A COMPARISON OF SEVERAL *IN VITRO* METHODS TO ASSESS THE IMMUNE RESPONSE TO BOVINE VIRAL DIARRHEA VIRUS (BVDV) CONTAINING VACCINES IN CATTLE

by

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(Under the Direction of Amelia R. Woolums)

ABSTRACT

Various methods of measuring immune responses to vaccination are available, but there is little consensus among researchers as to which method best characterizes the response and predicts the outcome following infection of vaccinated individuals. Most studies of immune responses employ a limited number of *in vitro* assays, usually dictated by the individual lab's expertise that provide a narrow view of the global immune response in each experimental context.

The objectives of this thesis were to: 1) employ an array of assays to measure cytokine and lymphocyte proliferative responses to vaccination of cattle with modified live (MLV) and inactivated (killed) BVDV containing vaccines, and 2) assess the degree of correlation of the assays results.

Thirty (30) seronegative calves were vaccinated (day 0) and boosted at day 14 with either MLV/MLV, MLV/killed, killed/killed, vaccines not containing BVDV, or left unvaccinated as sentinels. At days 0, 7, 14, 21, 28, and 49 blood was drawn, peripheral blood mononuclear cells (PBMC) isolated and serum collected. Following *in vitro* stimulation of the PBMCs with different BVDV strains and Staphylococcal enterotoxin B (SEB), proliferation, and cytokine mRNA and protein were assayed. Serum neutralizing titers to the BVDV strains were determined.

Whereas neutralizing antibody titers to the vaccinal biotype (type 1) were higher in calves primed with MLV vaccine than those raised to the type 2 or primed with the killed vaccine, no antigen-specific cytokine responses were observed. Proliferative responses to recall antigen were only observed when the NADL strain (type 1) was used to stimulate PBMCs. The discrimination of responses was best for the day 49 samples, which were collected following a period of naturally occurring respiratory disease and treatment.

The results emphasize the potential disparity of results generated in parallel from different assays of the same samples. Caution should be used when interpreting the results of any one of the individual assays in qualitatively and quantitatively assessing the immune response and its correlation to protection from disease.

INDEX WORDS: BVDV, MLV vaccine, Inactivated vaccine, Immune Responses, cytokine mRNA, IL-4, IFN-γ, Flow cytometry, RT-PCR, Blastogenesis, Serum neutralization, Virus neutralization.

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A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2004

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December 2004

ACKNOWLEDGEMENTS

I would like to extend special thanks to my mother and father (or perhaps, the mailman) for the few exceptional genes they gave me. I am also ingratiated to Adrian Reber for laying out the procedural groundwork of my thesis, and for the use of the graphics he so skillfully prepared. I am also thankful for Doug Donovon's participation with the statistical analysis. Additionally, I am very appreciative of Tats Okinaga's performance of the RT-PCR assays reported herein. Lastly, I would like to thank Rob Parks for his performance of the proliferation assays.

Most of all, I would like to express my gratitude to the members of my past committee, Phil Lukert, Donald Dawe, and Stephen Poet, and particularly my current committee, Amelia Woolums, David Hurley, and Zhen Fu for their extraordinary guidance and patience.

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CHAPTER 1

INTRODUCTION

The development of veterinary vaccines requires proof of vaccine efficacy and historically, simple *in vitro* serological assays combined with challenge experiments have been the gold standard. Serological assays demonstrate a functional immune response in animals vaccinated against some diseases. However, with other diseases, a measurable humoral response resulting from vaccination confers no protection or may even exacerbate disease subsequent to field exposure.

Challenge studies as a measure of vaccine efficacy can be problematic in that they require development of challenge models that mimic the pathological and immunological responses of the target species with the natural disease. In veterinary medicine, the target species are routinely used in challenge studies, but these studies are expensive in terms of dollars and animal suffering. Some diseases common in nature are difficult to replicate under experimental conditions. Furthermore, in an age of long-distance travel, pathogens can be introduced into geographically separated susceptible populations, necessitating “rapid response” vaccine development

programs to combat high morbidity and mortality or economic destabilization resulting from introduction of exotic infectious disease. Challenge models may take several years to develop and validate, and they don't meet the criteria for "rapid response".

Clearly, more rapid and less costly methods of assessing immune responses to vaccination are needed. Today, the depth of our understanding of immune responses to natural infection, and in response to vaccination has revealed the inadequacy of reliance upon serological methods alone. A more accurate picture of the quality and quantity of vaccine-induced immune responses can be achieved using a broader range of in vitro assays measuring cell-mediated immune responses.

In the veterinary field, no other viral disease causes as much economic loss to bovine production as infection with bovine viral diarrhea virus (BVDV). Its impact on the cattle industry has been well reviewed by Houe (1995), but recent discoveries about the degree of immunosuppression caused by the virus suggest the evaluation may have underestimated the economic losses associated with BVDV infection. Two genotypes, type 1 and type 2 are known, aned both share responsibility for the various manifestations of disease. Both inactivated and live vaccines containing BVDV type 1 and 2 are

commercially available, but little consensus exists among veterinarians and/or academics as to which best confers safe, protective immunity.

Several methods exist to assess the development of certain aspects of immune responses to vaccination. Proliferation of memory lymphocyte subsets in response to recall antigen is one measure of the behavior of the immune cells following secondary exposure. This can be accomplished by flow cytometry using vital dyes, or by incorporation of detectable nucleic acid analogues, such as bromodeoxyuridine (BrdU). Tritiated thymidine (^{3}[H]-thymidine) incorporation with subsequent detection by a scintillation counter is another common means of measuring antigen-driven proliferation. Identification of proliferation of lymphocyte subsets is possible by simultaneous staining of various subsets and activation markers such as CD2, CD3, CD4, CD8, CD21, MHC I and II, and CD25 with detection of incorporated BrdU.

Another method of monitoring immune responses is cytokine profiling. This method offers a qualitative feature that can be used to characterize a response as predominantly humoral (T_H2) or cell-mediated (T_H1). Upregulation of cytokine message (mRNA) can be determined using real-time reverse transcriptase polymerase chain reaction (RT-PCR) to determine the relative quantity of specific cytokine mRNA relative to housekeeping genes. Cytokine expression

can be determined flow cytometrically using intracellular staining with specific anti-cytokine antibodies conjugated to fluorochromes to measure the frequency of cells making cytokine protein. Relative frequency of cytokine expressing cells can also be measured using ELISpot assays. Secreted cytokines can be quantified by ELISA using tissue culture supernatant of activated lymphocytes, or by bioassay using responsive cell lines.

Antigen-specific effector cell activity can also be assayed. The conventional method entails loading autologous target cells with radiolabeled chromium (^{51}Cr) and antigen, and determining the relative level of ^{51}Cr release as a consequence of lysis by cytoxic T cells (CTL). Assays measuring lactate dehydrogenase (LDH) release have been substituted for the conventional CTL assays. In this way, total cytoxicity or CTL-mediated cytotoxicity can be measured and used to assess the cell-mediated immune response to vaccination.

Serum neutralization (SN) or virus neutralization (VN) assays offer a simple, inexpensive method to measure induction of systemic antigen-specific antibody production. However, different laboratories use different cell lines and different viral challenge strains at different concentrations to conduct SN assays. Each of these factors tends to decrease reproducibility and consistency between laboratories in presentation of results. Results of antibody-capture ELISAs generally

do not only measure neutralizing antibodies. Determination of a humoral response at mucosal surfaces (primarlily IgA) is fraught with even greater variability issues. Moreover, only a limited number of disease conditions are completely suppressed by a humoral response alone.

This thesis examines and compares several methods of measuring immune responses using the same set of samples from three vaccination regimens comparing two BVDV type 1 vaccines; one inactivated and one live. The studies presented here focus on cytokine responses of leukocytes isolated from blood of cattle primed/boosted using inactivated/inactivated, live/inactivated and live/live vaccine regimens as a primary topic. Proliferative responses, neutralizing antibody titers, and their degree of correlation with the cytokine assays were also determined.

Chapter 2

Literature Review

The Bovine Viral Diarrhea Virus (BVDV)

Bovine Viral Diarrhea Virus is the unfortunate name ascribed to the etiological agent of an "acute, contagious, and transmissible disease of cattle" first experienced in six dairy herds in New York in 1946. The point source of the outbreak may have been a lone 4-year old Red Devon cow imported from England two years prior and housed at a farm near Ithaca (Fox, 1996). This animal was the subject of the first of a series of routine visits by the Cornell Ambulatory Clinic to various dairy farms in the region. It and many of the cattle visited on March 3rd, 1946 succumbed to an affliction resembling winter dysentery. It is possible the rapid spread was fomite-mediated by the ambulatory crew.

By 1976, the BVD virus, along with the hog cholera viruses (now called Classical Swine Fever Virus, CSFV) and border disease of sheep had been categorized as a member of the Pestivirus genus within the Togaviridae family. This was based on its morphological and compositional characteristics as an enveloped, positive-stranded RNA virus. In 1984, the Flaviviridae family was created, in which the Pestivirus genus, including the prototype BVDV, became a member in 1991. The new classification was based on similarities of genetic organization, gene expression and means of replication with Hepatitis C virus (now hepacivirus) and the Flaviviruses.

BVDV Genome

The BVDV virion is spherical, 40-60 nm in diameter, and contains a single positive-sense strand of RNA ranging from 12.3 – 16 kb in size, depending on the strain (Lindenbach and Rice, 2001). It consists of one large open reading frame (ORF) bounded by 3' and 5' untranslated regions (UTRs, also referred to as non-translated regions, or NTRs). The 5' UTR is a 372 - 385 nucleotide stretch that is highly conserved within virus isolates and has been used to establish genotypic relatedness.

The 5' terminus lacks a methylguanosine cap structure, but the UTR bears extensive secondary structure that replaces the cap structure's function in initiation of translation. This region is known as the internal ribosome entry site (IRES). The IRES of Pestiviruses and Hepaciviruses is one characteristic that distinguishes them from the remainder of the Flaviviruses. BVDV IRESs can recruit 40S ribosomal subunits without the need for eukaryotic initiation factors and bind in such a way that the ribosomal P site is placed proximal to the initiation codon. Unlike other viruses possessing IRESs, BVDV IRES elements bind to the ternary complex eIF2/tRNA/GTP without the need of helper proteins, additional initiation factors (Pestova and Hellen, 1999; Sanderbrand et. al., 2000) or ATP (Hellen and Sarnow, 2001).

Critical for IRES function are the conserved 5' terminal tetranucleotide sequence and domains II and III, especially the pseudoknot formed by association of the IIIf loop and a region just upstream from the translational start codon (AUG). Conversely, the stability of secondary structures

downstream of the initiation codon negatively correlates with RNA binding at the 40S ribosomal subunit (Myers, et. al., 2001). The complementary sequence of the 5' terminal tetranucleotide sequence on the 3' terminus of the negative strand may be important for inititiation of the positive-strand RNA synthesis (Frolov, et al., 1998). Whereas Chon, et. al. (1998) demonstrated efficient IRES acitivity in replicons lacking stem-loops Ia and Ib, the Ia hairpin loop (the region containing the terminal tetranucleotide) was shown to be indispensable for BVDV viral genome translation, and the 3' complement of this region, called Ia(-), apparently forms a hairpin of which the loop is critical for RNA replication of positive strands (Yu, et. al., 2000). Additional sequences of the 5' UTR may act in the regulation of translation, replication of negative strands, or packaging.

The 3' terminus lacks a poly (A) tail; instead, three to six cytidine residues exist. The most conserved region of the 3' UTR possesses two hairpins, part of which may be important in minus-strand initiation.

Translation of the monocistronic genome renders a polyprotein ~4000 amino acids (a.a.) in length. The co- and posttranslational proteolytic processing of the BVDV strain NADL nascent polyprotein successively generates the cleavage products, NH_2-N^{pro}-C-E^{rns}-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH.

BVDV proteins

The first protein liberated from the polyprotein is the 168 a.a., 20kd nonstructural protein N^{pro}. This N-terminal protease is responsible for the autocatalytic cleavage at the N^{pro} – C (capsid protein) junction, a conserved

Cysteine-Serine site (Wiskerchen, et. al., 1991). Cellular ubiquitin C-terminal hydrolase can substitute for N^{pro} activity, and host signal peptidases participate in the cleavage of the remainder of the structural proteins. There is evidence that at least the 5' third of the N^{pro} gene is necessary for viral replication (Behrens, et. al., 1998).

The nucleocapsid protein (p14, C) is a highly conserved, 14 kd protein that is highly basic, owing to its high lysine content (21%). This 102-a.a. protein serves to package genomic RNA and associate with the host envelope. Although an adenovirus-vectored p14-based vaccine was shown to be successful in inducing cellular and humoral immune responses in mice (Elahi, et. al. 1999), it is not considered to be highly immunogenic.

The first of the envelope polypeptides, the E^{rns} (gp48, E0) protein is borne from a signalase-mediated cleavage within the ER lumen. This process generates a very hydrophilic protein lacking any hydrophobic anchoring region. This feature suggests a loose association with the viral envelope. In fact, its presence in high concentrations in infected cell culture supernatant indicates it is secreted. Immunostaining studies revealed that E^{rns} seen at the host cell surface was only in association with bound viral particles and not anchored in the plasma membrane (Weiland, et. al. 1999).

Although it is highly glycosylated and forms disulfide-linked homodimers, it can exhibit ribonuclease activity as an unglycosylated monomer. It possesses conserved domains with striking similarity to fungal and plant RNAses, with specificity for uridine residues. Sequence conservation with other pestiviruses suggests it plays an important role in

the viral life cycle, and the high antibody titers observed in infected cattle have some neutralizing properties.

The view that virus-associated E^{rns} is important for attachment is supported by competitive inhibition studies where excess E^{rns} added to culture supernatant blocked BVDV replication in permissive cell lines (Iqbal, et. al. 2000). This same study identified heparin, suramin, fucoidan and dermatan sulphate, but not chondroitin sulphate, karatan sulphate, dextran sulphate, poly-L-lysine, or mannan as cell surface glycosaminoglycans that have affinity for E^{rns}. The glycosaminoglycan binding sequence of E^{rns} was identified as a cluster of basic amino acids (KKLENKSK) located toward the C-terminus, but separate from the ribonuclease domain (Iqbal and McCauley, 2002).

The virus-dissociated form was shown to be capable of entering cells by virtue of a C-terminal extension of the RNase domain (Langedijk, 2002). The C-terminal extension contains two domains; one that shows sequence homology with another pore-forming peptide (magainin), and the other with the L3 loop of Ribotoxin. In this study, binding of E^{rns} was not species-specific, receptor mediated, and endocytosis was not energy-dependent. Its overall three-dimensional structure bears some similarity to mammalian RNase 6, and its dimeric nature and rare vicinal disulfides are features shared only with BS-RNase, a protein with known cytoxic and immunosuppressive properties (Langedijk, et. al. 2002). Bruschke, et. al. (1997) previously demonstrated that E^{rns} derived from Classical Swine Fever Virus (CSFV), a porcine pestivirus closely related to BVDV, induces apoptosis

in bovine lymphocytes, but not epithelial cells. Whereas, the binding of E^{rns} is not species-specific, competitive inhibition using CSFV E^{rns} of infection by BVDV isolated from cattle was higher than that of BVDV passed in a pig (Hulst and Moormann, 1997). This suggests selective pressure within a particular host governs the predominant E^{rns} phenotype.

Thus, in the virus-bound form, E^{rns} plays a role in non-specific attachment to a variety of host cells. In the secreted form, it exerts immunosuppressive effects by inducing apoptosis of lymphocytes, either by cross-linking transmembrane receptors with subsequent apoptosis-initiating signal tranduction, or by tranlocating to the cytoplasm and negatively influencing protein synthesis. Its role in the pathogenesis of BVDV 2 isolates causing severe hemorrhagic disease has been explored and one of the first attenuated strains was produced as a result of its inactivation (Meyer, et. al., 2002).

The E1 protein (gp25 or gp33) is a transmembrane polypeptide roughly 195 a.a. in length that possesses two or three consensus N-linked glycosylation sites and two hydrophobic regions that serve as a membrane anchor. It assists in the translocation of the E2 envelope protein, with which it forms disulfide bonds during heterodimer formation. It has received little attention, as it is believed to be immunologically unimportant. Convalescent cattle serum does not contain high levels of E1-specific antibody.

The major envelope glycoprotein, E2 (gp53), is 375-400 a.a. in length with a predicted mass of 41 to 45 kD. This class I transmembrane glycoprotein contains three or four consensus glycosylation sites. It is the

product of signalase-mediated cleavage of the nascent polyprotein at both the N- and C-termini. The C-terminus is anchored in the lipid bilayer by about 40 hydrophobic amino acid residues. Deposition of the envelope proteins E^{rns} and E2 occurs at intracellular membrane of the endoplasmic reticulum, and budding releases mature virions into the cisternae of the ER (Grummer et. al. 2001). Jordan, et al (2002a) extended these findings by suggesting BVDV is released via the Golgi apparatus in an way dependant on host ER glucosidase activity. In addition to its association with E1 and a noncovalent interaction with E^{rns}, the E2 glycoprotein can covalently link via disulfide bonds to form homodimers.

Unlike E1, the E2 protein is highly immunogenic. The immunogenicity of E2 is evident as a result of the high prevalence of E2-specific antibody in vaccinated and convalescent cattle serum. Because anti-E2 antisera is strongly neutralizing, it has been the protein of choice for many recombinant BVDV subunit and DNA vaccines. The gene for E2 has been employed in a Baculovirus expression system (Kweon, et. al. 1997), in fowlpox-vectored (Elahi, et. al. 1999), vaccinia-vectored (Toth, et. al. 1999), human adenovirus 5-vectored (Elahi, et. al. 1999), bovine herpesvirus 1-vectored (Schmitt, et. al. 1999), and bovine adenovirus 3-vectored (Baxi, et. al. 2000) vaccines. It has also been the expressed protein of choice in DNA vaccination of mice (Harpin, et. al. 1997), cattle (Harpin, et. al. 1999), of mice with co-delivery of an IL-2 expressing plasmid (Nobiron, et. al. 2000) and co-delivery of IL-2 or GM-CSF encoding plasmids (Nobiron, et. al. 2001). Recently, the E2 gene was used in an expression cassette, expressed as a

fusion protein coupled with complement protein C3d (Wang, et. al. 2004), resulting in strongly enhanced anti-E2 antibody production.

Genetic analysis of a highly conserved region of the 5' UTR led to a refinement of BVDV classification; the virus was designated as type 1 or type 2 based on PCR using specific primers. Early studies revealed that monoclonal antibodies specific for each type's E2 protein cross-reacted poorly. As an example, BVDV1, strain NADL and BVDV2, strain 890 have only 58% sequence homology at the E2 locus (Liang et al., 2003). Thus, it is clear that sufficient phenotypic differences exist between types 1 and 2, particularly at the E2 locus, to justify segregation into different "species" or genotypes. That E2 possesses the immunodominant and neutralizing epitope(s) is an important consideration in the design of vaccines against both BVDV1 and BVDV2.

For both types, the E2 region of the genome is one of three hypervariable regions of the BVDV genome (Donis, 1995), but within isolates derived from outbreaks, the neutralizing epitopes (1 for type 1, 3 for type 2) are highly conserved (Deregt, et. al. 1998). The apparent disparity of conservation in hypervariable regions may be explained, in part, by the maintenance of E2 phenotypes by immunotolerant, persistently infected carriers. Such animals serve as a continual source of herd infection, but any variant arising in the animal is uncloaked and eliminated by the internal selection pressures of the immune response (Hamers, et. al. 1998).

There exists ample evidence to suggest E2 plays a role in attachment. Hulst and Moorman (1997) demonstrated competitive inhibition of BVDV

infectivity with CSFV E2 protein, and that E2 binds a receptor distinct from that of E^{rns}. Anti-E2 monoclonals block infectivity of BVDV (Flores, et. al. 2002). A recent study provided evidence that it confers some degree of species specificity and cell culture tropism (Liang, et. al. 2003). This is contrary to another line of investigation using rabbit antibody raised to anti-E2 monoclonal antibody (anti-idiotypic antibody designated anti-D89) to mimic E2 and co-precipitate the 50 kDa membrane-bound receptor (Xue and Minocha, 1993). Unlike the E^{rns} receptor, this receptor was determined to be a protein of which affinity for anti-D89 was unaffected by *N*- and *O*-glycosidases (Xue, et. al. 1997). Anti-D89 bound to bovine and porcine cells, but also to nonpermissive monkey kidney, baby hamster kidney, equine kidney, and canine kidney cells (Xue and Minocha, 1996). If BVDV uses this receptor for viral entry via ligation with E2, some block in viral replication must exist in nonpermissive cell lines downstream of endocytosis. Trans-complementation studies using E2-deleted mutants eliminated E2 as a participant in viral replication (Reimann, et. al. 2003).

A small stretch of hydrophobic a.a. about 7kDa in size (p7), exists between the E2 and NS2 polypeptides. Cleavage of the E2-p7 is inefficient, and detectable quantities of the cleaved and coupled proteins are present in BVDV-infected cells. p7's high level of conservation (Elbers, et. al. 1996) indicates it is important, and cleavage of p7 from the nascent polyprotein is necessary for complete viral replication. RNA replication was shown to occur when E2-p7 cleavage was abolished, but not when p7-NS2 remained fused (Harada, et. al. 2000). Infectious virions were not produced unless p7 was

cleaved, presumably by signal peptidases. This suggests that the C-terminus of p7 acts as a signal sequence for tranlocation of NS2-3. The results of this study led to the hypothesis that p7 may participate in membrane permeabilization and viral exit as a member of the viroporin family of proteins. Elbers (et. al. 1996) demonstrated that the p7 protein co-sedimented with cell debris, but was absent in the purified virion.

The proteins encoded by the gene 3' to the p7 gene are designated NS2-3 (p125), for they are the coupled 2nd and 3rd nonstructural proteins (p7 could be considered "NS1"). The NS2-3 polypeptide is considered two ligated proteins because their homologs are expressed separately in athropod-borne flaviviruses (Donis, 1995), and in the cytopathic biotype of BVDV. The polypeptide is about 1300 a.a. in length, and possesses several functional domains. The N-terminal region is very hydrophobic, suggesting a role in membrane anchorage. The neighboring domain is a cysteine-rich zinc finger motif proposed to bind RNA. About 146 a.a. C-terminal to the zinc finger is a domain with serine protease activity responsible for cleavage of the remainder of the polyprotein into nonstructural proteins. The subsequent domain couples ATPase and helicase activities necessary for RNA replication.

BVDV NS2-3 protein has been intensively investigated because it is the site of genetic alterations that result in the separate expression of NS2 and NS3, or merely the duplication of NS3. The known means of NS2-3 cleavage are insertion of genetic sequences coding proteins that either provide or expose cleavage sites. Among the pestiviruses, BVDV is unique in that the appearance of NS3 corresponds to a switch from the noncytopathic biotype

(ncp), with which infection of cell culture produces no discernible impact, to the cytopathic biotype (cp) that induces cytopathic effect (CPE) *in vitro*. For CSFV and Border Disease Virus of sheep (BDV), the presence of NS3 does not necessarily correspond to the appearance of CPE. While the presence of cleaved NS3 protein confers cytopathogenicity to BVDV, NS2-3 is still expressed and is essential for viral replication.

The most contemporary model for NS2-3 ascribes a regulatory role in RNA synthesis and morphogenesis of the mature virion. Very early in the infection cycle (before 6 hrs p.i.), NS2-3 is almost completely cleaved, even in ncp BVDV (Lackner et al, 2004). This period corresponds to enhanced levels of RNA synthesis and intracellular accumulation. This processing, independent of genetic alterations, diminishes to undetectable levels by 9 hrs p.i. in ncp, but not cp BVDV. The functional attributes of the intact NS2-3 protein include a hydrophobic region (NS2) typical of membrane-spanning or anchoring sequences, sequences homologous to those found in capsid-binding motifs (NS3) of other Flaviviridae genera, cellular chaperone-binding regions (NS2), and a Zinc finger motif (NS3). A role in virion assembly, possibly by co-localizing capsid protein, envelope proteins, and genomic RNA at the endoplasmic reticulum membrane has been postulated (Agapov et al, 2004). Taken together, it appears that the NS2-3 protein is cleaved very early in the viral life cycle resulting in a burst of RNA synthesis. In the ncp biotype, this is followed by cessation of cleavage, thereby preserving the scaffolding nature of NS2-3 for viral assembly and budding.

Separate from the intact NS2-3 protein, the function of the NS2 protein is unclear. A subgenomic replicon (sg RNA) consisting of the first 3 a.a. of N^{pro}, then NS3, NS4A, NS4B, NS5A and NS5B was shown to be sufficient for RNA replication and induction of CPE (Tautz, et. al. 1999). Collett (1996) surmised that NS2 might play a recombingenic role; indeed, almost all genetic alterations occur in the region N-terminal to the first a.a. of NS3, a glycine residue (Gly-1590 of SD-1 strain). Even a single point mutation at position 1555 of the Oregon strain polyprotein, within the NS2 gene, was shown to impact NS2-3 processing (Kummerer et al, 1998). Substitutions at this position were thought to influence NS2 conformation, thereby altering NS2-3 cleavage efficiency and virus viability (Kummerer and Meyers, 2000). The NS2 conformation theory supports the notion that, either a strongly cytopathic or noncytopathic virus has the highest fitness, and NS2 plays a regulatory role in the viral RNA replication activity of NS3 that ultimately determines cytopathogenicity. Lackner, et al (2004) observed high sequence conservation with the NS2 of other members of the Flavivirdae family, of which it serves to autoproteolytically cleave NS2-3 into separate functional entities.

As mentioned above, NS3 (p80) is the product of proteolytic cleavage of the precursor NS2-3 protein. The NS3 protease domain consists of about 180 a.a. residues and shows considerable sequence homology to chymotrypsin. NS3 serine protease activity cleaves the remainder of the polyprotein into nonstructural proteins at cleavage sites determined by the N-terminal sequences of the products. These sites are flanked by an a.a.

with a small uncharged sidechain at P1' (e.g., serine and alanine) and a leucine at P1 (Xu, et. al. 1997). Like the NS3 a.a. sequence, these cleavage sites are highly conserved among the pestiviruses (Tautz, et. al. 1997). Serine at position 1842 (of NADL strain) is the critical nucleophilic residue of downstream proteolytic acticity (Xu, et. al 1997) and upsteam histidine and aspartic acid residues comprise the remainder of the catalytic triad (Tautz, et. al. 2000).

C-terminal to the protease domain are the domains having helicase and ATPase activities. The conserved DEYH sequence is critical for the coupled provision of these functions, with the N-terminal aspartate residue serving to bind and orient the Mg^{2+}-ATP substrate, and the C-terminal histidine linked to RNA duplex dissociation (Grassman, et. al. 1999). The actions of the helicase may serve to unwind the negative-strand intermediate-positive strand progeny RNA duplex, the double-stranded region of the 3' stem-loop structure immediately prior to, and the secondary structures of the 5' end at the conclusion of negative–strand replication, or both. Gu, et. al. (2000) studied point mutations in the Walker motifs, A (GKT) and B (DEYH), and an 18-residue deletion of motif VI to determine that helicase activity was necessary for negative-strand production.

The NS3 protein is apparently quite antigenic (Donis and Dubovi, 1987), but not immunogenic. Kwang (et al., 1991) found that cattle seropositive to a cytopathic strain had antibody reactive with NS3 in Western blots. The NS3 portion of NS2-3 confers antigenicity to the latter, but again, these antibodies are not neutralizing. A NS3-based genetic vaccine was

shown to prime mice cell mediated immune responses (CMI), but no neutralizing antibodies were generated (Reddy et al., 1999). Lambot (et al., 1997) found similar CMI responses to NS3 in cattle.

The NS4A (p10) protein is a highly conserved sequence 64 a.a. long that acts as a cofactor for the serine protease activity of NS3 (Xu et al., 1997). The cofactor domain was mapped to the central region and interacts with the N-terminal region of NS3 (Tautz et al., 2000). Mutational analysis demonstrated its importance in proteolysis and the maintenance of optimal protein precursor: mature protein ratio, essential for efficient RNA replication (Grassmann et al., 2001). The N-terminal one third is hydrophobic and the remainder acidic.

The NS4B (p38) viral polypeptide function remains unknown, but it may have a role in cytopathogenicity in that a single point mutation in the NS4B coding region generated an attenuated mutant (Qu et al., 2001). This finding, together with the results of cross-linking experiments prompted the suggestion that this protein may serve to co-localize NS3 and NS5A at the endoplasmic reticulum membrane. Indeed, NS4B has six hydrophobic stretches thought to span the ER membrane resulting in two ER lumenal loops and one cytoplasmic loop. Another theory extended by the finding that the mutation corresponded to increased NS2-3:NS3 ratio implicates NS4B as a player in NS2-3 processing; the higher ratio resulted in suppression of apoptosis.

The overall RNA-dependent RNA polymerase (RdRp) activity is split between NS5A (p58) and the NS5B (p75) proteins. This feature

distinguishes arthropod-borne flaviviruses, where NS5 possesses the functional domains of BVDV's NS5A and NS5B, from the pestiviruses and HCV. Using a yeast two-hybrid screen of cellular proteins, the NS5A protein of BVDV has been shown to interact with the alpha subunit of eukaryotic Elongation Initiation Factor-1 (eEIF1α) via a domain that exhibits conservation among all BVDV isolates assayed (Johnson et al., 2001). Typical functions of eEIF1α are participation in nascent polypeptide elongation, binding and bundling of F-actin, microtubule severing, ubiquitin-mediated proteolysis, as well as binding to ribonucleoprotein complexes. It also interacts within the UTRs of many viruses. Despite the sequence variability of the NS5A protein among divergent BVDV isolates, binding of eEIF1α and phosphorylation of serine and threonine residues by cellular kinases shows remarkable conservation (Reed et al., 1998).

The NS5B protein contains a canonical Gly-Asp-Asp motif present in other positive-strand viral RNA polymerases. That RdRp activity was associated with this sequence was confirmed *in vitro* (Zhong et al., 1998). While this activity was determined to have a terminal nucleotidyl transferase (TNT) activity, i.e. by addition of NTP to the 3' terminus of the negative-strand generating a covalently linked dimer, a free terminus was shown not to be a requirement for NS5B RdRp activity (Lai et al., 1999). TNT activity independent of the presence of a primer may be a mechanism to restore initiation sites destroyed by cellular exonucleases, much like the function of telomerases (Ranjith-Kumar et al., 2001). This activity, termed *de novo* synthesis, was shown to be dependent on the template sequence, AUAC-3'

(Kao et al., 1999). The primer-independent initiation was shown to be a result of GTP binding to the terminus, thereby mimicking a primer nucleotide (Choi et al., 2004). In addition, a role in virus assembly was attributed to the 5B-741 locus of the C-terminal portion of the protein. More specifically, this region may serve to direct newly synthesized RNA to C protein associated with the endoplasmic reticulum membrane for encapsidation (Ansari et al., 2004).

Viral Entry and Replication

Viral attachment to the plasma membrane is facilitated by several distinct interactions. As detailed earlier, E^{rns} confers non-specific attachment, but E2 is the principle ligand responsible for cell tropism. In the search for the key receptor of BVDV, Schelp (et al., 1995) precipitated 60 and 96 kD cell membrane proteins using a Mab (CA26) that inhibits BVDV attachment to susceptible cell lines. This group later postulated that this surface receptor was responsible for connection of the cell with cortical actin filaments (Schelp et al., 2000). Using a Mab with similar specificity, Maurer (et al., 2004) isolated a cell membrane protein with high sequence homology to CD46, the cofactor for factor I, an important negative regulator of complement activation on host cells. Use of anti-Low Density Lipoprotein receptor (LDLr) Mab and inhibitors of LDLr endocytosis to inhibit BVDV infection led Agnello (et al., 1999) to conclude that the LDLr was at least one of the BVDV receptors. Additional evidence for the LDLr's role in BVDV entry was the observation that an MDBK subclone resistant to BVDV infection (Flores and Donis, 1995) lacked LDLr. Using anti-anti-E2 antibodies, Xue and Minocha

(1996) were able to isolate a membrane protein of 50kd and propose it is the receptor for the E2 protein. Furthermore, virus particles opsonized with antibody could attach to and infect cells expressing F_c receptor (Flores et al., 2002). Entry and infection following binding to F_c receptor-bearing lymphocytes may be possible.

Subsequent to attachment, the virion gains entry into the susceptible cell by receptor-mediated endocytosis, presumably via the E2 receptor (Donis, 1995). Post-endocytotic events include pH-dependent fusion of the envelope with the endosomal membrane and delivery of the viral genome into the cytosol.

The viral RNA, unaccompanied by packaged viral proteins, autogenously recruits all the necessary factors required for the initiation of translation. Once the translation initiation factors have associated with the IRES, viral capsid and envelope proteins are synthesized, processed and associate with the ER membrane. Newly generated, cleaved nonstructural proteins and host cellular components are proposed to assemble into a functional membrane-bound replication complex at the 3' terminus of the genomic RNA and concomitantly initiate production of limited copies of negative-sense RNA. The 5' terminus of the newly synthesized negative-strand RNA folds into secondary stem-loop structures and modulates transcription of positive-sense progeny and "mRNA" (Yu et al., 2000). Because detection of positive-sense progeny was simultaneous to measurement of negative-sense intermediate production, second-step replication must occur immediately following synthesis of the negative-strand

template (Grassmann et al., 2001). The positive-sense progeny production rate increases rapidly 6 hrs post-infection (p.i.), attaining 1.5 X 10^4 copies per cell by 24 hrs p.i. (Gong et al., 1996). The rate of negative-strand synthesis remains constant throughout the replication cycle. The progeny genomic RNA strands then associate with the C protein, perhaps through an interaction with the 5' UTR. Following encapsidation, budding into intracellular vescicles completes viral maturation. Virion release is through exocytosis.

Plasticity of the Viral Genome

As BVDV is an RNA virus, it does not utilize host DNA polymerase and its proofreading exonuclease activity. This results in lower fidelity replication than that seen with DNA viruses. Base pair substitutions occur at a frequency of 1 per 10,000 nucleotide polymerized (Donis, 1995), translating to greater than one point mutation per viral RNA replication cycle (Bolin and Grooms, 2004). This results in establishment of quasispecies populations within a host following successive rounds of replication during infection. Some substitutions result in altered phenotype, reduced or enhanced efficiency, or have no impact at all. Intra-host genetic heterogeneity may explain such phenomena as vaccine escape, broad tissue tropism, differences in virulence and evasion of host antibody response. The latter is purported to be the means by which ncp BVDV infects the fetus of seropositive dams (Jones et al., 2002), sometimes resulting in persistent infection of the newborn calf. High genetic variation explains the rapid evolution of the

virus, the wide range of species-specificity exhibited by pestiviruses, and poses difficult challenges to vaccinologists (Domingo, 1998).

Despite the genetic variability observed among isolates and within quasispecies swarms, host selective pressures create a bottleneck that ultimately define the dominant viral phenotype. One such selection confers phenotypic stability of the dominant viral population circulating in persistently infected animals; variants of the phenotype present when immunotolerance was established are cleared, and can be found in virus-antibody complexes in the germinal centers of lymph nodes (Collins et al., 1999; Fray et al., 2000a).

By 1994, antigenic (Mab-based) and genetic (RT-PCR-based) differences of bovine isolates revealed the existence of two distinct genotypes (Ridpath et al., 1994). The high conservancy of the 5'UTR was demonstrated by mutational studies to be relatively intolerant to mutation (Becher et al., 2000), which allowed Ridpath to use this region in her genotypic analysis. The RT-PCR method, using primers specific to the 5' UTR was shown to be the most type specific and sensitive means of detecting virus in cell culture and clinical specimens (El-Kholy et al., 1998). Further refinement of BVDV 1 phylogeny was afforded by nested PCR of N^{pro}- (Vilcek et al., 2001) and E2-specific primers (Tajima, 2004). The findings of these RT-PCR-based studies underscored the genetic diversity of BVDV. The considerable genetic variability of BVDV 1 relative to BVDV 2 suggests it has been evolving longer. Indeed, most strains isolated prior to the early 1990's have been classified as BVDV 1.

Molecular Basis for Cytopathogenicity

In addition to the mutations formed by RdRp-mediated base pair mismatches, and permitted by poor proofreading capabilities, the RNA genomes of noncytopathic (ncp) BVDV are prone to drastic alterations resulting from recombination (Tautz et al., 1998). Recombinational events can be categorized as homologous, involving self-sequences, or as recombining with genetic material of host-cell or other BVDV strains (non-homologous). These alterations include insertions of host cellular sequences, deletions, duplications and rearrangements of viral sequences, insertion of superinfecting viral sequences, and a particularly influential point mutation. As alluded to previously, these recombinations result in the generation of a mutant swarm that causes cytopathic (cp) effect upon infection of susceptible cell lines. Invariably, the morphological changes induced by the cytopathic biotype correspond to the presense of NS3 protein expressed separately from NS2-3.

One of the most frequent causes of the emergence of the cp biotype from the ncp biotype via NS2-3 cleavage is insertion of (poly)ubiquitin-coding sequences within the NS2-3 gene. Insertions of sequences encoding a ubiquitin monomer result in expression of a polypeptide containing the cleavage site (Gly_{76} of NS2-3) recognized by a cellular protease, ubiquitin carboxyl-terminal hydrolase (Tautz et al., 1993). Actually, a complete ubiquitin monomer in the insert is not a strict requirement cytopathogenicity, as is observed with the RIT strain. Here, an N-terminally truncated ubiquitin linked to cellular ribosomal protein S27a provides the necessary cleavage site

(Becher et al., 1998). A cellular protein-coding insert was shown to add an ubiquitin-like protein, the bovine homologue of human Smt3B (Qi et al., 1998) in a recombinational event resulting in cp biotype. Insertion of another ubiquitin-like protein, bovine NEDD8, provides an additional type of cleavage site in the polyprotein (Baroth et al., 2000). The cellular enzyme responsible for the proteolysis of the recombinant polypeptide generated following insertion of cellular light chain 3 (LC3) of microtubule associated protein-encoding sequences was not identified (Meyers et al., 1998) until recently, when the protease responsible for cleavage of LC3-NS3 was characterized as a bovine cellular cysteine protease (Fricke et al., 2004). Additional cellular inserts inducing biotype switch, Gamma-aminobutyric acid (A) receptor associated protein (GABA(A)-RAP) and Golgi-associated ATPase enhancer (GATE-16), along with LC3 were shown to have ubiquitin-like homology, to insert in common locations in the BVDV genome, and to be processed in a way similar to ubiquitin (Becher et al., 2002). The common consequence of these insertions is the provision of a target for the cellular proteases responsible for NS2-3 processing.

Another sequence commonly inserted, referred to as “cINS” was first noted in the NADL strain and was shown to be ultimately causative for cytopathology (Mendez et al., 1998). This insert is generally 270 a.a. in length. Although it is conserved in its core sequences, in eight isolates recently analysed there existed variability in its 3’ and 5’ termini (Kummerer et al., 2000). A member of the DnaJ family of chaperones known to bind to Hsp70 proteins was found to contain an internal sequence (J-domain) with

high homology to the cINS found within the cp NADL genome (Rinck et al., 2001). The expressed protein of this mRNA-derived insert, subsequently termed Jiv90, was shown to act in *trans* to facilitate processing of the NS2-3 protein. That intracellular levels of Jiv90 protein bind to NS2 segment of NS2-3 and induce cleavage represented a novel means of NS2-3 processing. Even more novel is the location of inserts in strains CP8 and Nose; duplicate Jiv90 fragments are found inserted in the N-terminal region, instead of the NS2-3 region (Muller et al., 2003) in the former strain, and within the structural protein region in the latter (Nagai et al., 2003). As native Jiv is responsible for binding incompletely folded proteins and transferring them to Hsp's, it is possible that Jiv binding induces conformational changes in the BVDV polyprotein, thereby exposing cleavage sites for as yet unidentified cellular proteases.

It was noted that insertions of ubiquitin or ubiquitin-like sequences and DnaJ-like protein coding sequences occur with higher relative frequency in BVDV types 1 and 2, respectively. These type-biased insertions also showed a preference for insertion sites (Ridpath and Neill, 2000).

Liberation of NS3 by autoprotease activity can also result from duplication of the N^{pro} gene within the NS2-3 gene. It was theorized that the recombinant protein had an altered conformation that exposed cleavage sites for an unidentified cellular protease. This was one of the explanations for the conversion to a cytopathic biotype resulting from a 9 a.a. insertion in strain CP7 (Tautz et al., 1996). It was observed that for the Oregon cp strain of BVDV, no genetic alterations in the NS2-3 coding sequences could be found

(Kummerer et al., 1998). Rather, a few a.a. substitutions, particularly a switch from serine 1555 to phenylalanine, greatly reduced cleavage efficiency. Thus, in these instances, the post-translational modification of the recombinant protein, rather than transcriptional processes, is responsible for the yield of NS3 byproduct and cytopathic effect. This is suggested to be manifested by induction of a cleavable conformation.

Tautz et al (1994) characterized an association of a ncp BVDV and the defective interfering particle (DI9) resulting from a large internal deletion of structural protein coding genes and the N-terminal portion of NS2-3. The ncp BVDV helper virus provided replication and packaging functions for DI9, which was responsible for the observed CPE.

The broad array of recombinational events was postulated to arise from a "copy choice" mechanism in which a template switch from an intra-NS2-3 region to a viral or cellular template during negative strand synthesis occurs. It is proposed that inserts are the result of template switching during negative strand synthesis, followed by template switching during positive strand synthesis (Becher et al., 1999). It has been observed that open bulges in the nascent negative strand and the positive strand template duplex are common and that the RNA polymerase complex may cross over at these locations (Desport et al., 1998). Whereas it was first postulated that RdRp switches back to the viral RNA template after transcription of the insert (Meyers et al., 1991), it was later suggested that two successive recombinations in cp BVDV could alternatively explain improbable, multiple template switches (Meyers et al., 1998). This was supported by the finding

of several variants of a cp BVDV genome from a single outbreak of disease (Becher et al., 1999), which seemed to represent a progression of recombination from a progenitor genome. In fact, it was subsequently shown that recombination is an ongoing process in the course of infection, and a variety of additional recombinations occur among the primary recombinant population in an animal (Fricke et al., 2001). An entirely different mode of recombination independent of polymerase activity has been postulated and supported (Gallei et al., 2004).

There does not appear to be a "hot spot" in the viral genome as a recombination site. Although some homology is apparent among sequences flanking the insertion sites, only 3' terminus conservation is required (Ridpath et al., 1994). There is no detectable homology within the insertion sequence.

BVDV-induced Apoptosis

The aforementioned genomic alterations leading to the generation of a viral swarm capable of inducing cell death were originally surmised to be directly related to toxic properties of the NS3 protein, a lytic event. The CPE observed in cell culture was determined to be an apoptotic event by Zhang et al (1996) using the TUNEL and oligonucleosomal laddering methods. This study demonstrated that DNA fragmentation occurred only in cells infected with cp BVDV. These findings were corroborated and extended by Hoff and Donis (1997), who observed the nuclear morphological hallmarks of apoptosis *in vitro* and poly (ADP-ribose) polymerase inactivation. The affinity of BVDV for immune cells has been demonstrated (Adler et al., 1997).

Macrophages infected with cp, but not ncp BVDV primed uninfected macrophages for apoptosis, perhaps in response to expressed IFN-α, but likely in conjunction with another, yet unidentified factor (Perler et al., 2000). Furthermore, the high correlation of infection and apoptosis seen with adherent cell cultures was shown not to apply to immune cells isolated from cattle blood; whereas both monocytes and T cells ($CD4^+$ and $CD8^+$) undergo apoptosis when cultured together in the presence of cp BVDV, only in a small percentage of monocytes could viral antigen be detected (Lambot et al., 1998a). Again, this bystander effect was attributed to a soluble factor such as IFN-α. Cp BVDV was shown to induce oxidative stress during the onset of apoptosis (Schweizer and Peterhans, 1999). This observation may be attributable to the decrease in intracellular glutathione levels resulting in reduced antioxidant capacity. Reduced glutathione levels were also observed in a study focusing on an ER membrane-bound kinase (PERK) that senses accumulation of misfolded protein within the ER lumen. The findings of this study suggested that BVDV infection activates PERK, with subsequent downregulation of Bcl-2 expression resulting in oxidative stress and apoptosis (Jordan et al., 2002b). Redundancy of apoptotic signals in cp BVDV-infected cells appears possible, as the end-products of the intrinsic pathway of apoptosis have been demonstrated (Grummer et al., 2002).

During RNA virus replication, double-stranded RNA (dsRNA) intermediates are created. Aside from the secondary structures of cellular tRNA, intracellular dsRNA is a rare molecular species except during viral replication. Therefore, dsRNA is a reliable signal of viral infection exploited

by the innate antiviral immune response, including 2'-5' oligoadenylate synthetase (OAS or 2-5 A) / 2-5 A-dependent RNase (RNase L) system and double-stranded RNA dependent protein kinase (PKR). The latter was hypothesized to be an evolutionary advancement of the aforementioned PERK (Williams, 1999), which retained the function of detecting biological stress and phosphorylating common substrates. PKR was shown to be pivotal to induction of apoptosis resulting from a variety of stimuli (Der et al., 1997). Treatment of cells with dsRNA results in PKR-mediated increased expression of several players of apoptosis induction, Fas, FADD, and caspase-8 (Balachandran et al., 1998). Interaction of dsRNA with PKR results in phosphorylation of eIF-2α, leading to inhibition of mRNA and viral RNA translation (Srivastava et al., 1998). Activated PKR also phosphorylates IκB, ultimately liberating NF-κB. The former activity reduces the expression of protective genes as well as viral genes by sequestering key components of the translation machinery. NF-κB is a transcription factor responsible for inducing pro-apoptotic and survival genes (Gil et al., 1999). The simultaneous inhibition of the synthesis of anti-apoptotic and viral proteins, and upregulation of pro-apoptotic genes in response to the interaction of dsRNA with PKR is difficult to explain. One effect of PKR activation by dsRNA is cleavage (inactivation) of another transcription factor, STAT1, by either caspase-1 or –3 (King and Goodbourn, 1998). This may shift the balance of the survival/apoptosis equilibrium in favor of apoptosis. Indeed, repression of the synthesis of negative regulators of activation pathways was observed

following exposure to viral and synthetic dsRNA and shown to be involved in cross talk with other activation pathways (Iordanov et al., 2000).

Although the classic marker of cp BVDV is the expression of NS3, separate from the NS2-3 precursor, other features are distinctive. A particularly relevant occurrence in the course of cp, but not ncp BVDV infection is the accumulation of intracellular viral RNA. The levels of viral RNA were shown to correlate well with levels of NS3 expression (Vassilev and Donis, 2000). The cp NADL strain, for example, generates 26 times the amount of viral RNA as the ncp counterpart. In some cp BVDV strains, NS3 is expressed separately only by virtue of its duplication in the genome; NS23 is not cleaved at all. This suggests that cleaved NS2 protein is not solely causative for establishment of the cp biotype. Studies of subgenomic BVDV replicons led to the conclusion that NS2 functions as a *cis*-acting negative regulator of CPE (Tautz et al., 1999). NS2 protein in close contact with the NS3 component of the replicase complex may serve as a regulatory factor regarding RNA production. Noteworthy is the belief that the NS2 portion of the NS2-3 protein, by virtue of its hydrophobic a.a. content, may serve to anchor the entire replicase complex at the ER membrane. It was noted that despite higher levels of RNA synthesis observed in cp BVDV-infected cells, the level of protein synthesis was very similar to cells infected with the ncp BVDV counterpart (Vassilev and Donis, 2000). This may be attributable to RNA synthesis in a cellular compartment dissociated from the ribosomes. In other words, NS3 protein assembled in a functional replicase complex unrestrained at the ER membrane may result in unchecked RNA synthesis

distant from the membrane-bound packaging apparatus, and induction of apoptosis.

Schweizer and Peterhans (2001) have proposed that much of the viral RNA found accumulating in cells infected with cp BVDV might be double-stranded. This possibility prompted them to suggest that the PKR system may mediate apoptosis in infected cells. Exactly how the ncp biotype subverts the innate immune effectors initiating the apoptotic cascade has not yet been elucidated.

As important as the PKR system is in the innate antiviral immune response, many viruses have successfully evolved mechanisms designed to circumvent PKR activity. A close relative of BVDV, HCV-1, employs the NS5A protein to directly inhibit PKR-induced apoptosis (Gale et al., 1997). This genotype's NS5A protein possesses specific sequences conferring resistance to IFN treatment, termed interferon sensitivity-determining region (ISDR). It is proposed that HCV-1 NS5A binds to the dsRNA-binding region of PKR, thereby preventing dimerization and autophoshorylation (Gale et al., 1998). This inhibition was shown to be intact at NS5A:PKR ratios as low as 1:9. If BVDV NS5A, which is identical within ncp and cp pairs plays a role in PKR inhibition, the influence of much higher intracellular RNA levels on the stoichiometry of NS5A:PKR:viral RNA may be responsible for the differences between the biotypes in the induction of apoptosis.

Clinical Manifestions of BVDV Infection

BVDV is arguably the most economically costly pathogen to the cattle industry worldwide. The first and most notable disease associated with BVDV infection, mucosal disease, may be the least common malady. An equally insidious clinical outcome of BVDV infection appeared in the early to mid 1990s in Canada, chiefly Ontario. These outbreaks of hemorrhagic disease, characterized by severe thrombocytopenia and vasculitis, were almost exclusively caused by an acute infection with hypervirulent BVDV type 2 strains. It has subsequently been linked to bovine respiratory disease (BRD), featuring pyrexia and bronchopneumonia. Economic losses are probably under-estimated due to less tangible impacts such as reproductive failure and secondary infections permitted by BVDV-mediated immunosuppression. This wide array of clinical manifestations is a reflection of the phenotypic diversity characteristic of pestiviruses.

The emerging picture of Mucosal Disease (MD) is that it is the sum total of several consequences of BVDV infection invariably leading to death. The first requirement for the establishment of mucosal disease is the ability of the ncp biotype of either BVDV 1 or 2 to subvert the innate antiviral immune mechanisms of pregnant cattle. The inhibition of type I IFN production and the block of apoptosis of infected cells prevents effective clearance of the virus before it can infect the placentome. Transplacental passage of ncp BVDV circa 110 days of gestation places the virus in the fetus at a time when the adaptive immune system is establishing what is self-antigen. By the time the fetus is immunocompetent, T and B cell clones

reactive to self- and BVDV-antigen have been deleted by clonal selection. The establishment of immunotolerance to the infecting BVDV strain, the broad scope of tissue tropism, and the continued ability of the ncp biotype to block innate antiviral responses results in the birth of a thoroughly and persistently infected calf. Ultimately, the high recombinational rate of the viral genome, or the superinfection by a homologous, but cp strain abrogates whatever property the intact NS2-3 protein confers in terms of a cryptic existence. While the virus is uncloaked and evident to the apoptotic apparatus of infected cells, the adaptive immune response is still immunotolerant and helpless to stem the spread of the mutant swarm. With cytopathic BVDV infecting the same broad variety of tissues with impunity, it could be said that at the onset of MD, the animal essentially undergoes apoptosis. Dehydration as a consequence of profuse watery diarrhea resulting from loss of intestinal epithelial integrity and other complications leads to death.

Several lines of investigation support this model of MD. When cp and ncp pairs of the same strain and similar titer are used to separately infect susceptible calves, the ncp infection is more difficult to clear (Lambot et al., 1998b). That the primary infecting virus must be of the ncp biotype is underscored by studies demonstrating the lack of interferon production and induction of apoptosis (Schweizer and Peterhans, 2001) necessary to establish persistent infection in the fetus (Charleston et al., 2001a). Mechanisms responsible for ncp BVDV suppression of innate antiviral responses were extended that identified reduced Interferon Regulatory

Factor 3 (IRF-3) binding to DNA as a culpable block (Baigent et al., 2002) and the RNase activity of E^{rns} (Iqbal et al., 2004). Spillover of ncp virus was observed from acutely infected heifers first to the allantoic and amniotic membranes (Swasdipan et al., 2002) and then to lungs, spleen and liver of the fetus (Frederiksen et al., 1999b), but the distribution of BVDV antigen in fetal tissues was not nearly as widespread as that observed when the dam was persistently infected (Frederiksen et al., 1999a).

Persistently infected animals exhibit viral antigen (in descending order of permissivity) in lymphocytes and macrophages of the lymph nodes and spleen; thymocytes, intrafollicular dendritic cells, and macrophages of the thymus; lymphoid and epithelial cells of the ileum; mucosal epithelial cells and lamina propria of the abomasum; keratinocytes of the rumen, omasum, esophagus, gingiva, labia, tongue, and skin; lymphoid cells of the colon; bronchiolar epithelium of the lung; microglia of the brain (Bielefeldt-Ohmann, 1988a); and vascular epithelium (Liebler et al., 1991). The affinity of ncp BVDV for peripheral blood mononuclear cells (PBMCs) and keratinocytes make these tissues the choice for immunocytochemical diagnosis of persistent infection with BVDV (DuBois et al., 2000), but not acute infection (Liebler-Tenario et al., 2002). Ncp BVDV also shows a predilection for cells of the central nervous system (Fernandez et al., 1989), particularly the hippocampus, cerebral cortex, and spinal cord. Involvement of the eye includes microphthalmia, cataracts, leukocoria, retinal hemorrhages, chorioretinitis, retinal dysplasia, retinal detachment, optic neuritis and atrophy.

At the onset of MD, the cp biotype is detectable as a multifocal infection consistently present in epithelium and lymphoid tissues of the alimentary tract, including tonsils, lymph nodes, Peyer's patches and lymphoid nodules of the large intestine. The progression to diffuse infection of intestinal epithelia and pneumonic tissue corresponds with diarrhea (Liebler-Tenario et al., 1997) and BRD (Taylor et al., 1997), respectively. Nephritis and hepatic necrosis are occasional found upon necropsy.

It is noteworthy that some, but not all persistently infected calves exhibited stunted growth. In one study, average weaning weight of PI calves was 160 kg compared to 203 kg for BVDV-negative calves (Taylor et al., 1997). Many of these calves exhibited "growth arrest lines" associated with the epiphyses of the long bones that were commonly found with chronic dysplasia of the bone marrow. Murondoti (et al., 1999) suggested persistent infection involving the pancreas may directly or indirectly cause diabetes mellitis in emaciated cattle. Prolonged exposure to TNF-α as a result of chronic viral infection is known to induce cachexia in cattle (Bielefeldt-Ohmann et al., 1989), but BVDV-infected macrophages, the main producer of TNF-α, show a marked decrease in TNF-α production (Adler et al., 1996). Stunted growth may be solely the result of infection and dysfunction of tissues of the endocrine system.

One of the strongest clues that immunotolerance to, and generation of the cp strain from spontaneous mutation of the persisting strain was central to the establishment of MD was the observation that ncp/cp pairs isolated from cases were homologous in terms of antibody recognition (Brownlie,

1990). Homology of the infecting pair is the basis for variation in the infection-to-death timeframe observed. Early onset MD represents the classical, fulminating disease characterized by extensive lesions, diarrhea, dehydration and death. The virus pairs associated with early onset MD are virtually identical with the exception of NS2-3 cleavage efficiency. However, if slight antigenic differences exist between the original ncp and the new cp viruses, a chronic form of MD, termed late onset MD is manifested. This results from an immune response, albeit ineffective, that slows the pathology of MD. As opposed to early onset MD, the late onset disease shows more drastic lymphoid depletion, more widespread ulcerations and severe vasculitis (Liebler-Tenario et al., 2000).

The nature of the lesions associated with mucosal surfaces and intertriginous zones is intriguing. In a study of highly virulent ncp BVDV infection, apoptotic lesions were well correlated with the presence of viral antigen in lymphoid tissue (Liebler-Tenario et al., 2002). This did not hold for lesions of the digestive tract mucosa, where multifocal lesions did not match the pattern of diffuse viral antigen. Lesions of the vasculature were infrequent and may have developed from immune complex deposition in calves that were able to mount an immune response with incomplete clearance. Such vasculitis has been categorized as a type III hypersensitivity, whereby antibody-antigen complexes arise as a result of one of five possible scenarios (Desilets et al., 1996.) However, with the clinical presentations associated with MD, the erosive lesions of the oral cavity and gastrointestinal tract, especially the Peyer's patches are

associated with the appearance and unrestrained infection with cp BVDV. Although the cp BVDV strain can be isolated from a variety of tissues, lesions are only found on surfaces with high bacterial concentrations, such as the alimentary tract and the inter-digital zones. Induction of apoptosis by Type I interferon secreted by the cp BVDV-infected cells in regions of high endotoxin has been suggested to be the cause of the lesions associated with MD (Adler et al., 1997).

Unlike MD, the severe acute (SA) form of BVDV-related diseases features hemorrhagic and thrombocytopenic manifestations without the generation of co-infecting cp BVDV. Additionally, SA BVDV is strictly caused by strains of the type II genotype (Bezek et al., 1994), and does not always exhibit gross macroscopic lesions. The first experimental induction of SA BVDV infection documented several hematologic alterations, including lymphopenia, neutropenia, anemia, and thrombocytopenia (Corapi et al., 1989). Severe thrombocytopenia was correlated with multisystemic hemorrhaging and the detection of viral antigen in platelets (Corapi et al., 1990). The distinctive etiology of these infections may be attributable to the infecting strain's affinity for megakarocytes of the bone marrow, resulting in depletion of platelets (Ellis, et al., 1998) as a direct result of decreased production (Walz et al., 1999). Additionally, platelet aggregation was suppressed when infected with type II, but not type I BVDV (Walz et al., 2001). Differences in virulence among the type II isolates may be attributed to the variation in *in vivo* replication efficiency or tissue tropism, especially that of thrombocytes (Bolin and Ridpath, 1992). Close examination of a

recently reported attenuation event of a highly virulent type 2 strain (Deregt et al., 2004) may reveal the gene associated with SA hemorrhagic sydrome. Although it appears that the symptomatology of SA infections is more a direct consequence of viral replication, there is speculation that the disease, like MD is manifested as a result of the host's immune response (Liebler et al., 2002). In fact, in one notable outbreak in Canada, the most severe lesions belonged to adult cattle (Carman et al., 1998), that presumeably have more competent immune systems than calves (<6 months in age).

The level of contribution of BVDV to BRD is controversial. BRD is characterized by pyrexia, nasal discharge, dyspnea, and acute to chronic bilateral bronchopneumonia in animals 2 months to 1 year in age. Whether BVDV is a primary or secondary pathogen is an ongoing debate, but its isolation from a majority of respiratory tract disease cases highlights its relevance in the syndrome (Potgeiter, 1997). Indirect evidence of BVDV involvement in BRD is offered by the fact that calves arriving at the feedlot with pre-formed BVDV antibody titers are at reduced risk for BRD, while seroconversion after arrival correlates with higher risk of respiratory disease (Campbell, 2004). The percent pneumonic tissue of lungs infected with cp BVDV alone, *P. haemolytica* alone, or both sequentially (BVDV, then *P. haemolytica*) was found to be 2-7%, 15%, and 40-75%, respectively, with only the combination inducing severe respiratory disease (Potgeiter et al., 1984b). Odeon (et al., 1999) were able to cause severe acute fibrinous bronchopneumonia in 6-month old calves with intranasal inoculation of ncp BVDV type 2 isolate and dexamethasone. Although these calves were not

reported to be gnotobiotic at inoculation, the pneumotropism of the virus was demonstrated immunohistochemically in alveolar macrophages, subepithelial mononuclear cells and bronchiolar epithelium. However, the etiological agents causing the pneumonia were found to be *Pasteurella hemolytica* and *P. multocida.* This supports the notion that BVDV's role in BRD is chiefly one of immunosuppression (Liebler-Tenario, 2002). The pre-infection of lungs with BVDV has been shown to permit colonization and hinder clearance of infectious rhinotracheitis virus (IBR)(Potgeiter, et al., 1984a) and *P. haemolytica* (Potgeiter et al., 1985). Concurrent infections of BVDV with *Mycoplasma bovis* (Shahriar et al., 2002) and bovine respiratory syncytial virus (BRSV) (Elvander et al., 1998; Broderson and Kelling, 1999) demonstrate a synergism of pathologies. Again, the bulk of the evidence suggests BVDV infection is an important factor in BRD by virtue of its immunosuppressive effects, including those on bronchalveolar macrophages (Ellis, 2001). Welsh (et al., 1995) also documented impairment of alveolar macrophage functions that certainly impacts protective capability.

Although acute infection with type 2 genotype BVDV is responsible for much of the BRD seen in Canada, the type 1 genotype is isolated more frequently from BRD cases in the United States (Fulton et al., 2000). It is unclear if this observation is attributable to geographic predominance of one genotype over the other in outbreaks. A follow-up study revealed an elevated frequency of BVD 1 (subtype 1b) implicated in BRD cases (Fulton et al., 2002). In the southern part of Africa, a new type 1 subtype, 1d is the predominant pathogen of BRD cases (Baule et al., 2001).

Economic loss caused by various reproductive impacts of BVDV infection are said to be the most costly of all the clinical manifestations (Saliki, 1996). A broad continuum of outcomes, from embryo reabsorption to birth of a seropositive calf is possible subsequent to transplacental transmission. The outcome is determined by the gestational age of the fetus at infection (reviewed by Grooms, 2004). Losses are associated with opportunity costs of reduced conception rates, aborted fetuses, stillbirths, and calves born were various severe malformations.

Vaccinal cp BVDV was shown to transiently infect ovaries of heifers and adult cows following vaccination with MLV BVDV (Grooms et al., 1998). BVDV infection of ovaries has been linked to ovarian endocrine dysfunction culminating in reduced ovulation rates. This is a result of reduced estradiol (Fray, et al., 2000b) and LH surge suppression (McGowan, et al., 2003).

BVDV can be present in bull semen as a result of persistent infection of the testes, an immunologically privelaged site (Voges et al., 1998). Donor oocytes derived from persistently infected cows can be a source of early infection (Fray, et al., 1998). In artificial insemination systems, virus adhering to oocytes, infecting cumulus oophorus and oviduct cells, contaminating follicular fluid and FBS in uterine rinsate are all sources of virus (Givens and Waldrop, 2004).

Infection at the earliest stages of development is blocked by the zona pellucida, which protects viability of the morula and blastocyst. Once the zona pellucida is removed during early embryonic stages, various effects can be seen in terms of reduced conception rates.

Once implanted, the embryo is susceptible to transplacental transmission. At 30 to 100 days of gestation, development of persistent infection rates approach 100%. Persistent infection is only achieved by the ncp biotype of either genotype (Harding et al., 2002). When considering the impact of horizontal spread of virus to open cows whose estrous has been synchronized, it is surprising that persistent infection rates are as low as observed in intensively managed cattle operations.

Between 100 and 150 days of gestation, BVDV infection can have teratogenic consequences on the central nervous system and skeletal system. Central nervous system defects include microencephalopathy, hydrocephalus, hydranencephaly, porencephaly, cerebellar hypoplasia, and hypomyelination. Cerebral lesions of the pituitary gland and hypothalamus may reduce adrenocorticotropic hormone interferes with parturiton and may prolong gestation (Spensley, 2002). Eye involvement includes cataracts, microopthalmia, optic neuritis, and retinal degeneration. Mandibular brachygnathism is the most common skeletal malformation.

At 125 to 285 days of gestation, the fetus is protected from BVDV infection by a competent immune system. This is well illustrated by the occasionally high titers of BVDV-neutralizing antibodies found in FBS.

BVDV-induced Immunosuppression

As has been discussed in the context of BRD, BVDV causes immunosuppression and permits colonization and replication by opportunistic pathogens. Empirical evidence of immunosuppression, i.e., that seroconversion to BVDV accompanies outbreaks of other diseases and that

persistently infected animals have higher mortality rates and are at increased risks of pneumonia and enteritis has led to speculation that BVDV infection is a predisposing factor in many other pathologies. It has been observed that antigenic preparations, *Mycobacterium paratuberculosis* (PPD) and *M. bovis* (BCG) produced less pronounced immunological responses when the animal was recently vaccinated with MLV BVDV (Thoen and Waite, 1990) or acutely infected with ncp BVDV (Charleston et al., 2001b), respectively. Important bovine viral pathogens potentiated by concurrent BVDV infection, such as BRSV, have been shown to gain a foothold during delays in humoral response (Graham et al., 1998). Challenge experiments have demonstrated enhanced pathogenesis of BRSV (Broderson and Kelling, 1998) and bovine rotavirus (BRV) (Kelling et al., 2002) infections when co-inoculated with BVDV. Again, its suppressive role in respiratory tract infections can result in increased pathogenesis by bovine parainfluenza virus (PI3), IBR, BRSV, and *P. haemolytica.*

Several studies have demonstrated BVDV's predilection for leukocytes. One of the first demonstrations of viral antigen in PBMCs defined the frequency of infection at 5-36% (Bielefeldt-Ohmann et al., 1987) with B cells being permissive to infection without viral replication. This was later confirmed (Lopez et al., 1993); productive viral replication was only detected in monocytes and T cells expressing BoCD4, BoCD8 and γδ-TCR, but not B cells. However, in a study of leukocyte subsets permitting viral replication (Sopp et al., 1994), B cells comprised 11% of $p80^+$ cells, with γδ-T cells (11%), $CD2^+$ T cells (23%), and (35%) monocytes also exhibiting

fluorescence. Flow cytometric analysis of PBMCs from a persistently infected animal quantified the frequency of infection to be 3-21%, and determined this method to be suitable for diagnostic purposes (Qvist et al., 1990). Mononuclear cells also stained positive for BVDV antigen in lymph nodes (Wilhelmsen et al., 1990). Monocyte-derived macrophages expressed 20-30% viral antigen upon examination (Chase et al., 2004). A time-course experiment designed to track the dissemination of virus from the primary site to secondary tissues was performed (Bruschke et al., 1998a). This study identified leukocytes as the chief means of viral spread.

Immunosuppression is purported to be linked to ncp BVDV's circumvention of type I interferon that allows bystander viruses and bacteria to establish infections (Peterhans, et al., 2003). Infection of immune cells crucial to innate immunity that lead to altered function represents another means of rendering an animal immunosuppressed and more susceptible to bacterial and viral opportunists. This was clearly seen in cp and ncp BVDV-infected macrophages whose ability to respond to LPS with TNF-α (Adler et al., 1996) and to respond to PMA with superoxide anion production (Adler et al., 1994) was significantly impaired. MLV BVDV vaccination was shown to inhibit neutrophil-mediated antibody-dependant cell cytoxicity (Roth and Kaeberle, 1983). Inhibition of PHA-induced proliferation of PBMC by cp BVDV was shown to be due to reduced IL-2 production (Alturu et al., 1990) stemming from reduced IL-1 secretion by infected monocytes and inhibiton of mRNA production. The block in IL-1 activity was corroborated and anti-proliferative effects of BVDV infection documented (Jensen and Schultz,

1991). However, IL-2R expression is maintained in cells infected with both biotypes (Hou et al., 1998). BVDV-infected monocytes show marked reduction in leukotriene B_4, an important mediator in IL-1, IL-2 and IFN-α production (Atluru et al., 1992). Additionally, infected alveolar macrophages, an important frontline defense against bacterial pathogens of the lung exhibit reduced expression of FcR and C3R *in vitro*, as well as reduced phagocytic, chemotactic, and microbicidal activity *in vivo* (Welsh et al., 1995). Chemotaxis is an important consideration; the inability of infected monocytes to home to regions breached by potential pathogens is a major aspect of immunosuppression. Ketelsen et al. (1979) found chemotaxic responses of infected monocytes were reduced by 56%.

Antigen presentation by cells of the innate immune system is another function impacted by BVDV. Antigen presenting cells, with the possible exception of B cells, are subject to direct and indirect influences of BVDV infection. PBMCs isolated from calves infected with a highly virulent type 2 BVDV demonstrated reduced expression of surface MHC II and B7 molecules compared to uninfected controls (Archambault et al., 2000), but MHC I and B4 expression, and phagocytic capability were unaffected. As opposed to those of an acute infection, ncp BVDV-infected PBMCs isolated from a persistently infected calf were able to promote proliferation of $CD4^+$ and $CD8^+$ memory T cells derived from a seropositive MHC-identical calf (Glew and Howard, 2001). This suggests differences in virulence may be related to APC function. Biotype differences were not observed with regard to the altered mechanism of endocytosis by infected monocytes (Boyd, et al.,

2004), but phagocytic responses were inhibited by a cp and two highly virulent BVDV strains relative to two avirulent ncp strains (Chase et al., 2004). Importantly, monocytes and monocyte-derived dendritic cells (DC) are both permissive to both biotypes of BVDV, but DC are resistant to apoptosis when infected with cp BVDV (Glew et al., 2003). Infected monocytes were impaired with respect to stimulating uninfected $CD4^+$ T cells to proliferate, but DC were not. This has major implications in terms of long-term antigen presentation.

Like the cells involved in the innate immune responses, those of the adaptive immune response are also permissive to BVDV. Even the mild form of acute BVDV infection features lymphopenia. Early studies documented the characteristic reduction in circulating B and T cells in cp BVDV-infected cattle (Bolin et al., 1985), and impairment of immunoglobulin synthesis (Atluru, et al., 1979). Marshall (et al., 1994) examined the depletion of lymphocytes in a variety of lymphoid tissues by flow cytometric quantification. A similar study investigated the fate of circulating lymphocyte subsets, and similar depletion of T helper and cytotoxic/suppressor T cells was reported (Ellis et al., 1988). Whether lymphocytes were experiencing cell death and clearance or were being trafficked to or sequestered at the site of active infection was brought into question. As is typical in biology, the answer is likely “both”.

Immune Responses to BVDV Exposure: Natural Infection with BVDV

As described previously, immune responses to BVDV infection act at the innate and adaptive levels. Importantly, innate immune responses are intact in the fetus before the development of adaptive immune response.

Type I interferon has been shown to suppress BVDV replication and spread (Gillespie et al., 1985; Bielefeldt-Ohmann and Babiuk, 1988b; Sentsui et al., 1998). Cytopathic, but not ncp BVDV induces apoptosis, IFN-α production (Rinaldo et al., 1976; Charleston et al., 2001), and Mx gene products, and fails to produce productive infection in the fetus (Harding et al., 2002). Although ncp BVDV can subvert the innate antiviral immune responses of the fetus, this ability is diminished in calves (Muller-Doblies et al., 2002; Muller-Doblies et al., 2004). The high frequency of $\gamma\delta$ T cells present in circulation during the first year of life may be influential in staving off acute MD in a NK, or non-specific fashion (Bruschke et al., 1998b). Once immunocompetent, the fetus is able complement the innate immune system with an acquired response to mount a more effective immune response, even to ncp BVDV (Charleston et al., 2002). The acquired immune response comprises both cell-mediated and humoral responses (Chase et al., 2004).

That BVDV infection induces a strong antibody response in exposed animals initially led to the speculation that humoral responses confer protection from disease. Passively transferred maternal antibodies readily confer immunity to infection of newborn calves (Bolin and Ridpath, 1995). The half-life of maternally derived anti-BVDV antibody is between 20 and 23 days, with the time to seronegative status being between 118 and 192 days for BVDV 1, and between 94 and 158 days for BVDV 2 (Kirkpatrick et al., 2001; Fulton et al., 2004). Once maternal antibody has waned, the primary antibody response to BVDV exposure is one to three weeks, reaches plateau by three months, and remains high for at least three years (Fredriksen et al.,

1999). The isotype most associated with natural infection is IgG1, a T_H2-associated isotype. Anamnestic responses can generate very high titers, e.g. up to 1:8448 (Ross, 2003). Antibody to solubilized BVDV can even be generated *in vitro* from PBMC cultures derived from seropositive cattle (Larsson et al., 1990).

Immune Responses to BVDV Exposure: Vaccine Induced Immunity to BVDV

The protein most responsible for generation of neutralizing antibody is the E2 (gp53) protein (Bolin, 1993). Apparently, sufficient epitope similarities exist among the E2 protein of types 1 and 2 BVDV to elicit cross-reactivity as determined by cell-based *in vitro* serological assays (Fulton et al., 1997). While cross-neutralizing antibodies raised to inactivated BVDV1 vaccines diminish by day 140 post-vaccination, a single dose of MLV BVDV1 vaccine cross-reacted with a variety of strains, including BVDV2 (Cortese et al., 1998a). In challenge studies using BVDV2 challenge strain 890, a killed BVDV1 vaccine reduced clinical scores (Hamers, et al., 2003), while a single MLV dose of BVDV1 vaccine provided longterm serological responses, reduced clinical signs and nasal shedding, and elimination of viremia and leucopenia (Dean and Leyh, 1999; Fairbanks et al., 2003.).

However, the level of neutralization of the heterologous genotype of virus is generally less than that of the homologous immunogen (Jones et al., 2001; Fulton and Burge, 2001). Vaccination regimens using killed, then MLV BVDV1 vaccine at days 15 and 40-45 of life, respectively, reduced horizontal transmission of BVDV1 but not BVDV2 strains (Thurmond et al., 2001). A

serological analysis of the response to a killed vaccine containing two strains of BVDV1 cross-neutralized some, but not all BVDV2 strains (Hamers et al., 2002). The use of two antigenically distinct strains (genotypes were not disclosed) to develop an ISCOM vaccine generated antibody responses that were complementary, and cross-reacted with several field strains of BVDV (Kamstrup et al., 1999). A possible advantage of using a combination of both BVDV genotypes in a killed vaccine has been reported (DesCoueaux et al., 2003). They observed much higher heterologous and homologous antibody titers after administration of the combined vaccine, although the adjuvant in the bivalent vaccine (saponin) was different from that of the monovalent vaccine used for comparison (Aluminum hydroxide). A similar study employed an inactivated BVDV1/2 combination vaccine, attributing the high antibody titers observed to the high dosage of BVDV and novel adjuvants (Bay R 1005 and Polygen) used (Beer et al., 2000).

Again, the E2 protein of BVDV is the chief immunogen in BVDV serological responses, and its immunodominant characteristic has been exploited in vaccine development. An E2-expressing baculovirus recombinant was shown to elicit a humoral response to E2, but protection was not clearly afforded by this vaccine (Bolin and Ridpath, 1996). Recombinant fowlpox (Elahi et al., 1999a) and CMV-promoted human adenovirus type 5 (Elahi et al., 1999c) have been shown to induce neutralizing antibody to BVDV in the mouse model. Adenovirus type 3 vectored E2 raised E2-specific IgG and IgA in cotton rats (Baxi et al, 2000). E2 was successfully expressed by BHV-1 vector (Schmitt et al., 1999), and

further developed in a thymidine kinase-negative construct that elicited moderate seroconversion in cattle (Kweon et al., 1999). Experimental DNA vaccines expressing E2 induced neutralizing antibody to type 1 in mice (Harpin et al., 1997) and primed cattle to produce significant recall antigen proliferative responses, but did not fully protect against homologous challenge (Harpin et al., 1999). DNA vaccines co-expressing E2 and either IL-2 or GM-CSF showed promise in the mouse model (Nobiron et al., 2001), and conferred protection to challenge in cattle (Nobiron, et al., 2003). With the goal of fetal protection in mind, a multivalent baculovirus-vectored subunit vaccine possessing E2 proteins from three antigenically different strains of BVDV only partially protected ovine fetuses, but significantly reduced viremia in the ewes (Bruschke et al., 1999). In line with the successes of DNA vaccination, microparticle-bound BVDV RNA administered via gene gun elicited protective humoral responses to type 1 and 2 BVDV in cattle (Vassilev et al., 2001).

The incomplete protection observed during heterologous challenge following E2 subunit vaccination may be, in part, due to the fact that the E2 protein is the most variable protein within the BVDV polypeptides, confounding the observation of broad neutralization of several strains by some E2 containing vaccines. Furthermore, experiments with a vesicular stomatitis virus (VSV) vectored E2 vaccine suggested the expression of the dimeric form of E2 may generate more relevant antibodies with tenfold higher affinity to native E2 than the monomeric form (Grigera et al., 2000). The fact that cytokine adjuvancy, especially inclusion of IL-2 in conjunction

with DNA vaccination provides better protection may be attributable to its ability to stimulate clonal expansion of cells responsible for both cellular and humoral responses (Nobiron et al., 2000).

The importance of the cell-mediated immune response (CMI) is emphasized by the strong correlation of disease severity with degree of lymphopenia. It may be surmised that depletion of T cells of the thymus, spleen, and lymphoid tissue also corresponds to disease progression and severity. *In vivo* depletion of specific T cell subsets demonstrated the importance of $CD4^+$ T cells in the controlling the level and duration of viremia (Howard et al., 1992). Additional support for the role of CMI in controlling BVDV infection is provided by the finding that PBMC from seropositive, but not seronegative cattle exhibited a proliferative response to live, but not heat-inactivated BVDV (Larsson and Fossum, 1992). Moreover, T cell-mediated immune responses were purported to be responsible for protection against virulent challenge without seroconversion in calves that were exposed to live virus, either MLV vaccine or challenge virus in the face of high circulating maternal antibody (Endsley et al., 2003). This phenomena might extend to later in life as as it was shown that a MLV BVDV vaccine, administered once at day 0 did not induce a serological response on vaccination, but did induce a solid anamnestic response following vaccination with the same vaccine at day 140 (Fulton et al., 1995).

Closer examinations of the cell-mediated immune responses after BVDV exposure have focused on a few key players of the immune system. Whereas $CD4^+$, but not $CD8^+$ T cells were shown to be indispensable for

reduction of viral shedding during the acute phase of infection (Howard et al., 1992), generation of memory cells of both subsets was shown to occur, as antigen-specific cytotoxic lymphocyte ($CD8^+$ CTL) activity (Beer et al., 1997) and mixed T_H1/T_H2 responses were observed in PBMC cultures of convalescent- phase cattle (Rhodes et al., 1999). The latter study documented proliferation and cytokine profiles indicative of T_H2 and T_H1 biases in purified $CD4^+$ and $CD8^+$ T cells, respectively. The $CD4^+$ response was characterized in detail and shown to be MHC II-restricted, recognizing several cross-reactive determinants (Collen and Morrison, 2000). A study of the $CD4^+$ responsiveness (lymphoproliferation) of persistently infected cattle to peptides demonstrated immunotolerance at the level of the CMI response (Collen et al., 2000). The determinants most immunogenic were derived from the NS3 and the conserved regions of E2 proteins (Collen et al, 2002). That soluble (i.e. exogenous) recombinant NS3 elicited a proliferative response in inoculated cattle PBMC cultures supports the MHC II restriction model (Lambot et al., 1997). IFN-γ and IL-4 mRNA production and BVDV-specific proliferation were shown to be higher in seropositive cattle irrespective of pregnancy status (Waldvogel et al., 2000). Glew and Howard (2001) used BVDV-infected monocytes isolated from a persistently infected calf to demonstrate that both $CD4^+$ and $CD8^+$ T cells are activated in seropositive, but not naïve calves, indicating that both arms of the adaptive immune response are employed in response to BVDV exposure. Additionally, several peptide regions exist in the viral polyprotein that could serve as candidates for presentation on bovine MHC I alleles (BoLA-A11, -A20, -HD1,

and -HD6) that appear to correspond to the C, E^{rns}, E2, and NS2-3 regions (Hegde and Srikumaran, 1996; Chase et al., 2004). Taken in total, this information suggests that memory $CD4^+$ T cells have a helper function in mounting the humoral response required to reduce systemic viral spread during the acute phase of infection, but that both $CD4^+$ and $CD8^+$ memory is established, and respond with expansion of both T_H2 and T_H1 activity upon secondary exposure.

Striking a balance between T_H2 and T_H1 responses has been the goal of several vaccine development strategies. The role of E2 in driving a humoral response is well documented, but several studies have highlighted the CMI response to E2-based vaccines. The cellular immune responses induced by the fowlpox-vectored E2 construct were characterized by high output of IFN-γ by mouse PBMC stimulated with NADL strain of BVDV1 (Elahi, et al., 1999a). The recombinant human adenovirus-5 E2 vaccine primed mouse PBMC to proliferate in response to the NADL strain; the IFN-γ response to NADL was similar to that promoted by the fowlpox recombinant (Elahi et al., 1999c). These vaccines essentially establish a low-grade viral infection that mimics the events of a bona fide BVDV infection, with presentation of viral peptides in MHC I encoded in the construct. As with most intracellular infections, the classic T_H1 response predominates.

DNA vaccines customarily induce both humoral and cellular immunity by making newly synthesized antigen accessible in MHC I and II contexts. Naked DNA encoding E2 was shown to stimulate bovine PBMC to proliferate in response to the Singer strain of BVDV1 (Harpin et al., 1999). The

liposomal DNA encoding E2 used in this study generated a strong humoral, but no proliferative responses. The animal most protected from challenge in this study produced high antibody titers in conjunction with CMI responses, underscoring the need for a balanced response. Use of T_H1-biased cytokine constructs (IL-2 or GM-CSF) enhanced the cellular responses to E2-encoding DNA vaccines as evidenced by the production of neutralizing IgG2a isotype antibody typically associated with complement-mediated killing of virally infected cells (Nobiron et al., 2001). Splenocytes from vaccinated mice demonstrated antigen-specific proliferation. This approach produced desirable results in cattle, where lymphoproliferation was enhanced and the incidence of viremia in vaccinates was half of that of the control group (Nobiron et al., 2003). As with the mouse model, GM-CSF was the most relevant cytokine. A striking difference from the mouse model was the lack of augmented neutralizing antibody production associated with cytokine adjuvancy, and the predominance of the IgG1 isotype in the antibody pool.

An adenovirus-vectored BVDV nucleocapsid vaccine has been tested in mice (Elahi et al., 1999d). Although the antibody response was non-neutralizing, PBMC proliferation and strong IFN-γ in response to both type1 and 2 BVDV was observed. No IL-4 or IL-2 was detected. The same vector carrying the NS3 gene was used to stimulate murine PBMC with BVDV types 1 and 2 (Elahi et al., 1999e). Type 1, NADL strain elicited a very strong IFN-γ response, while stimulation with type 2, 125 strain demonstrated moderate IFN-γ expression. No IL-4 was detected, nor was proliferation observed after stimulation with either genotype. Previously, the same target protein was

used in a Semliki Forest virus vector with similar results (Reddy et al., 1999). Antibody produced was non-neutralizing, but proliferative and cytotoxic responses were demonstrated upon stimulation with heat-killed BVDV strains NADL and NY-1.

The emerging trend seems to indicate that subunit vaccines based on the highly variable, but highly immunogenic E2 protein elicit the best humoral responses, but those targeting highly conserved proteins, such as NS3, provide better cross-protective cellular immune responses. This may be the basis of the success of MLV BVDV vaccines in conferring protection against heterologous challenge. Cortese (et al., 1998b) demonstrated that a MLV type 1 BVDV vaccine protected calves from virulent type 2 challenge, but they were unable to assess the aspect of the response correlating best with protection. PBMCs from vaccinated cattle (and cattle seropositive at vaccination) did not differ greatly in terms of proliferation or IFN-γ secretion when compared with the unvaccinated calves. Although heat-inactivated virus was used to stimulate cultures for these assays, target cells infected with live virus were used in CTL assays, which similarly showed no difference in response between vaccinates and unvaccinated controls. This study exemplifies the difficulty in determining which aspect of the overall immune response best correlates with reduction of adverse clinical outcomes of BVDV infection.

CHAPTER 3

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Chapter 4

A COMPARISON OF SEVERAL *IN VITRO* METHODS TO ASSESS THE IMMUNE RESPONSE TO BOVINE VIRAL DIARRHEA VIRUS (BVDV) CONTAINING VACCINES IN CATTLE

1. Abstract

Current paradigm dictates that inactivated vaccines, bacterins, and toxoids generate a humoral immune response characterized by secretion from T helper cells of T_H2 cytokines, including IL-4, among other cytokines. The same paradigm asserts that live virus vaccines (MLV) establish a limited infection at the site of deposition that mimics natural infection and elicits an appropriate cell-mediated immune response. T_H1 cytokines are most influencial in the development of a cell-mediated immune response, including IFN-γ. Commercially available BVDV vaccines come in both inactivated (killed) and MLV forms. If the paradigm holds for BVDV vaccination of naïve cattle, the predominance of T helper lymphocytes will express either IL-4 or IFN-γ after inactivated vaccine- or MLV-immunized cattle, respectively.

The study undertaken as reported here employed a method of detecting cytokine production in PBMC cultures isolated from cattle vaccinated/boosted with MLV BVDV (MLV/MLV), killed BVDV (killed/killed), or both (MLV/killed) upon *in vitro* re-stimulation with one of three strains of live reference BVDV. Both vaccines contained the type 1 genotype BVDV. Staphylococcal enterotoxin B (SEB) was used as a positive control stimulant,

and culture media was used as a negative control. PBMCs were isolated at primary vaccination, and days 7, 14, 21, 28, and 49. On day 3 post-*in vitro* stimulation, cells were fixed and permeabilized, and stained by direct immunofluorescence using anti-IL4- and anti-IFN-γ-phycoerythrin (PE) conjugates. Flow cytometric analysis was used to determine the frequency of cytokine-producing (orange fluorescent) cells. Parallel re-stimulated cultures were established to determine the level of IL-4 and IFN-λ mRNA transcription, and antigen-specific proliferation (by tritiated thymidine incorporation). Additionally, serum neutralization (SN) assays were conducted on the weekly serum samples.

The results of the SN assessment showed time-dependent increases in antibody titer in the groups vaccinated with MLV vaccine. The titers of the group vaccinated with only killed vaccine were significantly lower at the termination of the experiment. Antibody response was biotype-dependent, with higher titers to BVDV 1 than BVDV 2 virus. Assays of IL4 and IFN-γ mRNA transcription and translation, as well as proliferation generated results that were variable in profile, and did not discriminate among the vaccine applications used. The results suggest that either immune responses induced by vaccination with BVDV-containing vaccines do not conform to the classical T_H1/T_H2 paradigm, or some aspect of the assays did not permit discrimination of cytokine induction and expression.

INDEX WORDS: BVDV, MLV vaccine, inactivated vaccine, immune response, cytokine mRNA, IL-4, IFN-γ, flow

cytometry, RT-PCR, blastogenesis, serum neutralization, virus neutralization.

2. Introduction

Bovine Viral Diarrhea Virus is a complex pathogen of ruminants worldwide. Its pathogenicity is related to its broad tissue tropism in the infected animal, induction of damaging host responses, and probably a direct mechanism of virulence. Measures used to attempt to control and eradicate BVDV have included management practices to curb spread and eliminate persistently infected animals from susceptible herds (Kelling et al., 2000), and 50 years of vaccination. The increasing frequency of cattle with elevated antibody titers to BVDV suggests exposure to field strains has not been diminished despite 50 years of vaccination programs (Chase et al., 2003). This suggests that current vaccines may be insufficient or inadequate in conferring sufficient herd protection, or effective fetal protection.

Design of more efficacious vaccines must be based on information on the types of immune responses that confer effective protection. While serological responses are the easiest to monitor, they may misrepresent the overall functional immune responses induced by vaccination or field exposure. This was recently exemplified by Endsley et al (2004) when they demonstrated that exposure of calves to BVDV in the face of high maternal antibody, induced no detectable serological response, but led to priming for protection from virulent challenge after colostrum-derived antibody had waned. They were able to assess the activation of the cell-mediated immune

response and correlate it to protection of neonatal calves. A more complete evaluation of the overall immune response requires methods allowing measurement of innate and adaptive effector mechanisms, proliferation, activation markers, and cytokine profiles.

T cell cytokine profiles are indicative of one of two major branches of the cellular immune response; either a T_H1 or T_H2 type response is usually mounted in response to vaccination, experimental challenge or natural field exposure. Cytokines predominating in a T_H2 response are IL-4, IL-5, IL-10, and IL-13. Those important in a T_H1 response are IL-12 and IFN-γ. While these responses are not completely mutually exclusive, the bias can determine the progression or outcome of disease.

Peptide-specific $CD4^+$ T cells express IFN-γ upon ligation of the T cell receptor (TCR) with exogenous-peptide-bound MHC II molecules on antigen presenting cells (APC), such as dendritic cells or macrophages. IFN-γ serves to increase expression of MHC II molecules on other monocytes, macrophages, and vascular endothelial cells. IFN-γ also induces expression of MHC I molecules, and several key players in the endocytic proteosomal pathway. It also activates macrophages for enhanced microbial killing, and induces immunoglobulin class switch to IgG2a.

IL-4 performs a similar function in B cells, i.e. up-regulation of MHC II molecules, upregulation of immunoglobulin gene transcription, and translation of pro-proliferative cytokines. Its pair cytokine in the T_H2 cascade, IL-10, antagonizes the actions of IFN-γ. A combination of T_H2 cytokines, including IL-4 and IL-5, causes immunoglobulin class switch from

IgG to IgE or IgA. More importantly, they promote the expansion and differentiation of T_H2 subset of $CD4^+$ T cells.

It is clear that tailoring a vaccine to thwart certain pathogens requires a fundamental knowledge of the immune responses that are most effective in pathogen clearance, as well as the nature of the immune response elicited by a specific vaccine. Such an endeavor requires proof of concept, such that measures of immune response are correlated with protection from virulent challenge. This study represents the first step towards developing an assessment strategy to be employed in challenge studies. The objectives of the study outlined here are to: 1) to compare various measures of CMI to assess trends in response to various BVDV-containing vaccines, and 2) compare the measures of CMI with serological data. The measures of immune responses addressed in the current study may one day be routine components of the composite picture of the global immune response to vaccination.

3. Materials and Methods

3.1 Animals, Vaccines and Study Design

Thirty (30) Angus-cross breed calves between 6 and 9 months old were maintained in open fields at the University of Georgia beef herd teaching facility (Rose Creek Farm) prior to vaccination. Forage was available for grazing and water was provided *ad libitum*. The animals had been screened for anti-BVDV 1 and 2 antibodies to ensure all animals used in

the study had undetectable levels of anti-BVDV antibody. Calves were identified with a numbered ear tag.

The vaccine groups were randomly arranged as follows:

MLV/MLV	MLV/Killed	Killed/Killed	Unvaccinated Controls	Contact Sentinels
4 steers 2 heifers	3 steers 3 heifers	3 steers 3 heifers	2 steers 4 heifers	6 calves unknown sex

The vaccines used were patterned after typical commercially available vaccines. The MLV vaccines consisted of lyophilized BVDV (NADL strain), parainfluenza virus, type 3 (PI-3), and infectious bovine rhinotracheitis virus or bovine herpesvirus, type 1 (IBR or BHV-1). This vaccine required reconstitution with sufficient sterile H_2O diluent to render the titer of the BVDV fraction at 5.0 $\log_{10}$ $TCID_{50}$/dose. Calves received 2 ml of this vaccine subcutaneously. The killed BVDV vaccine consisted of an inactivated preparation of Singer strain (liquid) adjuvanted with Carbopol used to reconstitute the MLV PI-3 and IBR lyophilized cake. Calves in this group also received 2 ml subcutaneously. Unvaccinated control calves were vaccinated with the H_2O-reconstituted PI-3/IBR containing vaccine only. Animals received vaccine at days –1/0 (V1) and 13/14 (V2) of the study.

Shortly after the primary vaccination, these animals were moved to two open paddocks approximately 1 hectare in size, where they remained for the duration of the study. To prevent exposure of controls and killed vaccine recipients to shed vaccinal BVDV of the MLV group, animals were divided

between the two paddocks based on the vaccine administered; those receiving MLV BVDV vaccine were kept separate from unvaccinated controls and calves receiving the killed BVDV vaccine. Sentinels commingled with the killed and control groups. Upon booster vaccination, those receiving the killed vaccine (MLV/killed) were allowed to commingle with the killed/killed group, unvaccinated controls and sentinels. This was permissible based upon the assumption that calves of the MLV/killed group had ceased to shed vaccinal virus subsequent to the primary vaccination. The two paddocks had sufficient distance between them to ensure no nose-to-nose contact could be made between divided groups.

3.2 Samples Collected for *In Vitro* Assays

To maintain the workload at a manageable level, sampling and sample processing were split into two sessions (consecutive days) each week. On the first day of primary vaccination, the first twelve animals through the chute were systematically assigned to “Group A”, with three representatives for each vaccine group. Group A was cattle vaccinated on day –1; Group B calves were vaccinated on day 0. The order of sampling of the two groups was alternated each week during the course of the study.

Animals were restrained in a manual head gate, and bled via the jugular vein. Approximately 50 ml of veinous blood was drawn into a 60-ml syringe containing 1 ml of porcine heparin solution (1% v/v in phosphate-buffered saline, or PBS), and mixed to prevent clotting. Additionally, two vacutainer “red top tubes” of blood were collected for serum. The blood

samples were maintained cool, and were transferred to the lab as soon as sampling was completed.

Blood collected for serum was centrifuged and the serum harvested. Serum was heated to inactivate complement by immersion in a hot water bath at 60°C for 30 min. It was aliquoted, labeled, and stored at -20°C until SN assays could be performed on all samples at one time.

The heparinized blood was used for isolatation of PBMCs. The whole blood was centrifuged at 3000 rpm for 20 min. The buffy coat was aspirated and added to 40 ml of 1% heparin in PBS in a sterile, 50-ml conical centrifuge tube. This was layered over 10 ml Histopaque 1083, and centrifuged for 45 min at 3000 rpm. The layer of PBMCs was aspirated and rinsed in 10 ml of PBS. Following centrifugation (15 min at 3000 rpm), the supernatant was decanted and the cells suspended in PBS. A cell count/viability assessment (trypan blue exclusion) was performed, and the cell suspension adjusted to 6 X 10^6 viable cells/ml in RPMI supplemented with 2 mM L-glutamine, 2 mM sodium pyruvate, 50µg/ml gentamicin, and 5% (v/v) equine serum (HS), hereafter referred to as "complete RPMI". These components are essential as L-glutamine has been shown to enhance proliferative and cytokine responses *in vitro* (Chang, et al., 1999), and HS contains no non-specific BVDV inhibitors or anti-pestivirus antibody. This suspension was used to seed wells of 96-well and 48-well, flat-bottomed, tissue culture-treated plates at 100 µl/well and 200 µl/well, respectively.

Viral stocks of BVDV NADL (cp type 1, $TCID_{50}$/ml = 6.80 $\log_{10}$), Singer (cp type 1, $TCID_{50}$/ml =7.15 $\log_{10}$) and 890 (ncp type 2, $FAID_{50}$/ml =7. 40

log_{10}) were diluted 1:20, and added to appropriate wells of the 96-well and 48-well plates at 100 μl/well and 200 μl/well, respectively. Unstimulated control cells received no virus; rather, the same volume of complete RPMI was added. SEB-stimulated control cells received the same volume per well of an SEB solution (0.2μg/ml in complete RPMI). SEB is known to stimulate both $CD4^+$ and $CD8^+$ T cells with equal proportion in an MHC II- and Vβ-dependent fashion (Sjogren, 1991). The cultures, which were used for mRNA RT-PCR, intracellular cytokine staining, and proliferation assays were placed in a humidified incubator maintained at 37°C, 5% CO_2 tension.

3.3 Intracellular cytokine staining

Preliminary work performed by Dr. A. Reber (Food Animal Health and Management Program, University of Georgia) established optimum parameters for intracellular cytokine staining (ICS). To summarize, he compared stimulation with Concanavalin A (ConA) or SEB, for 24 hr or 48 hrs, blocking exocytosis with either Brefeldin A or monensin, and anti-IFN:FITC and anti-IL-4:PE dilutions of 1:200, 1:100, 1:50, 1:25, and 1:12.5. His analysis resulted in the conclusion that the best results were achieved by stimulating the cells for 3 days with SEB, blocking exocytosis with monensisn 6 hrs pre-fixation, and use of anti-cytokine fluorescent conjugates at 1:25 dilution. Additionally, to reduce “noise” inherent in two-color flow cytometry, the decision was made to reduce the voltage of the unused photomultiplier tube (PMT) to zero. Prior to the initiation of the study, both conjugates used were switched to PE conjugates.

According to the optimized protocol, on day 3 post-stimulation, at 6 hr pre-fixation, monensin was added to each well of the 48-well plates to achieve a final concentration of 2μM. This reagent has been shown to block exocytic function of the Golgi apparatus (Tartakoff, 1983). The next step involved centrifugation at 2000 rpm for 10 min, removal of the spent media, vortexing (to disrupt the pellet), and suspension in 200μl of PBS. The cells were then transferred to duplicate wells of non-tissue culture treated, U-bottom 96-well plates and centrifuged. Following gentle decanting of the supernatant, the cell pellet was resuspended in 100μl /well of 2% (w/v) paraformaldehyde (PFA) in PBS. Following storage at 4°C, the PFA was decanted and the pellets washed with PBS as before, the fixed cells disruptively vortexed, and suspended in 200μl /well of PBS with 0.5% (w/v) bovine serum albumin (BSA) and 0.1% (w/v) NaN_3 (PBA). The following day, the second set of samples was processed identically.

Cells were stained using the commercially available staining kit, Leucoperm (cat# BUF09B, Serotec, Oxford, UK). On weeks 1 and 2, samples were centrifuged to remove PBA, and rinsed with PBS as before. Pellets were resuspended in 50μl /well of Leucoperm-fixation reagent A and incubated for 15 min at room temperature.

On week 3, a change in fixation and storage method was implemented. Although the Leucoperm reagent A does not disclose its ingredients for proprietary reasons, it was obvious from the odor that it contained PFA. Therefore, we concluded that fixation with the in-house fixative was unnecessary and proceeded to fix the cells only with Leucoperm

reagent A. Regardless of the number of fixations, the cells were washed twice as before with PBA. After decanting the wash solution and disruptively vortexing, 50µl /well of each direct conjugate diluted 1:25 in Leucoperm Permeabilization Reagent B was added to one of the duplicate wells derived from each stimulation treatment for each animal. The cells incubated with conjugate for 45 min, at room temperature, in the dark. The cells were washed three times thrice with PBA, disruptively aspirated, and transferred to 12 X 75 mm plastic test tubes containing 200µl /tube of PBA. The samples were held a 4°C until the flow cytometric analysis. Flow cytometric analysis was performed the following day using EPICS XL-MCL flow cytometer, (Coulter Cytometry), with the discriminator set to exclude debris from the analysis. Gating on forward scatter was established to include monocytes and lymphocytes in the analysis. When possible, at least 10,000 events were acquired for each sample. Analysis of data was performed using the FlowJo software, version 4.5. Statistical analysis was performed by week with each treatment group compared to the control group using Dunnet's multiple mean comparison (significance accepted at $p<0.05$).

3.4 Blastogenesis

Parallel cultures, set up as described above for flow cytometry were used for antigen-specific proliferation assays. PBMCs were stimulated in 96 well plates (6 X 10^5 cells/well) for 5 days. Six hours prior to harvesting, wells were pulsed with ^{3}[H]-thymidine (0.2 µCi/well). Cells were lysed after the 6-hour incubation with distilled water and the double stranded nucleic

acid harvested onto glass fiber filters. Cellular proliferation was determined by measurement of the incorporation of ^{3}[H]-thymidine into cellular DNA using a scintillation counter, and expressed as a percent of incorpation in SEB stimulated cells. Analysis was performed by week against the control group using Dunnet's multiple mean comparison (significance accepted at $p<0.05$).

3.5 Cytokine mRNA by RT-PCR

PBMCs were stimulated in 96-well plates ($6X10^5$ cells/well) with for 3 days, after which cells were suspended in RNAlater (Ambion) and stored at –20° C for later analysis. Upon analysis, cells were thawed and total RNA was isolated with Absolutely RNA 96 Microprep Kit (Stratagene) and quantified with RiboGreen RNA quatitation reagent (Molecular Probes). RNAs were reverse transcribed into cDNA with the cDNA Archive Kit (ABI) at 25°C for 10 min and then 37° C for 2 hr. Real-time PCR was performed for IL-4, IL-10, IL-12, IFN-γ and 18S rRNA (internal reference) with self-designed, bovine specific primers and TaqMan probes. All the reaction components were added using a HydraAW (Robbins) into 384-well plates and each sample was prepared in triplicate. The reaction was 1x master mix without AmpErase UNG, 900 mM of each primer, 250 nM of TaqMan probe and cDNA corresponding to 1 ng of total RNA in a 10 ml reaction volume. PCR cycling and detection was performed in ABI 9600HT. The reaction cycle was 50° C for 2 min, 90° C for 10 min and 50 cycles of 90° C for 10 sec and 60° C for 1 min. The data was collected and analyzed with SDS 2.0 (ABI). The threshold was set manually for each gene. Ct values were normalized against 18S in

each cell treatment and expressed relative to unstimulated controls. Analysis was performed by week against the control group using Tukey-Kramer multiple mean comparison (significance accepted at $p<0.05$).

3.6 Serum Neutralization Assay

Heat-inactivated serum (and plasma on days 6 and 7 post-V1) were assigned random numbers for blinding. The sera were loaded into row A of tissue culture-treated 96-well plates at 100µl/well. Minimum Essential Media (MEM) containing 30 µg/ml gentamicin was loaded into all other wells at 50µl/well, and into all wells of two plates designated for back-titration for each challenge virus. The test and control sera were serially diluted twofold generating a range of dilutions from undiluted to 1:128. Control sera were BVDV antibody positive FBS (positive control), reference BVDV-negative sera (negative control), and HS (internal control). The challenge viruses, NADL, Singer, and 890 strains were diluted to target a $TCID_{50}$/50µl of 125 (the GMT of 50 – 300 $TCID_{50}$/50µl). To all test plates, NADL, Singer, and 890 suspensions were added to columns 1-3, 5-7, and 9-11, respectively at 50µl/well. MEM was added to columns 4, 8, and 12 at 50µl/well, representing serum cytotoxicity controls. The serum / virus interaction was allowed to proceed at room temperature for at least 30 min. In the back-titration plates, 50µl/well of virus was added to column 1, and serially diluted twofold from 1:2 to 1:1024 (2^1 to 2^{10}). Two columns were left uninoculated as cell controls. To all wells of all plates, 100µl/well of an MDBK cell suspension (40,000 cells/ml) in MEM supplemented with 10% (v/v) HS were

added. The plates were incubated for 5 days (NADL and Singer), and 7 days (890) at 37°C, 5% CO_2 tension.

On day 5 plates were examined microscopically and the plate lids scored over wells exhibiting CPE. The plates were replaced in the incubator until day 7, at which time the plates were fixed with ice-cold 85% (v/v) acetone, and incubated at –20°C for 10 min. The acetone was removed and the plates were allowed to air-dry. Direct fluorescent antibody conjugate (FA, American BioResearch, Inc., Seymour TN, USA), diluted 1:50 in PBA was added at 50µl/well. This was allowed to incubate in the dark at room temperature for 30 min. The plates were rinsed three times and examined with an FA microscope for typical apple-green fluorescence characteristic of FITC. The plate lids were scored over wells exhibiting positive fluorescence. Antibody titers were calculated using the Spearman-Karber method of titer determination. Analysis was performed by week against the control group using Dunnet's multiple mean comparison (significance accepted at $p<0.05$).

4. Results

4.1 Study Events

All animals were determined to be seronegative prior to and at V1. Sentinel calves were not bled and tested for BVDV neutralizing antibodies until the end of the study. All were seronegative, inferring that exposure to extraneous or vaccine-virus shed BVDV was not a confounding factor. Although injection sites were not palpated to detect reactions, no gross lesions were obvious at injection sites.

Bleedings at days –1/0, 6/7, 13/14, and 20/21 were uneventful, except for an occasional escape and retrieval. However, the bleeding at week 5 was postponed as many of the calves exhibited clinical signs of BRD. Depression, mucopurulent discharge, and coughing were observed. The decision was made to treat the animals for bacterial respiratory disease and bleed at a later date. The justification for this was related to the prediction that the calves would be responding to pathogens with high cytokine expression, resulting in high background and reduction of discrimination in our assays. The attending veterinarian, Dr. D. Ensley treated the cattle shortly thereafter with a combination of Micotil™ (6 ml/animal) and also with Ivomec™, as the calves had never been treated with an antihelminthic. On days 48/49 (week 8), the clinical signs of respiratory disease in the calves had resolved, and the final bleeding was done.

On day 6 post-V1, a decision was made to reduce the workload by using plasma, a byproduct of the PBMC isolation that was usually discarded, as SN samples. Thus, on day 6, no clotting-activated bleeding was performed. Upon heat-inactivation, a cloudy precipitate formed. The precipitate, presumeably BSA was centrifuged out, and the plasma collected and aliquoted. This aroused concerns that such manipulations might perturb the antibody content and activity. Therefore, we reverted to using serum. To detect any differences in centrifuged plasma versus serum, both samples were processed on day 7 using the blood of the calves sampled that day. SN results were inconclusive toward that end in that both sample types were negative for BVDV antibody.

4.2 Serum Neutralization Results

The $TCID_{50}$/50µl for each challenge virus was 652, 218, and 90 for NADL, Singer, and 890 strains, respectively. Although 652 is higher than targeted, the positive control serum titer was close to the expected value. Therefore, that portion of the assay was considered valid. The $TCID_{50}$/50µl for the other viruses was within the targeted range. The results of the humoral response to BVDV NADL, Singer, and 890 strains are depicted in Figures 1, 2 and 3. The results serve to demonstrate that the vaccines were functionally immunogenic.

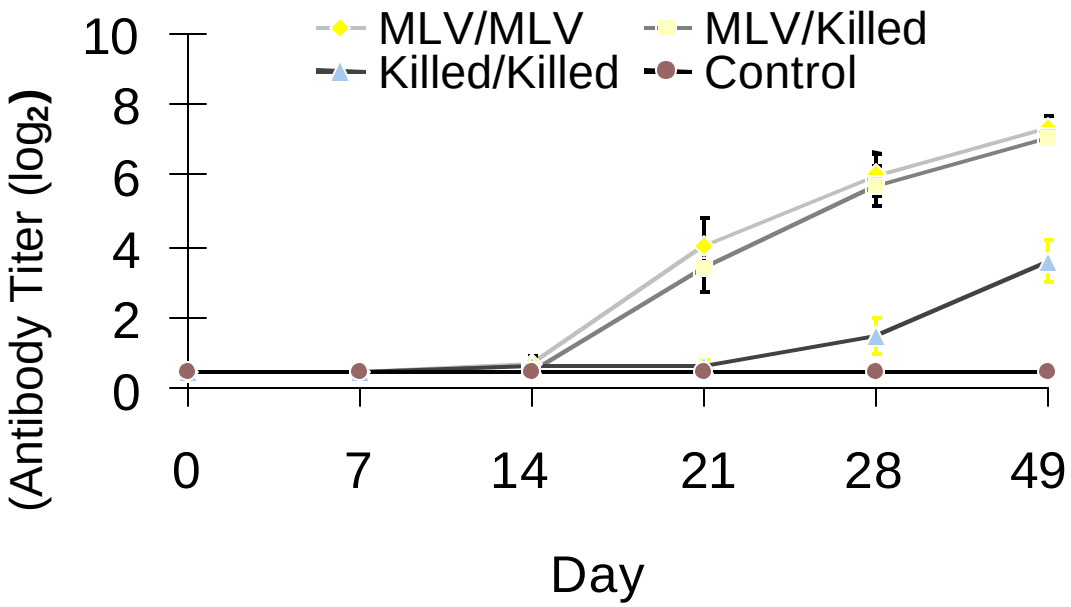

Figure 1. Serum Neutralizing Antibody Titers to NADL Strain

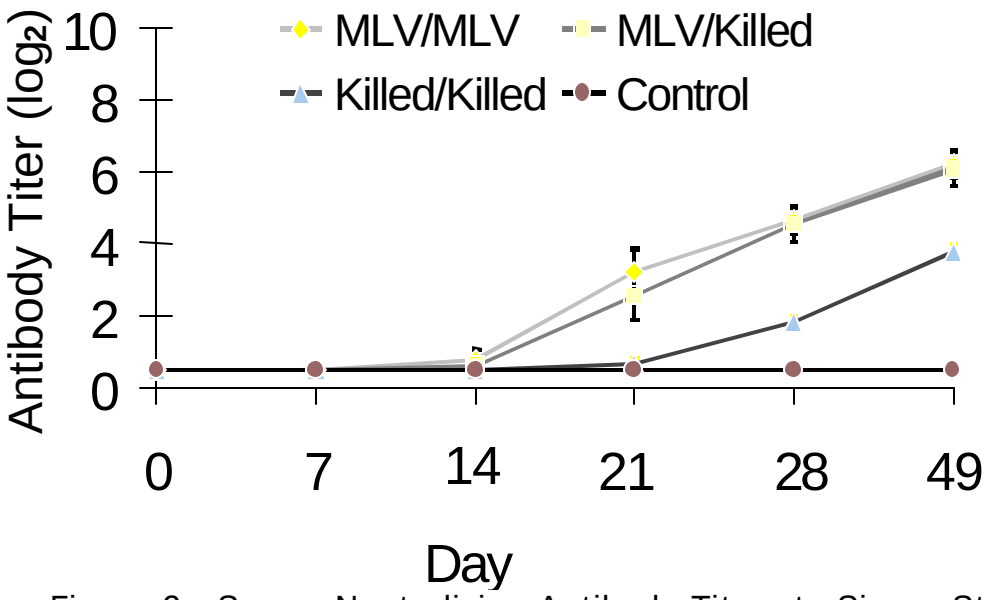

Figure 2. Serum Neutralizing Antibody Titers to Singer Strain

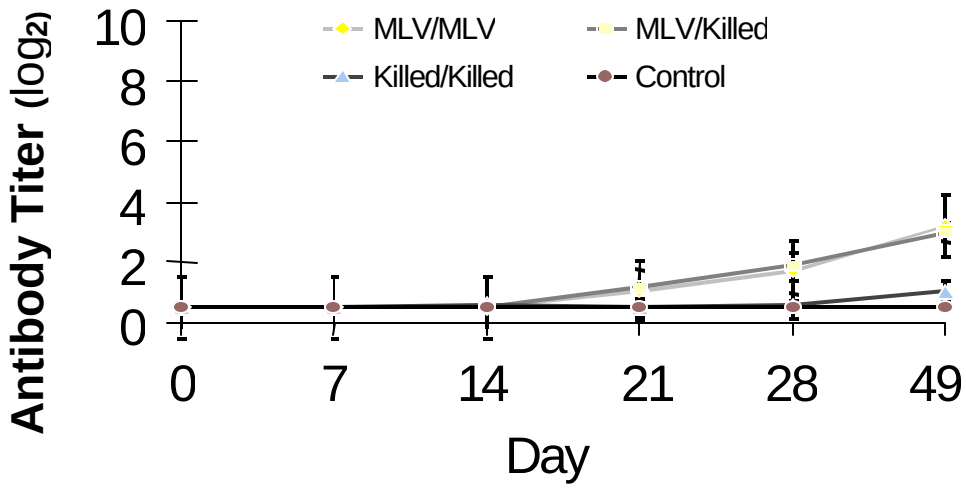

Figure 3. Serum Neutralizing Antibody Titers to 890 Strain

4.3 Intracellular Cytokine Staining

The prolonged period of time required for analysis of week 1 samples resulted in poor sample quality and the data was excluded from analysis. The intracellular cytokine profile did not parallel the profile of the humoral response for any of the stimulating viruses or cytokines. No discernible

trends of cytokine expression in response to recall antigen were evident. There was no significant difference in intracellular cytokine expression between treatment groups at any time. However, while not significant, a consistent finding was the higher level of cytokine production at week 8 in the MLV/MLV group relative to the other groups. The results are depicted in Figures 4, 5, 6, 7, 8, and 9.

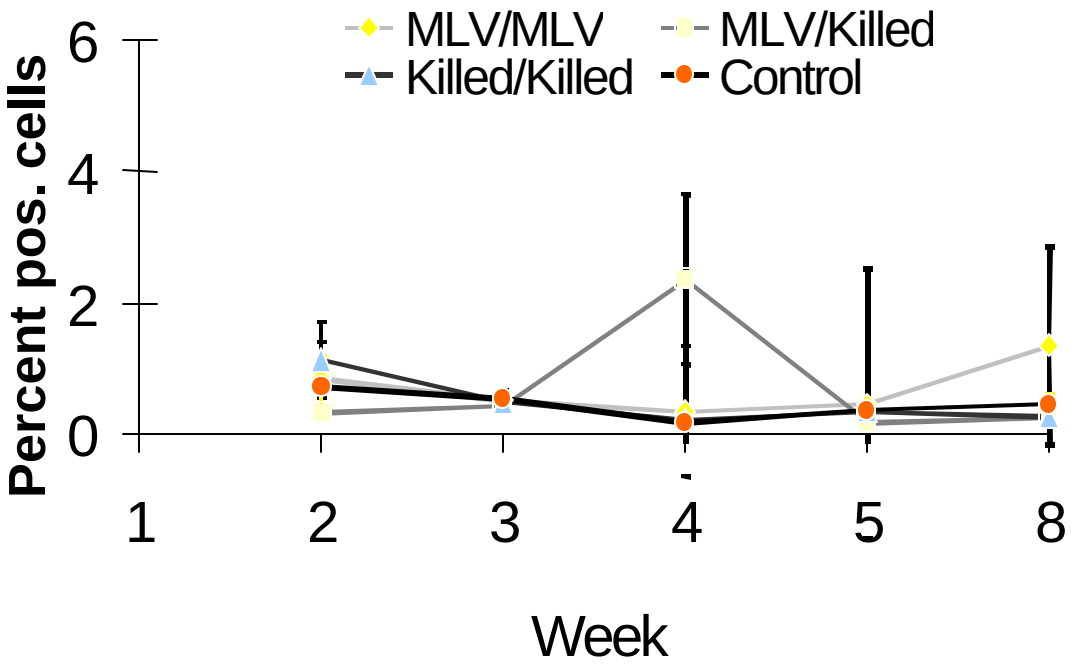

Figure 4. Frequency of Intracellular IL-4 Positive Cells in Response to NADL Strain

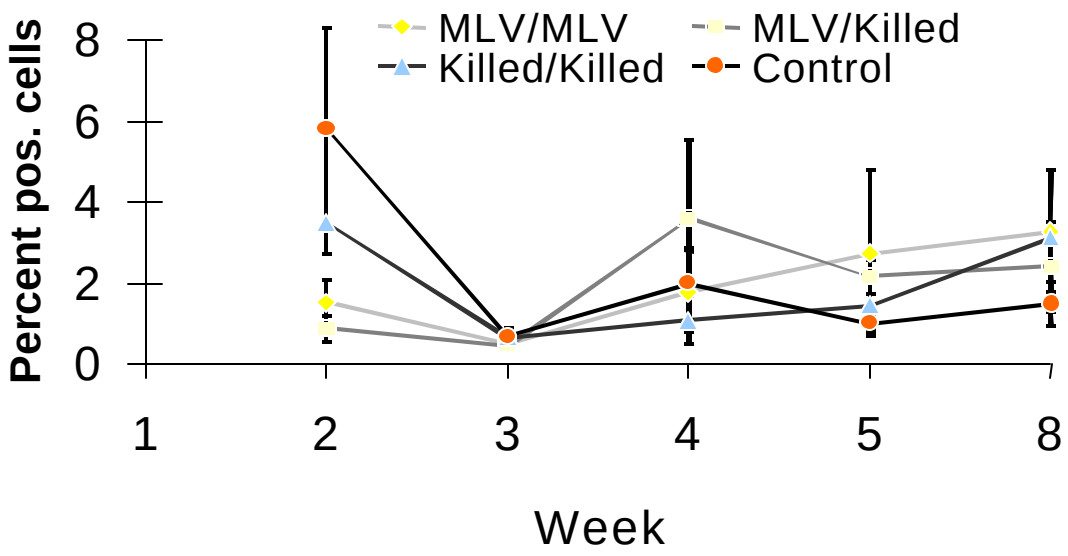

Figure 5. Frequency of Intracellular IL-4 Positive Cells in Response to Singer Strain

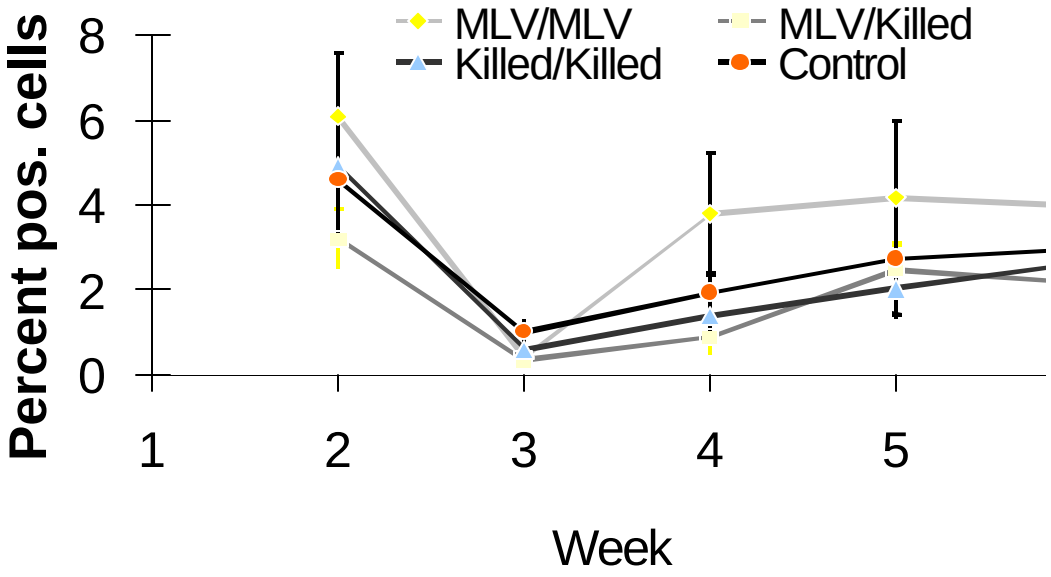

Figure 6. Frequency of Intracellular IL-4 Positive Cells in Response to 890 Strain

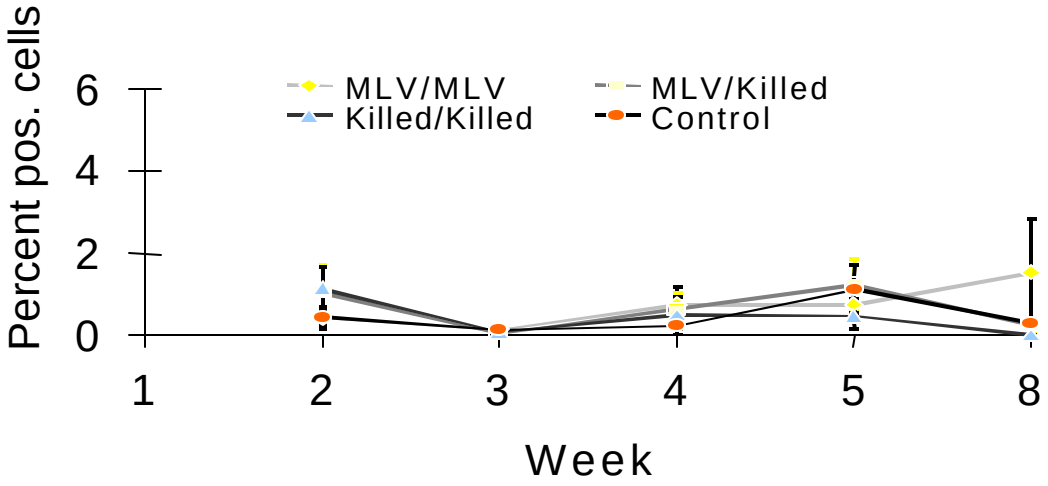

Figure 7. Frequency of Intracellular IFN-γ Positive Cells in Response NADL Strain

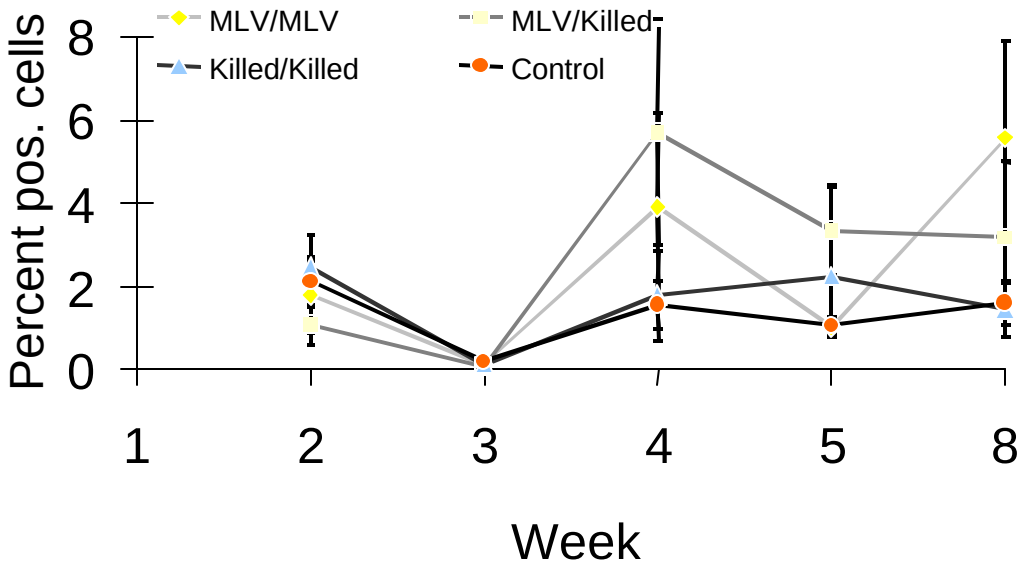

Figure 8. Frequency of Intracelluarl IFN-γ Positive Cells in Response to Singer Strain

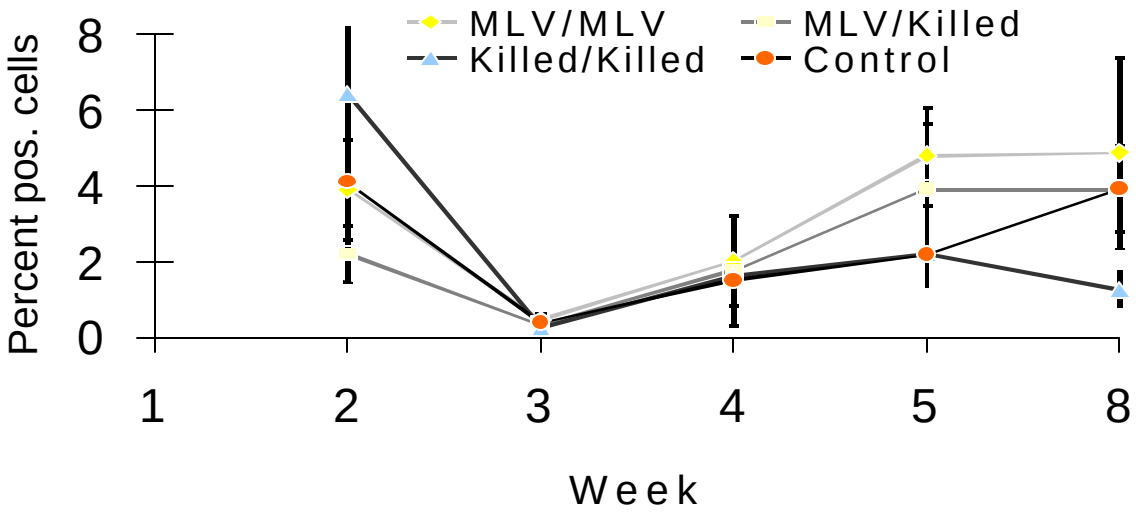

Figure 9. Frequency of Intracellular IFN-γ Positive Cells in Response to 890 Strain.

4.4 Cytokine mRNA

Figures 10, 11, and 12 present the data generated by the IL-4 mRNA in RT-PCR assay for NADL, Singer, and 890 stimulation, respectively. The mRNA profiles for IFN-γ are presented in Figures 13, 14, and 15. There were no significant differences in the level of IL-4 or IFN-γ mRNA detected in response to the viruses. Transcription was consistently the lowest, albeit not significantly, for the PBMCs stimulated with the NADL strain at all time points.

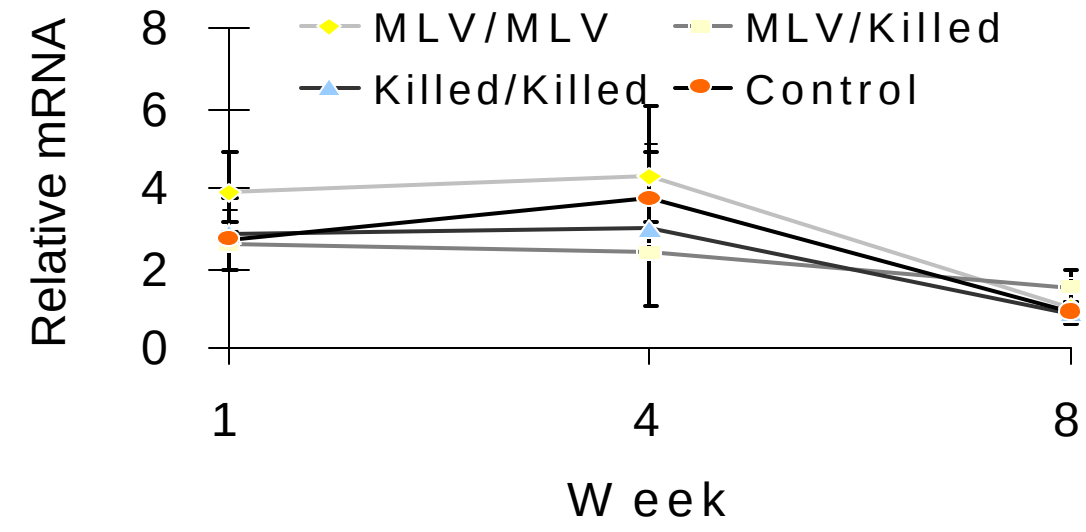

Figure 10. Relative IL-4 mRNA in Response to NADL Strain

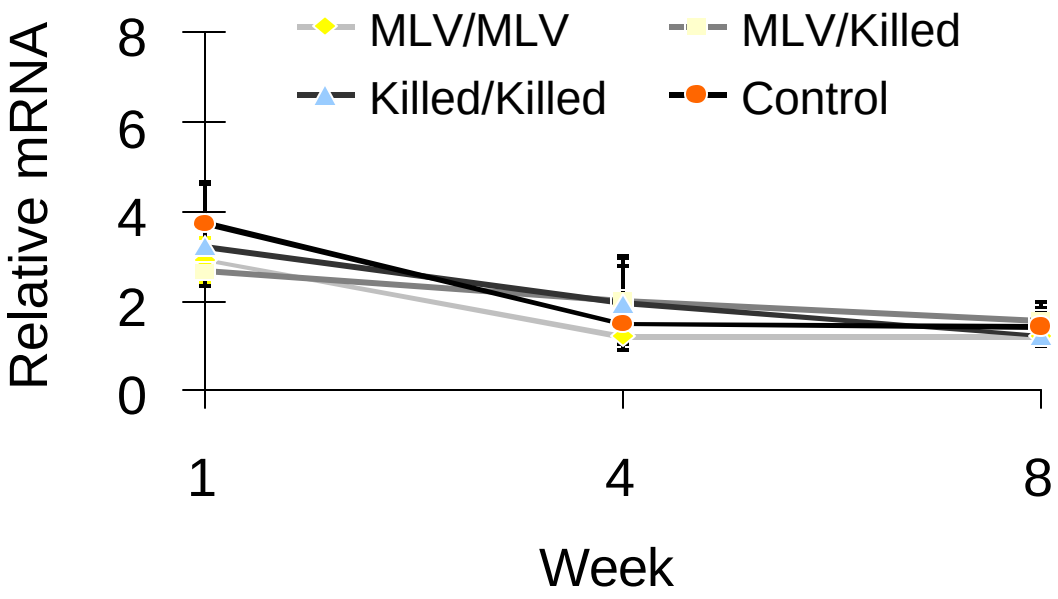

Figure 11. Relative IL-4 mRNA in Response to Singer Strain

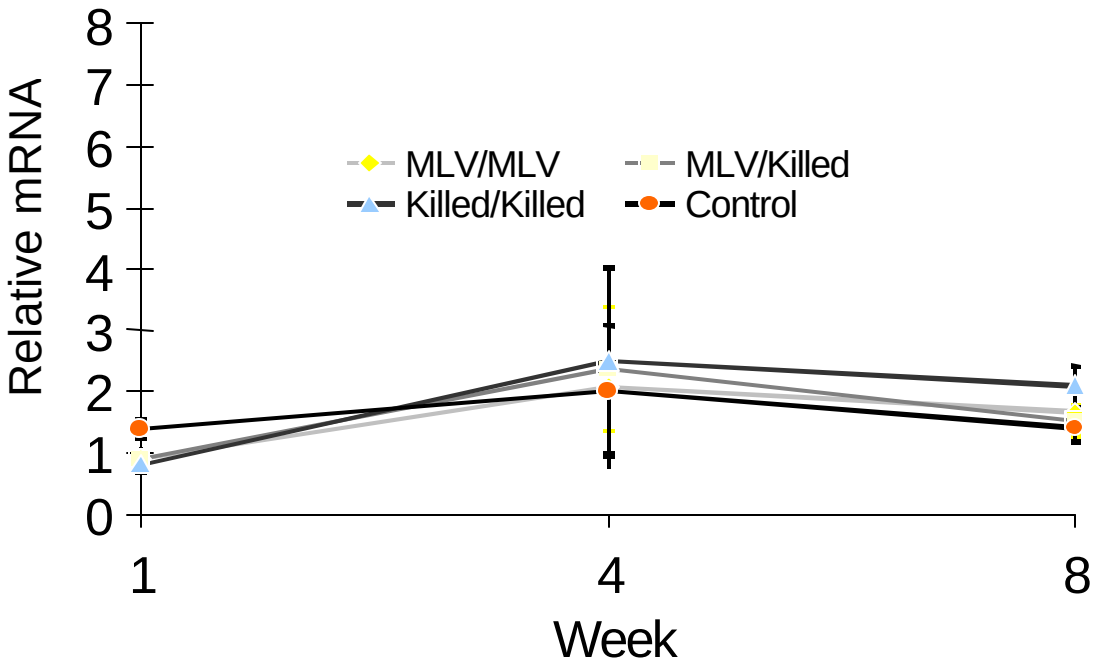

Figure 12. Relative IL-4 mRNA in Response to 890 Strain

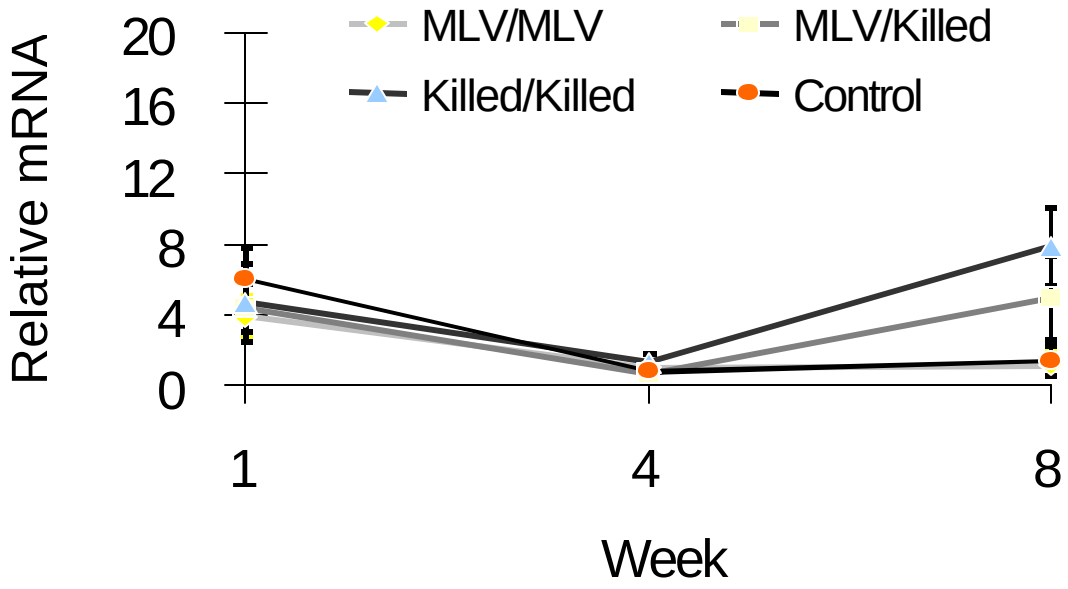

Figure 13. Relative IFN-γ mRNA in Response to NADL Strain

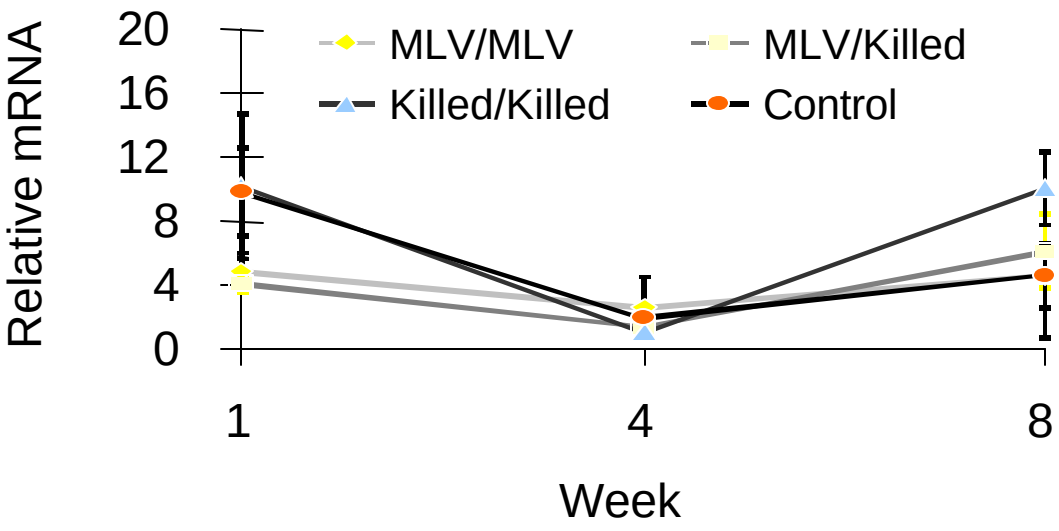

Figure 14. Relative IFN-γ mRNA in Response to Singer Strain

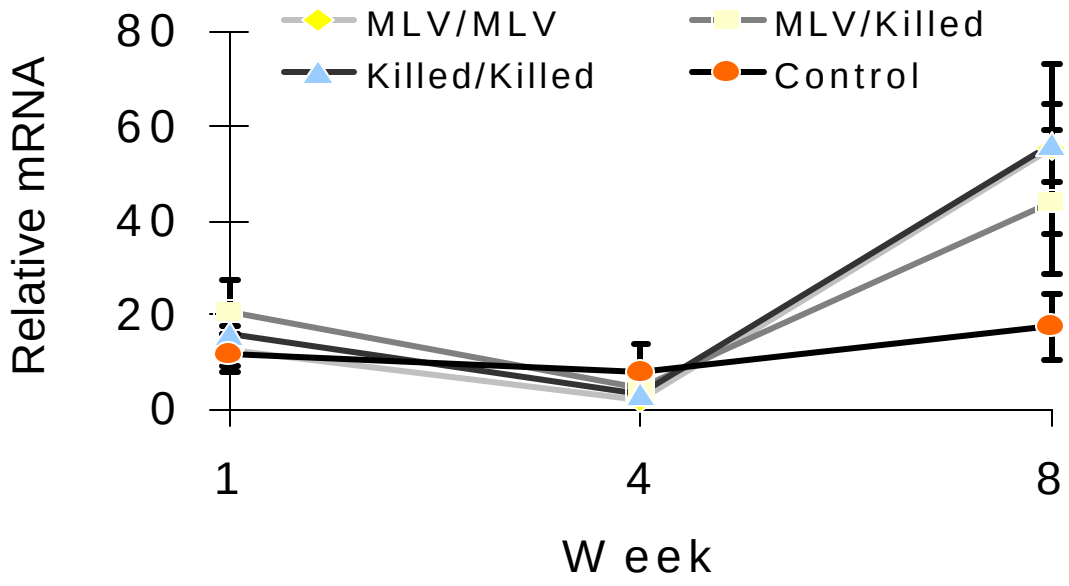

Figure 15. Relative IFN-γ mRNA in Response to 890 Strain

4.5 Proliferation

Proliferation as measured by [^{3}H]-thymidine incorporation is depicted in Figures 16, 17, 18, 19, 20 and 21. With the exception of the proliferative response to NADL stimulation at week 8, the PBMCs showed no significant increases as a result of exposure to the viruses used. PBMCs collected at week 8 from calves primed with MLV, when stimulated with the NADL strain demonstrated significant increases in proliferation relative to the control-vaccinated animals (Figure 17).

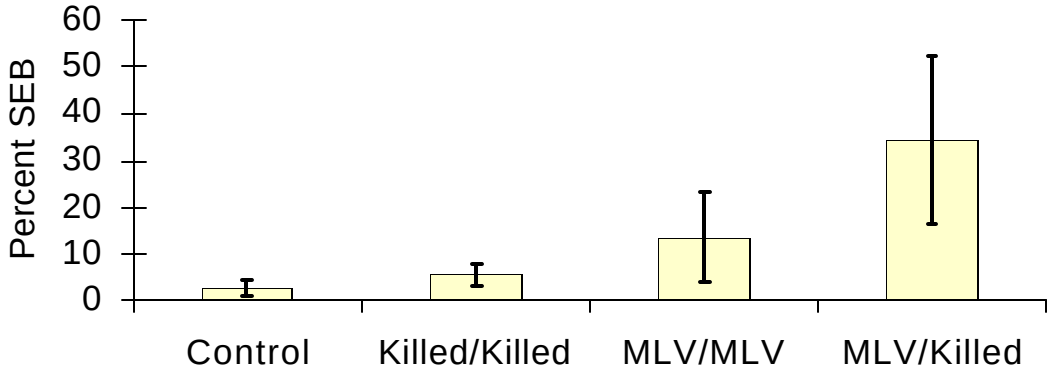

Figure 16. Proliferative Response to NADL Strain at Week 5 post-V1

(expressed as percent of SEB stimulation)

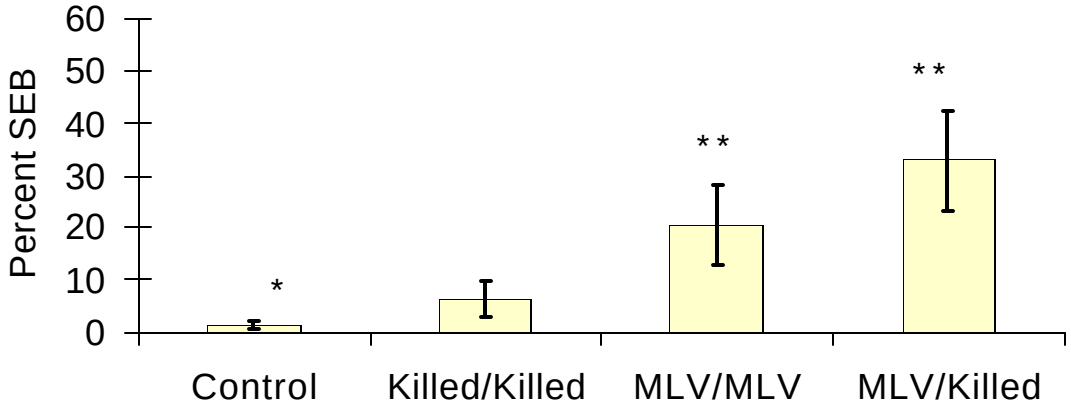

Figure 17. Proliferative Response to NADL Strain at Week 8 post-V1)

(expressed as percent of SEB stimulation)

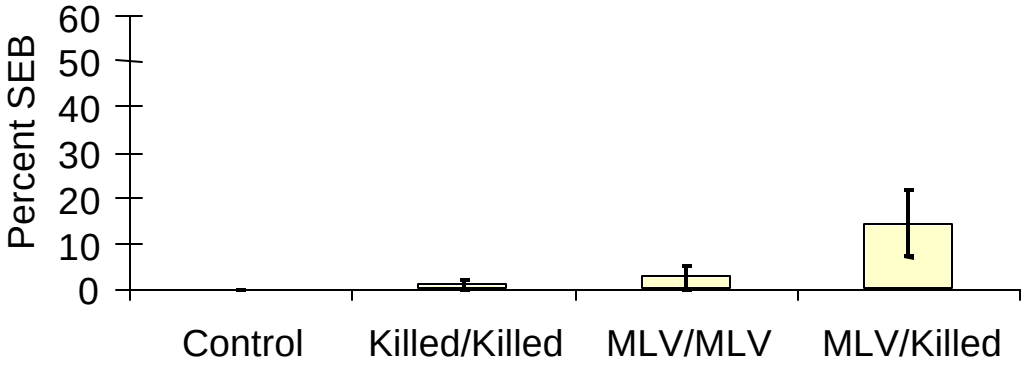

Figure 18. Proliferative Response to Singer Strain at Week 5 post-V1

(expressed as percent of SEB stimulation)

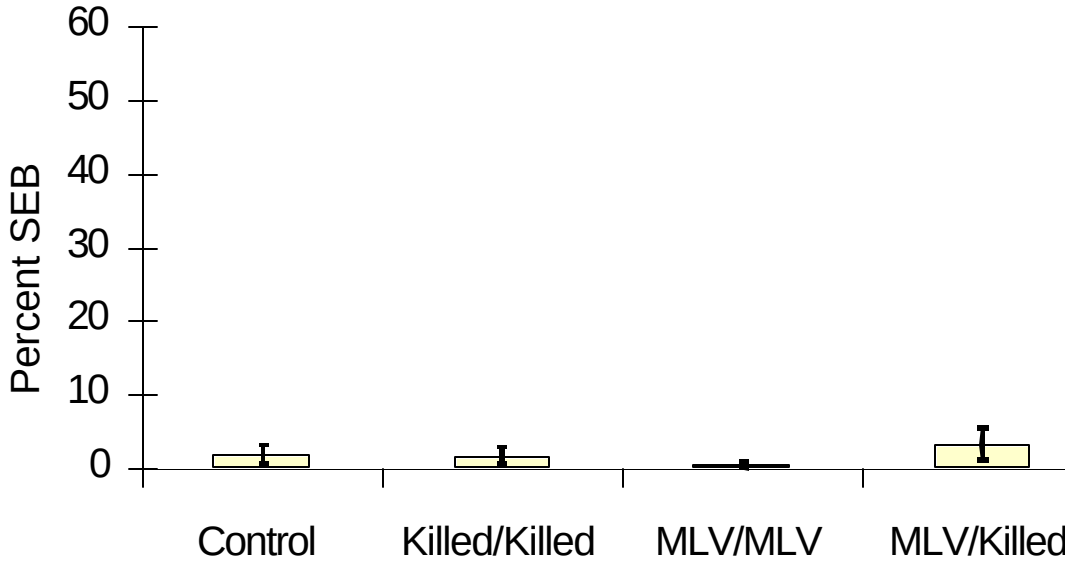

Figure 19. Proliferative Response to Singer Strain at Week 8 post-V1

(expressed as percent of SEB stimulation)

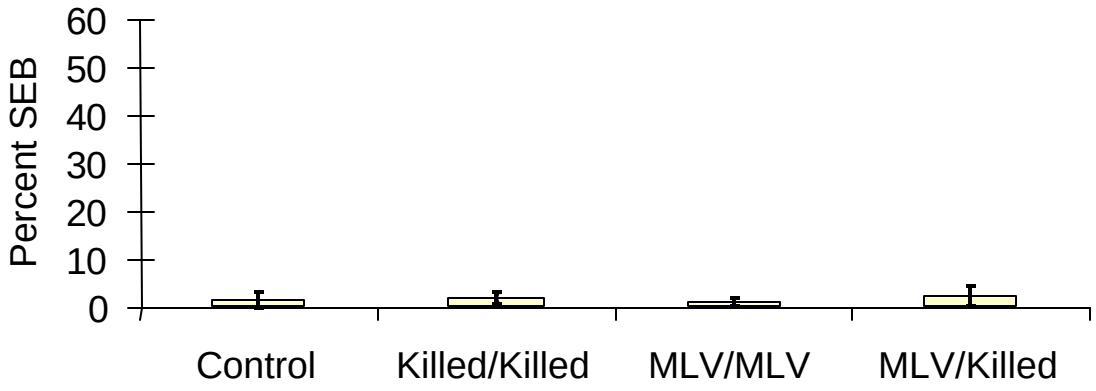

Figure 20. Proliferative Response to 890 Strain at Week 5 post-V1

(expressed as percent of SEB stimulation)

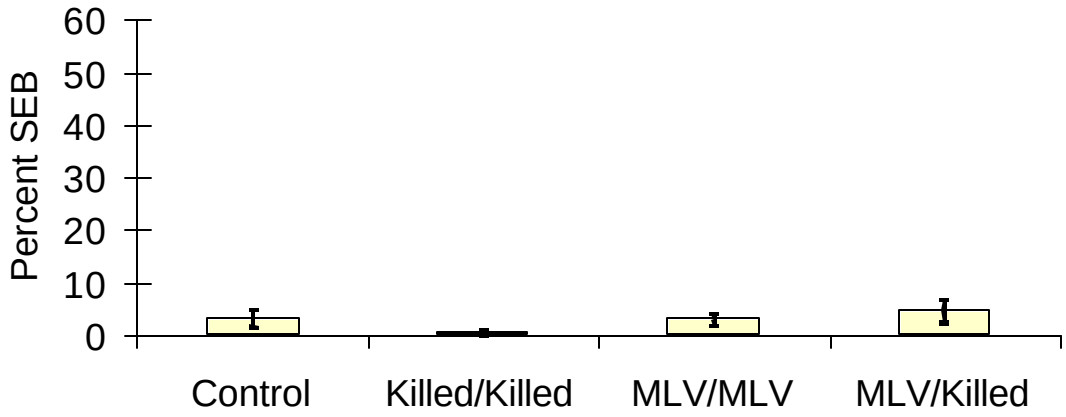

Figure 21. Proliferative Response to 890 Strain at Week 8 post-V1

(expressed as percent of SEB stimulation)

5. **Discussion**

As is evidenced by the apparently stochastic responses of PBMCs to vaccination, little correlation in CMI response profiles is evident from this set of data. With the exception of proliferative responses to NADL stimulation, there did not appear to be a pattern of cellular response to vaccination; responses were equal to or higher than pre-vaccination values and were not significantly higher than responses of non-BVDV-vaccinated controls. Furthermore, the correlation between IL-4 expression profiles and serological results was weak.

The uncertainty of the results presented here is compounded by the question: Are the assays not sensitive enough to distinguish between vaccinates and controls in terms of cytokine production and proliferation, or are the cattle responses accurately reflected in the data, but are highly variable? An alternative question might be directed toward the use of a stimulating agent (BVD virus) that infects and potentially perturbs the assay respondents. Stated differently, did culture of PBMCs of vaccinated and control animals with infectious virus alter the cytokine profile by virtue of intracellular inhibition of protein synthesis or activation signals?

Because the SN data conforms to expected results, the quality of the vaccine cannot be brought into question, nor the can the method and route of administration. Intramuscular and subcutaneous routes are equivalent, at least in terms of protection (Fairbanks et al., 2003). That the titers to BVDV 2 were lower than those of the homologous biotype was also expected

(Fulton and Burge, 2001). The data suggests that with respect to serology, the strain used to prime elicits the predominant humoral response.

However, the morbidity that was evident midway through the sampling may very well have contributed to the variation in responses observed. Such variation could be attributable to the variable degree of respiratory disease from animal to animal. Simultaneous sampling of over-responders and the occasional healthy non-responder could render any assay of immune responses non-discriminating.

Of the data generated by this study, the week 8 responses may be the most credible. The level of cytokines observed across groups may have been influenced by the response to the pathogens associated with the respiratory disease that developed up to week 5. As the calves had not been treated with anthelmintic, a high intestinal parasite load may have elevated the baseline and non-vaccinated control IL-4 levels. The resulting high background levels of IFN-γ and IL-4 may have reduced the discrimination of the BVDV-specific response. By week 8, these factors should have been minimized by the treatment for bacterial infection and intestinal parasites, thereby permitting the cytokine response to return to baseline levels. Such conclusions are difficult to draw because neither the nature of the ailment the calves experienced as observed at day 34, nor the internal parasite load at the initiation of the study were well documented or diagnosed.

One common observation of this study was the low levels of cytokine detected at week 3. As the volume of samples processed was high, and the “fresh” integrity of the samples was paramount, flow cytometric analysis of

all the study samples in one session was not possible; PBMCs had to be analyzed immediately subsequent to treatment and staining. This restriction deprived us of analyzing the cytokine production of samples collected across all study dates under the same test conditions. Therefore, the results obtained at day 20/21 may have been unique to that session; i.e. the unusually low values observed may have been due to some error in sample handling and not truly representative of the immune response of the cattle. In retrospect, it would have been desirable to process PBMCs of the sentinel (non-vaccinated) group. This would have allowed us to determine if the low responses were artifactual or simply a return to baseline response following primary vaccination. One would expect that the non-BVDV-vaccinated animals would have exhibited an equal degree of non-responsiveness following stimulation with BVDV. Additionally, the nature of the observed response of all groups could be better characterized if the "bystander" effect of the IBR/PI3 MLV vaccination could be teased apart from the BVDV vaccination-specific response. Although unlikely, that the observed reactivity of all groups could be a result of cross-reactivity at the T cell level (Selin et al., 1994.) cannot be entirely ruled out. This assessment could also have been achieved by analyzing the sentinel PBMCs in parallel with the vaccinates.

Disparity in response profiles is reported in the literature. For example, close examination of SEB stimulation detected temporal discrepancy in cytokine production and proliferation as measured by Bromodeoxyuridine (BrdU) incorporation (Mehta and Maino, 1997). Cytokine

production and proliferation did not occur simultaneously. The use of an experimental BVDV NS3 vaccine induced a strong IFN-γ response in the absence of proliferation (Elahi, et al., 1999). Furthermore, flow cytometric analysis of IL-10 correlated poorly with ELISA results in a study of immune responses to HCV infection and progression of disease (Sobue et al., 2001).

The question of the appropriateness of employing a test system to measure immune responses that results in perturbation of the participants by virtue of the stimulation process should be addressed in light of the experimental results generated here. Our model is fundamentally different than most employed in research in that the techniques described here don't involve polyclonal stimulation of the cells with, for example, PMA/ionomycin, PHA, or α-CD3 Mab. Because, as with the assays described herein, the cells (monocytes and lymphocytes) are productively infected and are not induced to proliferate with mitogens makes this assay more reflective of the *in vivo* model. Rhodes et al., (1999), Collen and Morrison (2000) and Larsson and Fossum (1992) all recognized the difficulty in observing proliferative responses without the use of live virus for stimulation. The experiment most similar to the assays we performed employed IL-2 as a means of effector cell expansion for up to 25 days (Beer, et al., 1997). BVDV is well known to infect macrophages and spread via the haematogenous route to secondary lymphoid tissues; infection of blood-derived monocytes is representative. Monocytes are APCs, so presentation by MHC class I and II are involved in cells infected with ncp BVDV (Glew and Howard, 2001).

Additionally, cells infected with cp BVDV undergo apoptosis. This results in an externalization of phosphatidylserine (PS), normally sequestered on the inner leaflet of the cell membrane. Phagocytes possess receptors for PS and act to bind and engulf apoptotic cells. This serves to clear infected cells, limit the toxic aspects of cell lysis following necrosis, and potentially present viral antigens derived from the phagocytosed cell in the context of MHC II molecules.

The most significant finding of this study was the proliferative response evoked by the NADL strain of PBMCs isolated from calves vaccinated with MLV (NADL) and boosted with killed BVDV vaccine. The proliferation induced in PBMCs isolated from vaccinated animals upon in vitro stimulation with NADL, but not a type 2 BVDV (125) has been reported (Elahi et al., 1999). Neither elicited IL-4 production. Endsley (et al., 2002) noted reduced CD25 expression in cells isolated from BVD 1 vaccinates when exposed to BVD 2 *in vitro*. A proliferative response to the Singer strain was noted in DNA-vaccinated calves, but the type 2 used to stimulate failed to do the same (Harpin et al., 1999). Why the MLV-boosted group did not show equivalent response in not clear. Nor is the response elicited by the Singer strain. It may be that the response is strain-specific, but immunodominant determinants of Singer are not homologous enough to be recognized by NADL-primed lymphocytes.

Total lack of responsiveness has also been reported. Cortese (et al., 1998) found no significant difference in unvaccinated and vaccinated (with NADL strain) calves' PBMCs in terms of proliferative response and IFN-γ

secretion. However, live virus was not used as a stimulating agent in blastogenesis assay, rather the virus was heat-inactivated. Larsson and Fossum (1992) also heat-inactivated the virus used in stimulation assays, but the treatment incompletely inactivated the virus, as it retained $10^{1.3}$ $TCID_{50}$/20μl. Use of heat-killed virus evoked an expansion of $CD4^+$ T cells (Collen and Morrison, 2000), so lack of proliferation based on the infectivity status of the stimulator is unclear.

The lack of proliferative response to BVDV 2 observed in this study is in agreement with previous research. This may be attributable to the lack of homology between the immunodominant component of the type 1 vaccinal BVDV (NADL) and the stimulating BVDV type 2 (890) epitopes. Alternatively, infection of the PBMCs *in vitro* with ncp BVDV might inhibit activation signals. Infection with a virulent ncp BVDV, as is strain 890, was shown to cause a decrease in surface expression of MHC II and B7, but not MHC I or B4 (Archambeault, et al., 2000). Using serial analysis of gene expression technology (SAGE), Neill and Ridpath (2003) documented several instances of gene downregulation in ncp BVDV2-infected MDBK cells, including α- and β-tubulins, cytochrome c, malate dehydrogenase, cytochrome oxidase VIIc1, and ADP/ATP tranlocase A2. Hence, a certain degree of non-responsiveness in terms of cytokine induction may be a result of viral infection of the test cells. However, these findings do not explain the lack of responsiveness to Singer strain, a cp type 1 strain that shows cytopathic effect intermediate between NADL and ncp 890 strains. It has been shown that although ncp and cp BVDV inhibit DNA synthesis of PBMCs, only cp

strains inhibited translation of proteins involved in metabolic activities other than activation signals (Hou et al., 1998). Other genes impacted by cp BVDV infection involve immune regulation, apoptosis, and signaling plasma membrane proteins (Risatti et al., 2003).

While one might suspect that the use of monensin would cause a transient reduction of MHC turnover (and viral budding), concentrations between 0.01 and 100μM were shown to reduce surface expression only slightly (Jung et al., 1993). Accordingly, a flow cytometric method of CTL activity of BVDV-exposed PBMCs demonstrated a 42% increase in MHC II expression (Beer et al., 1996). A similar model to the one employed in this study was used to examine BVDV–specific responses of T cells (Rhodes et al., 1999). In that model, ncp-infected monocytes were used to stimulate T cells for enhanced IFN-γ and IL-4 synthesis as measured by ELISA, as well as proliferation. The roles of $CD4^+$ and $CD8^+$ T cells were elaborated in a test system similar to the one described here (Collen, et al., 2002).

The differences in proliferative response to cp and ncp BVDV may be linked to the differenct kinetics of proliferation, as seen by Collen and Morrison (2000), in which blastogenesis occurred earlier with PBMCs from calves infected with cp biotype than the ncp (3-4 vs. 6-8 weeks, respectively). Also, lymphoproliferation was maximal 84 days post-exposure, but was suppressed prior to day 21. Similarly, the kinetics of cytokine production is uncoupled; stimulated human PBMCs demonstrate peak IFN-γ at 10-12 hrs, while IL-4 production is maximum at 4 hrs post-stimulation (Prussin and Metcalfe, 1995). Because IL-4 tends to peak and decline,

whereas IFN-γ levels remain elevated after induction, particular attention should be paid to the kinetics of the more limited window of IL-4.

In conclusion, assays very similar to our protocol have demonstrated functional immune responses to BVDV infection and vaccination, but they required mitogens. The inconclusiveness of our results, with the exception of the one lone significant response, may be a reflection of the high background of leukocytes from moribund animals, or that the assays used were not sensitive enough to identify responses in animals that were vaccinated but not challenged. It may also be that the assays required further optimization. Double-fixation and prolonged storage prior to flow cytometric analysis may have had deleterious effects on the signal and condition of the samples.

It would have been interesting to examine modifications of the test system in parallel to the established procedure. In particular, how would the response be characterized if infected cells and viruses were opsonized by antibody or complement? In light of dendritic cells' resistance to infection (Glew et al, 2003), how would the response be elaborated with DC as the predominant APC? Also, if viral interference is a possibility when using a high multiplicity of infection (MOI) as was used in this study, it would be more representative and relevant to infect cells with lower MOI and culture longer. The impact of combining Brefeldin A and monensin to permit accumulation of cytokines to enhance signal could be explored.

The use of SEB as a control was appropriate, especially as a positive control for IFN-γ responses. SEB alone induces mainly IFN-γ when used alone, but induces mainly IL-4 when IL-4 is added during primary stimulation

(Hoiden, et al., 1993). PMA with ionomycin, which is a good dual positive control, could have also been used.

The described shortcomings notwithstanding, the use of flow cytometric analysis to document immune responses to experimental vaccines could yet prove to be a valuable tool for future vaccine development. Its greatest utility, however, could be to characterize the type of immune response that best confers protection or amelioration of clinical disease in challenge studies or as a preface to vaccine development.

6. References

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