV-induced IFN-\(\gamma\) production followed a kinetic course similar to CD25 expression in CD4\(^+\) and other T cells. The data not only shows that this inactivated BRSV vaccine is capable of priming for IFN-\(\gamma\) production, which was reported for a similar commercial vaccine (Ellis et al., 2001), but indicated that it may in fact be superior to a particular MLV vaccine in that regard. Formalin-inactivated human RSV (FI-HRSV) and BRSV (FI-BRSV) vaccines, both with alum adjuvant, have been shown to exacerbate disease upon subsequent infection (Kim et al., 1969; Gershwin et al., 1998). It has been theorized that such vaccines skew the immune response toward a T helper 2 (Th2) bias, thus undermining the important CTL response and promoting the recruitment of granulocytes to the lung (Graham et al., 2000). An older theory proposes that non-neutralizing antibodies elicited by formalin-
inactivated RSV is a root cause because they are non-protective yet favor type III hypersensitivity reactions (Murphy et al., 1986). Calves that received a FI-BRSV vaccine developed non-neutralizing antibody to the virus and weak IFN-γ responses during subsequent virus challenge, results which appear to fit both theories (Gershwin et al., 1998; Woolums et al., 1999). The strong IFN-γ response observed after vaccination with inactivated BRSV vaccine in the present study, combined with the fact that CD8+ T cells were primed against the virus, suggests that this commercial vaccine does not have Th2-biasing properties. SVN antibody responses in this study are complicated by the fact that calves were seropositive at vaccination, but our earlier study indicated that an inactivated BRSV vaccine could induce SVN titers as high as a MLV vaccine (Sandbulte and Roth, in press). Taken together, these findings offer assurance that some commercial inactivated BRSV vaccines are low risks for disease enhancement.

The effectiveness of an inactivated BRSV vaccine in driving T cell responses, particularly by CD8+ T cells that potentially correspond with CTL, is contrary to dogma. It is generally understood that CD8+ T cells are only primed by an antigen that is processed and presented through the endogenous pathway, as with components of intracellular pathogens. The antigen cross presentation mechanism attributed to professional antigen presenting cells offers one potential way for inactivated BRSV to access the endogenous pathway (Kurts, 2000). Certain adjuvants may have properties that facilitate antigen crossing into the cytoplasm (Vogel, 2000). Aside from these general possibilities, it is possible that Paramyxoviruses have unique properties that favor their antigen presentation to naïve T cells, even when inactivated. Heat-inactivated Sendai virus, which is also a member of this virus family, was shown to prime for CTL responses in mice (Liu et al., 1995). One could
speculate that the fusion (F) and attachment (G) glycoproteins retain a conformation, even when virions are inactivated, that mediates entry across the plasma membrane of a host cell.

A different line of thought was opened by a recent study that compared the effects of live and inactivated BRSV on bovine monocyte-derived dendritic cells in vitro (Werling et al., 2002). Incubating live BRSV with the dendritic cells led to greater IL-10 gene transcription, but less transcription of IL-12p40 and IL-15 genes than inactivated virus. If such a scenario is also true in vivo, it is conceivable that inactivated BRSV antigen creates a microenvironment that is more favorable to priming Th1-type T cell responses than live BRSV.

Acknowledgements

The authors thank Tom Skadow for his essential role in animal handling and technical assistance, and Shawn Rigby for flow cytometry expertise and operation of the Epics Altra cytometer in the ISU Flow Cytometry Facility.

References


Chapter 8. General Conclusions

Discussion.

The studies described in this dissertation have generated several novel findings about immune responses to bovine respiratory syncytial virus (BRSV) in calves. New knowledge has been gained about the immunostimulatory effects of several vaccines and about the influence of pre-existing antibody upon immune responses to BRSV vaccines and natural BRSV exposure. Progress was also made in understanding interactions between bovine lymphocytes and modified vaccinia virus Ankara (MVA), which is a potential vector for BRSV vaccines.

In the first project, which is described in Chapter 4, it was learned that T cells could be primed against BRSV independently of an antibody response. In certain calves, CD4⁺, CD8⁺, and γδ TCR⁺ T cells were sensitive to activation by in vitro exposure to BRSV, even though no neutralizing antibody or IgG specific for the virus were detectable. Furthermore, upon vaccinating calves with modified-live BRSV, individuals with primed T cells were able to mount anamnestic virus neutralizing antibody responses. It would be consistent with these results that a natural BRSV infection had occurred in those calves in the face of maternal antibody, leading to suppressed antibody production but normal T cell priming. An important implication of this work is that seronegative calves should not be considered to be immunologically naïve to BRSV. Another potentially important impact of this discovery is that seroconversion may not need to be seen as a necessary correlate for effective immunization. Functional immunity to BRSV might be achievable by vaccinating calves at younger ages, while maternal antibody to BRSV is still predominant.
The priming of CD4⁺, CD8⁺, and γδ TCR⁺ T cells by modified-live and inactivated virus vaccines given to BRSV-seronegative calves was investigated in the second major project, which is described in Chapter 5. This work showed that commercial BRSV vaccines belonging to both categories are capable of priming all three phenotypes of T cells for in vitro recall responses. The data indicated that T cell responses to an adjuvanted, inactivated BRSV vaccine can in fact be higher and longer-lasting than responses to modified-live virus (MLV). Of particular interest was the priming of CD8⁺ T cells, because the inactivated virus vaccine induced at least as strong a response in that subset as MLV. Data obtained with cells from an animal in the inactivated virus vaccine group indicated that the in vitro recall response by CD8⁺ T cells was MHC class I dependent. Data demonstrating γδ TCR⁺ T cell responses to the vaccines tested in this experiment (and others within the dissertation) provide new knowledge to the field, as well. The author is not aware of other studies that monitored the priming of bovine γδ TCR⁺ T cells in response to BRSV vaccines or infection. Whether these cells contribute to protective immunity against BRSV is uncertain, but these results suggest that previously reported lymphocyte blastogenesis data for CMI responses to BRSV probably reflected a considerable amount of γδ T cell activity.

The interactions of MVA with bovine lymphocytes were the focus of studies in the third project, which is described in Chapter 6. MVA could potentially be used as a vaccine vector for BRSV genes, as it has been used to vector genes from many pathogens. It was learned that young calves inoculated with recombinant MVA constructs would develop CD4⁺, CD8⁺, and γδ TCR⁺ T cell responses and neutralizing antibody titers to the MVA backbone. More surprisingly, it was also learned that T cells from many normal (non-MVA-immunized) cattle are activated by incubation with a relatively high level of MVA. This phenomenon was most
often observed in γδ TCR+ T cells. Low, fluctuating titers of MVA neutralizing activity were measured in sera from some normal cattle, but it was not clear that this was due to antibody from a previous exposure to a poxvirus. No statistical correlation was found between T cell sensitivity and serum neutralizing activity to MVA. The observations of seemingly “innate” reactivity by bovine T cells with MVA may have practical importance. This reactivity might lend an adjuvant effect to recombinant vaccines for BRSV or other targets. However, it is also a drawback to experiments in which recombinant MVAs would be used as in vitro recall antigens to probe for recall responses to individually vectored proteins. Our experience indicated that the vector-specific T cell response tends to create a background that causes difficulty in detecting responses to the heterologous protein.

The fourth project, which is described in chapter 7, expanded upon the earlier study of T cell responses to modified-live and inactivated BRSV vaccines. Whereas the calves tested in the prior study were seronegative to BRSV, the ones tested this time had low to moderate levels of serum virus neutralizing antibody, presumably of maternal origin, at the time of vaccination. As before, a modified-live and an adjuvanted, inactivated BRSV vaccine were evaluated in this experiment, but both of them were obtained from different commercial sources than in the previous experiment. Using a flow cytometry system that could measure overlap in the expression of CD8 and γδ TCR, it was possible to discern the two single-positive subsets from each other and from the double-positive subset. It was determined that vaccines could prime for in vitro activation of CD4+, CD8+ γδ TCR−, CD8+ γδ TCR+, and CD8− γδ TCR+ T cells in response to BRSV in the presence of pre-existing antibody. Statistically significant responses by the latter three subsets were measured in calves receiving either form of vaccine. It is valuable to know that in vitro CD8+ T cell responses to
BRSV following immunization with inactivated virus are representative of both the CD8$^+$ γδ TCR$^-$ and CD8$^+$ γδ TCR$^+$ phenotypes. If all of the response had been attributable to CD8$^+$ γδ TCR$^+$ T cells, then it would have been doubtful that any classical cytotoxic T lymphocyte precursors were primed by the vaccine. Another key finding from this study was that vaccines prime for BRSV-specific IFN-γ production, and this activity corresponded rather closely with in vitro CD25 expression in T cells.

**Recommendations for further research.**

Quantifying the reactivity of various types of T cells with BRSV at the in vitro level, as was done in the studies of this dissertation, is a promising approach to monitoring immune responses. The techniques that my colleagues and I have developed and reported upon should be adequate to follow up with some useful and interesting experiments. The concept of T cell priming in the presence of passive antibody should be pursued in more depth. The studies described in Chapter 4 offered circumstantial evidence that infection in the face of maternal antibody had led to T cell priming without antibody production, but the concept was not proven. A good experimental design would be to feed newborn calves defined amounts of pooled colostrum from seropositive and seronegative dams before vaccination at an early age. Modified-live and inactivated BRSV vaccines should both be tested, since the latter is thought to be less sensitive to passive antibody interference. Antibody titers and in vitro T cell responses should be monitored until the point when passive antibody has waned. B cell priming should also be monitored by a flow cytometry-based assay similar to that used for T cells. Then a challenge infection or secondary immunization can be administered to test the
hypothesis that CMI and anamnestic antibody responses to BRSV can be primed in calves with high maternal antibody titers.

In terms of making long term progress in understanding BRSV immunity, more in-depth methods will be important. It will be very difficult to draw firm conclusions about vaccine effectiveness until there have been more comprehensive studies that draw associations between T cell activation, T cell effector functions, and protection from challenge infection.

Challenge studies have demonstrated fairly conclusively that CD8$^+$ cytotoxic T lymphocytes (CTL) are a vital part of protective immunity, and that aspect of immunity should be a major focus of research to come. Traditional CTL assays are cumbersome for monitoring immunity in large numbers of cattle. It would be worthwhile to invest in studies to link CTL activity with parameters that can be tested more efficiently. Ideally, the assay we already developed for CD25 expression on CD8$^+$ T cells would be sufficient. However, other antigen-driven responses by CD8$^+$ T cells may correlate better. Good candidates would be IFN-γ or granzyme production, which could be analyzed by flow cytometry.

At this point, the literature is lacking in studies that tested the roles of CD4$^+$ helper T cells and γδ T cells in protecting cattle from virulent challenge. An area of particular interest for studying these cells would be the production of cytokines, such as IL-4, IL-5, IL-10, and IFN-γ. The cytokines produced by helper T cells and γδ T cells might reflect shifts in the Th1-Th2 balance and help to predict the development of protective versus pathogenic immune responses. An attractive method for analyzing cytokine production within the distinct T cell subsets would be with intracellular staining and flow cytometry.

Innate immune cells are likely to be important in the induction of immune responses to BRSV infection or vaccination. The potency of adjuvanted, inactivated BRSV vaccines in
priming for T cell responses, as described in parts of this dissertation, is indirect evidence that early stimulation of innate immune cells might be highly influential. Studying the dendritic cells (DC) and natural killer (NK) cells immediately following exposure to BRSV could lead to a better understanding of their roles, and help to predict development of protective immunity. Sampling these cells would probably be most successful in lymph or draining lymph nodes. Useful in vitro parameters for testing DC might include the uptake of viral antigen, modulation of co-stimulatory molecules, and production of IL-12 or chemokines. Useful parameters for testing NK cells might include upregulation of activation markers, production of IFN-γ, and cytotoxicity.

Given successful methods for testing some of these additional parameters, a series of powerful experiments could follow. Modified-live and inactivated virus vaccines, plus any number of new-generation recombinant vaccines could be administered to cattle and followed up with challenge infection. A few experimental models of BRSV infection were reported in recent years to cause virulent infection in calves, and would be ideal tools for this purpose. Presumably, there would be differences between vaccine treatments in terms of the in vitro immune responses that they prime for. Discovering which of those parameters correlate positively and negatively with the severity of disease would greatly facilitate continuing progress in the design and evaluation of new BRSV vaccines.
Acknowledgements

I would like to thank numerous people at Iowa State University for opportunities, guidance, and friendship during the five years that I have spent in graduate school. My major professor, Jim Roth, offered me the opportunity to join an excellent laboratory and work on interesting research projects, then gave me encouragement to enter the Ph.D. program. I have learned a great deal from him about scientific thinking, planning, and presentation. During my career, I hope I will be able to emulate his thoughtful, humble, and confident manner in dealing with people and challenges. Other people in Dr. Roth’s lab and office have helped me very much, including Tom Skadow, Dagmar Frank, Ratree Platt, Janice Endsley, Dawne Buhrow, and Naomi Backous. They and the rest of the group have always provided a great family-like atmosphere to work in. I am also grateful for the contributions of the other members of my POS Committee – Doug Jones, Randy Sacco, Steve Sorden, and Mike Wannemuehler. They have been generous with their time, advice, and supportive words, both in the formal duties of supervising my graduate studies and in everyday settings. Many other terrific teachers have shaped me, starting at Whittier Elementary School in Winfield, Kansas, and continuing through college at Kansas State and graduate school at Iowa State. The most important people in my development as a person and my progress in academics have been my parents, Glen and Jane. They both taught me to think, learn, work hard, and take part in a wide variety of activities. Their continuing care and support mean a great deal to me. Most of all, I thank God for life, grace, and opportunities to serve.