PATHOGENESIS OF LEUKOPENIA IN CALVES EXPERIMENTALLY INFECTED WITH NON-CYTOPATHIC TYPE II BOVINE VIRAL DIARRHEA VIRUS

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by
ROBERT DARREN WOOD

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This study is an investigation of the pathogenesis of the hematological abnormalities observed in calves experimentally infected with non-cytopathic (ncp) type II bovine viral diarrhea virus (BVDV). Calves were intranasally inoculated with a virulent strain, 24515, or a low virulence strain, 11Q. Calves inoculated with the virulent strain developed anorexia, diarrhea and respiratory signs. Calves inoculated with the low virulence strain exhibited no overt clinical signs. Fevers were significantly higher in 24515-inoculated calves. Serial peripheral blood and bone marrow samples were obtained before and after virus inoculation and monitored for changes in the circulating and bone marrow cell populations, respectively. Bone marrow samples were evaluated for the presence of viral antigen in hematopoietic cells with immunocytochemistry using the monoclonal antibody 15C5. Calves inoculated with both viruses developed leukopenia, neutropenia, lymphopenia and thrombocytopenia to varying degrees. White blood cell counts were significantly lower and persisted longer with the virulent 24515 strain. The bone marrow myeloid maturation pool decreased concurrently with development of leukopenia in animals inoculated with either virus but depletion persisted longer in 24515-inoculated animals. The bone
marrow proliferation pool increased in proportion to the decrease in the maturation pool in 11Q-inoculated calves, but took 4 days longer to increase in 24515-inoculated animals. Viral antigen in bone marrow cells was sparse and transient in 24515-inoculated animals but was present when peripheral blood counts were lowest. Viral antigen was never demonstrated in 11Q-inoculated calves. Megakaryocytes were the predominant cell type exhibiting positive staining. These experiments demonstrated that infection with ncp type II BVDV strains of varying virulence result in leukopenia. The virulent isolate caused a delay in the production of myeloid cells which could potentially compromise the ability of the host to satisfy tissue demands for neutrophils and contribute to secondary bacterial infections. Infection of myeloid precursor cells appeared to contribute to this delay. Ability to delay production of bone marrow myeloid cells and infection of megakaryocytes may enhance virulence, contributing to the ability of certain ncp type II BVDV strains to induce severe disease.
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CHAPTER 1

Literature Review and Study Objectives

1. Introduction

Examination of peripheral blood is commonly performed during evaluation of illness in cattle and is used frequently as part of the database that leads to discovery of the underlying process. Although specific diseases are rarely diagnosed solely using a complete blood count (CBC), viral diseases produce a wide range of alterations in blood parameters, which may suggest considering some diseases over others. In addition, the CBC often is helpful in distinguishing infectious from non-infectious disease.

Understanding the pathogenesis of specific alterations in blood parameters contributes to an understanding of the disease process and therefore improves ability to treat and offer prognosis. Serial observations are important when determining prognosis. Leukopenia can exacerbate the primary disease process by predisposing the animal to secondary infections. This can prolong recovery from disease and can contribute to ongoing losses from decreased production and fertility. Furthermore, predisposed by leukopenia, secondary infections may result in the death of an animal that might have recovered from the initial disease process.

Thrombocytopenia occurs frequently in viral diseases and if severe enough can result in hemorrhage. Severe hemorrhage obviously can contribute to the outcome of the disease. Anemia is uncommonly observed in most viral infections unless severe hemorrhage occurs. Furthermore, because of the 160 day mean lifespan of a bovine red blood cell, anemia due to infection of erythroid precursors is usually not appreciated during the course of an acute viral infection. Exceptions include the more chronic viral infections such as equine infectious anemia virus and B19 parvovirus of people.

Concurrent examination of a bone marrow sample can provide an explanation for alterations in peripheral blood parameters and may be useful in diagnosing disease. There may be
no changes, non-disease-specific reactive changes or disease-specific, i.e. diagnostic, abnormalities in the bone marrow. Most viral diseases induce non-disease-specific alterations in bone marrow cell populations.

2. Pathogenesis of Leukopenia

Leukopenia is a decrease in the circulating population of leukocytes, and may involve changes in the number of neutrophils or lymphocytes depending on the relative size of the two populations. Many mammalian viral diseases induce at least a transient leukopenia.

Lymphopenia can result from increased destruction of cells, decreased production of cells or redistribution of cells within and outside of vascular spaces. Lymphocytes (and other infected cells) can be destroyed as a result of cytotoxic products induced by viruses. Viruses may also directly invade lymphocytes, which may result in direct cytotoxicity. Alternatively, virus-infected cells may be eliminated by the immune system after recognition of altered surface molecules. This would be reflected as increased apoptosis.

Decreased production of lymphocytes in the lymphopoietic organs may occur as viruses infect progenitor cells, which do not develop into mature lymphocytes, or develop incorrectly and are removed.

Lymphocytes normally circulate between the primary and secondary lymphoid organs and the peripheral blood. Viral infection may cause a redistribution of these cells, such that fewer are in circulation because they are required either in the lymphoid organs or in other tissues as part of viral defense and elimination.

Lymphopenia from viral infection may be partially due to stress of disease, causing increased blood cortisol levels. Hypercortisolemia causes lymphopenia, due to lymphotoysis in blood and lymphoid tissues and increased margination or emigration of lymphocytes from
Alterations in the relative proportions of subsets of lymphocytes occur in several viral diseases. Neutropenia results from similar mechanisms. Cells can be destroyed directly or indirectly by viruses or their products resulting in decreased numbers in circulation. Neutrophils also can enter tissues or be lost to a body cavity if there is chemotactic demand. This results in a decreased transit time in the peripheral blood.

Decreased production of neutrophils may occur in the bone marrow, either as a direct result of viral infection of precursor cells, or by removal of these cells as a result of surface molecule alterations. Mature neutrophils themselves are rarely infected with virus. Neutrophils have a short lifespan and antimicrobial mechanisms that do not easily allow infection. Viral particles can be coated by antibody and phagocytosed as immune complexes by neutrophils and other cells.

Neutrophils can be redistributed, moving from the circulating pool to the marginated pool as demand in tissues or body cavities increases. This latter effect tends to be an acute change, as seen with endotoxicosis. Neutrophils leave the circulating pool and enter the marginal pool, sequestering in the microvasculature, especially in the lung.

Chemicals such as epinephrine and cortisol actually cause increases in circulating neutrophils and are not a cause of neutropenia. Neutrophilia due to epinephrine results from an increase in the circulating pool because of a decrease in the marginal pool. Excess exogenous or endogenous cortisol causes an increased mobilization of marrow granulocyte reserves to the peripheral blood, as well as decreased margination.

3. Neutropenia in Viral Infection

Neutrophils often are found in very early virus-induced lesions but are quickly replaced by mononuclear cells. Neutropenia is a common finding in viral diseases, and may be associated
with decreased production in the bone marrow. Cytologically, this is seen as myeloid hypoplasia.

Many viruses are cytocidal and cause lysis of the target cell via induction of cell membrane damage, and by altering protein synthesis, DNA replication and cytoskeletal properties.

Viruses can result in bone marrow failure by one or more of three mechanisms: 1) direct inhibition of or cytotoxicity to hematopoietic progenitor cells, 2) direct inhibition of or cytotoxicity to marrow stromal cells required for hematopoiesis, or, 3) stimulated production of cytokines or cytotoxic lymphocytes that inhibit the production of or destroy hematopoietic cells.

The first mechanism is demonstrated by the parvoviruses, especially feline panleukopenia virus, which are well known for their tropism for hematopoietic cells, and often result in decreases in circulating neutrophils and lymphocytes. The pathogenesis of feline panleukopenia involves direct infection and destruction of mature cells, and inhibition of bone marrow myeloid progenitors, resulting in marrow hypocellularity.

Infection with dengue virus results in bone marrow suppression by all three mechanisms. Dengue viral antigen is easily detected on the surface of infected myeloid cells. Therefore, these cells may be targeted as “innocent bystanders” in an attempt to eliminate virus. A study of dengue-2 infection in long-term bone marrow cultures showed infection of marrow stromal cells. This suggested that viral infection of cells of the hematopoietic inductive microenvironment might result in decreased production of the myeloid series, at least transiently. Furthermore, dengue-specific T-cells result in the release of bone marrow suppressive cytokines.
4. Special Considerations in the Evaluation of Bovine Hematology

Calves and other young ruminants are born with a neutrophil:lymphocyte (N:L) ratio similar to most other species, but by the fourth or fifth day of life, lymphocytes begin to predominate. However, in the mature animal, the blood N:L ratio is much lower than observed in many other domestic animals; lymphocytes outnumber neutrophils. As a result, severe neutropenia and left shifts can develop more quickly than in most other species. Therefore, severe neutropenia with a left shift does not imply the same severity of the inflammatory response in cattle as in other species.

Some authors suggest that bovine bone marrow has less marrow granulocyte reserve than other animals. This may explain why cattle quickly develop a left shift when there is a severe peripheral demand for neutrophils. In cattle, leukopenia that persists for more than 3 to 4 days indicates inadequate myelopoiesis to meet peripheral demand.

5. Thrombocytopenia in Viral Infection

Thrombocytopenia results from increased destruction or use of platelets, from decreased production, or by sequestration. Platelets can be consumed rapidly during disseminated intravascular coagulation (DIC) or because of immune-mediated destruction. Both these phenomena may occur secondary to viral infection. For example, platelet-associated IgG and IgM were demonstrated in foals infected with equine infectious anemia virus.

Bone marrow production of platelets may be decreased if viruses infect megakaryocytes, resulting in either cell death or ineffective thrombopoiesis. Splenic sequestration can result in lower numbers in the peripheral blood. Sequestration is rarely, if ever observed with viral
infection. As with the myeloid series, more than one mechanism may contribute to thrombocytopenia during viral infection.

6. Hematology of Flavivirus Infections

Flaviviruses are single-stranded, positive-sense RNA viruses.23 There are three genera in the family Flaviviridae; genus Flavivirus, genus Pestivirus and genus Hepacivirus.24 Dengue virus and yellow fever virus are important pathogenic flaviviruses. The only virus classified in the genus Hepacivirus is hepatitis C. The genus Pestivirus contains three viruses, each important in veterinary medicine: classical swine fever virus (hog cholera); border disease virus; and bovine viral diarrhea virus (BVDV). Infection of cells by viruses in the family Flaviviridae usually results in cytolysis.25 Non-cytopathic strains of BVDV are an exception, at least in vitro.

Hepatitis C virus of the genus Hepacivirus is associated with mild transient bone marrow suppression and rarely, aplastic anemia.26

In contrast, dengue fever virus uniformly produces thrombocytopenia and neutropenia in people.16,27 Mechanisms of hematosuppression include both direct viral cytolysis and immune-mediated destruction of progenitor cells, resulting in decreased production.27 During dengue hemorrhagic fever, hypocellularity of all bone marrow cell lines occurs from days 1-4 post-infection, with increasing cellularity from days 5-8, and a return to normal bone marrow cellularity after day 10.28 A lymphocytic infiltrate in the bone marrow, which likely represents immune system activation against virus infected cells, is also present from days 5-8 post-infection.

The primary cell targets of dengue virus are those of the monocyte-macrophage system, but other hematopoietic cells often also are infected.28 Four dengue serotypes exist that vary in their ability to infect different cell lines. For example, dengue 2 is much less efficient than dengue 4 at infecting erythroid cells, but infects cell lines of both the myelomonocytic and
lymphocytic lineages. Simultaneous suppression of both megakaryocytes and myeloid cells suggests that there is infection of a pluripotential stem cell, or concurrent infection of both lineages.

Alterations in stromal marrow cells and their secretions, in addition to direct viral cellular destruction, may contribute to hematosuppression. Furthermore, only low numbers of stromal cells may need to be infected to signal the entire marrow environment to decrease proliferation in an attempt to minimize infection of rapidly dividing cells by virus. This may be considered a protective mechanism from the perspective of limiting spread of virus and means that very quickly (3 to 5 days post-infection) production of hematopoietic cells can be down-regulated. As infected cells are eliminated by specific immune responses, the signals inhibiting production are reduced and a rebound in mitosis occurs.

7. Hematology of Pestivirus Infections

Pestiviruses are known for their ability to produce marked hematologic abnormalities. Classical swine fever virus (hog cholera) results in marked leukopenia and in thrombocytopenia which may be severe enough to result in overt hemorrhage. Border disease of sheep also may result in lymphopenia and neutropenia. The hematology associated with BVDV infection will be discussed in detail in the next sections.

In hog cholera and border disease, mechanisms for the development of some hematological abnormalities have been studied. Classical swine fever viral antigen can be demonstrated by immunohistochemistry in 2.5-9.0% of megakaryocytes in the bone marrow, along with morphological changes suggestive of necrosis. However, infection of megakaryocytes could not account for the severe thrombocytopenia induced by the disease, since overall megakaryocyte production in the bone marrow was unimpaired. Alterations in the myeloid series associated with classical swine fever virus infection were not assessed.
Lymphocyte subsets have been evaluated in sheep with border disease and in cattle with BVDV infection. The destination of lymphocytes leaving the peripheral blood during viral infection is still unclear. However, reductions in both T- and B-cells occurred during infection with both viruses. Early in infection, CD4+ cells may be diminished while CD8+ cells may also decreased or may remain relatively unchanged. Towards the end of active infection (10-12 days post-infection) CD4+ cells returned to pre-inoculation levels, while CD8+ cells increased significantly over pre-infection values. Neutrophils and lymphocytes may leave the circulation and migrate to sites of viral replication.

Pestiviruses have been documented to infect lymphocytes, which may be killed by natural killer cells or cytotoxic lymphocytes.

The kinetics of cell production during infection has not been closely examined. Characterization of the hematological and bone marrow kinetics of domestic animals with viral diseases is poorly understood.

8. Introduction to Bovine Viral Diarrhea Virus (BVDV)

Bovine viral diarrhea virus (BVDV) is a pestivirus in the family Flaviviridae. BVDV has recently assumed increased significance in the bovine industry due to outbreaks of a seemingly new form of the disease that began in 1993 in Ontario, Quebec and the Great Lakes States. The disease occurred in veal calves as well as in older animals and caused morbidity and mortality significant enough to result in alarming production losses.

BVDV has been classified into two biotypes, non-cytopathic (ncp) and cytopathic (cp), based on the effect in cultured cells. Cytopathic effect can range from minor changes in the cell structure to cell dysfunction, cell lysis or cell transformation. Cytopathogenicity in cultured cells has no bearing on the ability of BVDV to cause disease in an animal. Non-cytopathic strains typically have been associated with a subclinical self-limiting disease, but they also are
responsible for persistent infections and reproductive problems in infected cattle. Cytopathic strains, associated with mucosal disease, are considered to result by mutation from a non-cytopathic strain persistently infecting an animal. These strains have an additional protein, p80, generated by proteolysis of the larger non-structural protein, p125, permitting differentiation of biotypes by molecular methods.

More recently, BVDV has been divided into two genotypes, types I (a and b subtypes) and II, based on differences in the 5'-untranslated region of the genome. Type I strains have been used in diagnostic tests, in research and for most vaccine production. Type II strains are isolated predominantly from fetal bovine serum, persistently infected animals and animals with acute BVD and hemorrhagic disease. There also is a vaccine based on a type II strain.

BVDV infection may result in a number of distinct clinical syndromes, depending on the host, the virus and epidemiologic circumstances. Manifestations may include: 1) subclinical infection with viral clearance, 2) acute disease of the intestinal, respiratory and/or hematopoietic organs, 3) chronic disease with multiple organ involvement, 4) fetal infections and reproductive disorders, 5) persistent infections and 6) mucosal disease.

Type II strains have been known since the early 1980s. However, it wasn’t until the late 1980s that animals with signs resembling mucosal disease or a hemorrhagic diathesis were associated with ncp BVDV. In 1993 in Ontario, outbreaks of acute diarrhea, anorexia, fever, depression and overt hemorrhage were linked to ncp type II strains of BVDV. The outbreak appeared to be due to an evolution in virulence of strains that previously caused only subclinical or mild disease and did not result in appreciable morbidity or mortality. Outbreaks of severe acute BVDV began to increase and cause unprecedented losses from death of animals, abortions and decreased milk production. Many herds experienced widespread disease in animals of different age groups, including neonatal calves, older calves and adult cattle. Affected older calves (> 6 months) and adult cattle tended to exhibit more gross and microscopic alimentary
lesions.\textsuperscript{36} Type II strains seem variable in their ability to cause disease, since ncp type II viruses also can be isolated from animals that have not shown signs of overt disease.

9. Hematology in BVDV Infection

There are numerous case reports and experimental studies documenting the hematologic abnormalities associated with BVDV infection.\textsuperscript{29,53-57} Even the earliest descriptions of the disease include leukopenia as part of the clinical syndrome.\textsuperscript{58} Subclinical BVDV infections are associated with a mild fever and transient leukopenia of a few days duration.\textsuperscript{57} Severe acute BVDV infection typically causes mild to severe leukopenia characterized by moderate to severe neutropenia and mild to moderate lymphopenia. Mild to marked thrombocytopenia is a frequent feature of animals affected with this form of BVDV.\textsuperscript{29,57,59} If the platelets are sufficiently decreased to result in hemorrhage, a regenerative, blood-loss anemia can be found. Non-regenerative anemia also has been observed with ncp type II BVDV infections.\textsuperscript{60} Not only are absolute numbers of cells decreased, but function of both lymphocytes, neutrophils and platelets may be affected.\textsuperscript{61-63}

Immunosuppression from leukopenia may permit secondary or opportunistic infections, or prolong the course of disease.\textsuperscript{62} The mechanisms are not well understood, but may involve viral replication in cells of the immune system.\textsuperscript{64} Concurrent infections frequently include bovine respiratory syncytial virus (BRSV)\textsuperscript{65,66}, parainfluenza-3 virus (PI-3)\textsuperscript{67} and bovine herpesvirus-1.\textsuperscript{67} There is ongoing debate about the role of BVDV in causing or contributing to respiratory disease in cattle.
10. Pathogenesis of Hematological Abnormalities in BVDV Infection

BVDV infection is established by inhalation, with viral replication initially in the oronasal mucosa and oropharyngeal lymphoid tissue. Virus is disseminated widely via leukocytes circulating in lymph and blood. Viral replication continues in cells in lymphoid tissues, in circulating leukocytes and in the bone marrow. The bone marrow may be an important site of viral replication and subsequent dissemination throughout the body. Cell tropism may vary with viral strain. Most of the genetic diversity is from variation within the major envelope glycoprotein 53 (gp53) which likely accounts for different cell preferences.

Leukopenia appears to coincide with episodes of fever. As discussed previously, several mechanisms can contribute to the development of these abnormalities. No detailed studies have been performed to determine the reason for the leukopenia that develops in BVD. An assessment of bone marrow production during infection would help elucidate the mechanism(s) involved. The few previous studies have produced conflicting conclusions. These reports are limited by examination of the bone marrow only at post-mortem or at a few specified intervals during infection. In one report, bone marrow at post-mortem examination (10–12 days post-inoculation) was hypoplastic, with necrosis and minimal granulopoiesis, with a predominance of immature forms. Another study found a large number of BVDV-positive myeloid precursor cells in several calves at necropsy on day 10 post-infection. Bone marrow cellular kinetics during infection has not been adequately studied to determine if there is a change in production of myeloid cells.

Much work has been done to answer the same questions about platelet numbers. Recently, a model of a type II ncp BVDV infection that results in overt hemorrhage has been reported and the same authors suggest that there may be a defect in platelet function which could contribute to hemorrhage. However, decreased production in the bone marrow as a mechanism for thrombocytopenia has not been thoroughly examined.
The current literature is confusing with respect to the causes of thrombocytopenia in BVDV infection. Megakaryocyte hyperplasia, normal megakaryocyte populations, necrosis and dysmegakaryopoiesis have been reported. Walz et al suggested that assessment of mean platelet volume (MPV) can help discriminate between thrombocytopenia due to peripheral destruction or lack of production as reported in humans with HIV infection and swine with classical swine fever virus infection. They demonstrated a significant decrease in MPV in BVDV-infected versus control animals. Immunoglobulin was not demonstrated on the surface of platelets of BVDV-infected calves, suggesting that thrombocytopenia was not due to immune-mediated destruction.

Megakaryocyte hyperplasia in other experimentally-infected calves suggested peripheral consumption of platelets and leukocytes as a mechanism for cytopenia. But, a correlative study of peripheral blood platelet counts and concurrent bone marrow cytology and histopathology has not been reported.

Infection of calves with a cytopathic isolate did not result in a significant decrease in platelets, compared to the effect of several ncp isolates. Those authors suggested that only ncp BVDV strains have the ability to induce thrombocytopenia.

Disseminated intravascular coagulation has been evaluated as a cause for thrombocytopenia during BVDV infection. Coagulation parameters assessed included prothrombin time, partial thromboplastin time and fibrin degradation products. Observed values were within reference intervals in all animals. Therefore, DIC was unlikely as a mechanism for thrombocytopenia in infected calves.

11. Effect of Age

The age of the animal may have an important impact on the outcome of disease. Most experimental BVDV studies use neonatal calves or those < 3 months of age. Minimal
evaluation of BVDV infection in older calves and mature animals has been done. Very young animals sometimes are more susceptible to pathogens since their immune systems have not yet matured. Yet with acute BVD, all age groups may develop severe clinical disease.

12. Virulence Factors

Virulence refers to the relative capacity to cause disease. Individual virulence factors of a particular virus may contribute to the development of severe disease. There are usually multiple factors for any one virus, in those viral systems where virulence factors have been characterized. The susceptibility or resistance of the host (usually multifactorial) is also a very important factor in the outcome of viral infection. It is the interaction of specific virulence factors of the agent with host factors that results in the overall outcome of viral infection. A comparison of virulence factors requires that variables such as infecting dose, age, sex, health of animals and immune status must be controlled. Commonly, viral virulence has been assessed using lethal dose (LD) as an index. Assessment of the relative severity of histopathologic lesions also is frequently used in evaluations of virulence.

The classic virulence factor in BVDV is the production of the protein p80/NS3, characteristic of a cytopathic strain that mutates in an animal persistently infected with a ncp strain. Type II non-cytopathic strains vary in their ability to cause disease but the most important virulence factors are poorly understood. Potential variability at the genomic level and cell tropism also likely are important virulence factors. Genomic differences contributing to virulence have been identified in many viruses.

In order to infect a specific cell type, a virus must express attachment proteins complementary to receptors on target cells. These proteins can be host and cell specific or may permit the invasion of many cell types. Carbohydrates, lipids and proteins all can serve as attachment molecules. If a virus has the capability to infect multiple cell types, this is an
obvious advantage to the virus, since it provides for efficient dissemination throughout the body. Flaviviruses circulate free in plasma\textsuperscript{81} and as cell-associated virus. Variations in virus-macrophage interactions also may account for differences in virulence between viral strains and for differences in host resistance.\textsuperscript{8} For example, feline infectious peritonitis (FIP) virus infection relies on the ability of the virus to replicate in macrophages.\textsuperscript{8}

There are a few reports examining the virulence of non-cytopathic strains of BVDV. One group infected neonatal calves with two different ncp type II BVDV, which resulted in only mild clinical disease, proving that not all ncp type II strains were highly virulent.\textsuperscript{68} Another study found differences in disease produced by two different ncp strains, as well as variability in the severity of clinical signs, hematologic abnormalities and lesions induced among calves infected with the more virulent strain.\textsuperscript{71}

13. Cell Tropism of BVDV in Bone Marrow

Immunocytochemical studies on bone marrow have been performed in an attempt to delineate cell tropism of BVDV. Monoclonal antibodies to various glycoprotein components of the virion have been used to detect the presence of virus, and 15C5 is a monoclonal antibody in wide use for both research and diagnostic purposes. It labels glycoprotein 48(E0), a viral structural protein important in construction of the capsid and other components of the virus.\textsuperscript{82,83}

During infection, type II ncp-BVDVs have a wide tissue distribution, including cells of the bone marrow, where megakaryocytes express viral antigen, as do ‘mononuclear cells’, stromal cells and myeloid cells.\textsuperscript{53,54,68,69,72} Infection of megakaryocytes may be a mechanism for viral dissemination, since virus has been demonstrated in platelets in the peripheral blood.\textsuperscript{54} The ability of a particular BVDV strain to adhere to and infect megakaryocytes could be considered a virulence factor. This has not been extensively studied immunohistochemically and observations tend to be based upon a single post-mortem sample. However, Spagnuolo \textit{et al} demonstrated that
myeloid cells expressed BVDV antigen 3 to 10 days post-inoculation and that megakaryocytes expressed viral antigen on all but day 3 post-inoculation.69

14. Summary

To date, no reports document which cells are infected in type II ncp BVDV throughout infection and at what time the cells become infected. A study of the kinetics of the hematological abnormalities, concurrent with bone marrow cytology, would help delineate the mechanisms of leukopenia associated with BVDV infection. Serial immunocytochemical studies on bone marrow could help determine when bone marrow cells become infected and what cell lines are susceptible. A comparison of BVDV strains of varying virulence could determine if cell tropism in the bone marrow is related to development of severe disease.

Therefore, in an attempt to better understand the pathogenesis of the hematological changes in severe acute ncp type II BVDV, the following objectives were outlined:

1) Documentation of sequential changes in the peripheral blood leukocytes during infection

2) Description of sequential changes in the bone marrow cell types and how they relate to the circulating populations of leukocytes

3) Show if the appearance of virus in various bone marrow cell types relates to observations on the cells of the bone marrow and leukocytes in peripheral circulation

4) Discover if the virulence of BVDV strains influence bone marrow morphology and circulating leukocytes
15. References


47. Fulton RW, Saliki JT, Confer AW, Burge LJ, d'Offay JM, Helman RG, Bolin SR, Ridpath JF, Payton ME: 2000, Bovine viral diarrhea virus cytopathic and noncytopathic biotypes and type 1 and 2 genotypes in diagnostic laboratory


CHAPTER 2

Pathogenesis of leukopenia in calves experimentally infected with non-cytopathic type II bovine viral diarrhea virus

Introduction

Bovine viral diarrhea virus (BVDV), a pestivirus in the family Flaviviridae, is a common bovine pathogen and responsible for worldwide economic losses. The virus is categorized into non-cytopathic and cytopathic biotypes based on effects on cultured cells and into genotypes I and II based on genomic differences. Genotype I has further been subdivided into types Ia and Ib.

BVDV is well known for its association with mild acute diarrhea, mucosal disease, persistent infection and reproductive disorders. Since the early 1990s, it has been implicated as the cause of severe acute signs, including fever, anorexia, diarrhea and overt hemorrhage in young calves and adult cattle in North America. This severe acute manifestation is caused primarily by non-cytopathic type II strains of BVDV. Previously, these strains were associated with a self-limiting mild disease, and the more recent association with severe clinical signs appears to represent an evolution toward increasing virulence. Subsequently, an alarming increase in morbidity and mortality due to BVDV has been associated with the appearance of these more virulent strains.

The pathogenesis of acute infection with non-cytopathic type II BVDV is incompletely understood. Hematological abnormalities are a consistent feature during infection and contribute to clinical signs and disease outcome. An understanding of the pathogenesis of these abnormalities would enhance understanding of virus-host interactions.

Flaviviruses are capable of inducing marked hematological changes during infection. Dengue is well known to cause severe leukopenia and thrombocytopenia in people. Classical swine fever virus (hog cholera) and border disease of sheep also induce leukopenia and thrombocytopenia. A delay in adequate production of hematopoietic precursor cells in the
bone marrow has been implicated as a major cause of leukopenia and thrombocytopenia in these diseases.\textsuperscript{12,17}

Non-cytopathic type II acute BVDV infections also are associated with the development of leukopenia and thrombocytopenia.\textsuperscript{11,18} Leukopenia is characterized by neutropenia and frequently lymphopenia. While much work has been done, our understanding of the pathogenesis of thrombocytopenia is still unclear.\textsuperscript{19-23} The pathogenesis of leukopenia has received little study.\textsuperscript{18} Severe neutropenia can increase an animal's susceptibility to secondary bacterial infections, while lymphopenia may contribute to immunosuppression and prolong morbidity.

Since other related viruses have been implicated in decreased bone marrow production, it is suspected that BVDV may have a similar effect. Here, we examined the role of increased peripheral utilization of leukocytes as an alternative, or complement to, decreased production in mediating leukopenia. The development of leukopenia during experimental infection with two non-cytopathic type II BVDV strains of differing virulence is initially described. Secondly, serial bone marrow cytology and histopathology was used to examine the effect of BVDV infection on myeloid cells as an explanation for observed leukopenia. Finally, identification of virus in hematopoietic cells using immunohistochemical methods was performed in an attempt to detect an association with leukopenia. Experimental studies often use a purified viral product for animal inoculation, but we sought differences between the effects of unpurified virus stock and purified virus. Furthermore, an evaluation of strains of differing virulence provided an opportunity to identify if variation in tropism for cells of the hematopoietic system is an important factor in the development of severe disease.
Materials and Methods

1. Animals and Experimental Design

Six to eight-month-old Holstein steers (n=21) were housed in groups of three in an isolation facility. Calves were obtained from a commercial supplier\(^a\) and were negative for BVD virus and antigen. Routine virological studies were performed according to standardized methods at a centralized laboratory\(^b\). Experiments were conducted in accordance with the *Guidelines For The Care And Use Of Experimental Animals* established by the Canadian Council on Animal Care under protocols approved by the University of Guelph Animal Care Committee. Animals considered healthy on physical examination after 5 to 7 days of acclimation in isolation were admitted to the study.

The steers were assessed daily for temperature, pulse, respiration and a series of subjective clinical parameters including attitude, appetite, hydration status and fecal consistency (Appendix 1). Blood samples were taken on alternate days for 8 to 10 days prior to the day of virus inoculation (day 0) for development of baseline complete blood count (CBC) parameters. After blood samples were obtained, calves were given an intramuscular injection of xylazine hydrochloride\(^c\) at a dose of 0.25mg/kg.\(^d\) Once sedated to the point of immobility when placed in lateral recumbency, the sternum was shaved and surgically prepared using a chlorhexidine/isopropyl alcohol solution\(^d\). The ventral midline was located by palpation and a stab incision through the skin was made with a #15 scalpel blade. A Jamshidi bone marrow biopsy needle was introduced and 4 to 6 ml of bone marrow was aspirated into a syringe containing 0.5 ml 1% EDTA. The needle was re-introduced and a core biopsy was obtained from the opposite side of the sternum and fixed in B5. Four to five bone marrow samples were obtained prior to virus inoculation to establish baseline cytology and histology for each calf.
On day 0, each calf was given an intranasal inoculation (1 ml/nostril) of medium (Earle’s minimal essential medium (EMEM) supplemented with 10% horse serum and antibiotics) containing virus. Control animals received the same volume of medium without virus. The treatment groups, virus strain and titer administered are given in Table 1. Bone marrow samples were not collected on the day of virus inoculation. Blood and bone marrow samples were collected every second day until euthanasia on day 14. This resulted in seven serial blood samples and six serial bone marrow samples for each calf post-inoculation (pi). Bone marrow samples were not collected for treatment group 7; these calves were only included in the hematological evaluation. Serum from clotted blood samples and EDTA-anticoagulated blood were collected at the same intervals for viral serology and virus isolation, respectively.

Calves were assessed daily throughout the infection period for the clinical parameters previously mentioned on page 30. Calves were euthanized on day 14, or earlier if certain criteria were fulfilled. These criteria included pyrexia ≥ 41°C, complete anorexia or diarrhea for 2 days without improvement, or inability to rise for more than six hours. Calves were killed humanely by an intravenous barbiturate overdose. Complete necropsies were performed immediately on all calves. Gross lesions were recorded and tissues (Appendix 2) were collected and fixed in 10% neutral buffered formalin for histological evaluation.

2. Virus

Two type II non-cytopathic BVDV strains were chosen for use in these experiments; virulent 24515 and less virulent 11Q. The 24515 strain was isolated from the fetus of a morbid cow during the outbreak of acute BVDV in Ontario in 1993. The 11Q strain was isolated from a persistently infected feedlot animal in Manitoba. This herd was experiencing intermittent diarrhea and pneumonia in calves and adult animals.

Both viruses were passaged three times in Madin-Darby bovine kidney (MDBK) cells cultured in EMEM to produce an unpurified (up) virus stock. Unpurified virus stock was purified
(p) by limiting dilutions to result in a single viral clone.\textsuperscript{25} Titres of $10^7$ TCID\textsubscript{50}/ml (groups 2,3,5,7) and $10^5$ TCID\textsubscript{50}/ml (groups 4,6) were obtained. Supernatant containing virus at the above titres was used to inoculate calves intranasally (Table 1).

3. Hematology

Samples for complete blood counts were processed within 4 hours of collection. Automated analysis with a hematology analyzer\textsuperscript{6} and a manual 100 cell differential count were performed. CBC parameters measured are listed in Appendix 3. Values for total leukocyte, segmented neutrophil, lymphocyte and platelet counts were compiled. The daily means for each group were plotted. The pre-inoculation and post-inoculation values were examined to evaluate alterations during disease. Mean values from all calves prior to inoculation with virus (n=21) were used to establish age-specific threshold values for the different cell types (Table 2). For the purposes of this study, cytopenia was defined as a statistically significant difference (p<0.05) in the post-inoculation absolute cell count compared to the mean of the individual animal’s pre-inoculation values and/or as being outside the above established threshold values.

For each animal, the difference between the mean of pre-inoculation values and the post-inoculation nadir was calculated as the reduction in absolute cell count. These data were used to assess differences in the severity of cytopenia between viruses and groups of animals.

4. Bone Marrow Cytology

Bone marrow aspirates obtained in EDTA coated syringes were used to make smears for cytological analysis and immunocytochemical staining.

Bone marrow was ejected onto slides, excess blood was tipped off, and marrow granules were captured and spread on another slide. Three smears from each sample were rapidly air-dried, Wright’s stained\textsuperscript{h}, coverslipped and stored until cytological evaluation was performed.
Each sample of three slides was encoded for blind analysis. A bone marrow differential count of 1000 cells was performed on each Wright's stained sample. Cells were identified based on morphologic criteria described elsewhere. Cells of the megakaryocyte, erythroid and myeloid lineages, other cells, and subjective assessments of granule cellularity and marrow granulocyte reserve (MGR) were recorded (Appendix 4). A ratio of myeloid to erythroid cells (M:E ratio) was calculated based on the total number of cells/1000 in each lineage. The cells of the myeloid series were divided into the proliferation (myeloblasts, promyelocytes and neutrophilic myelocytes) and maturation (neutrophilic metamyelocytes, neutrophilic bands and segmented neutrophils) pools for analytical purposes. Pre- and post-inoculation bone marrow cytology parameters were compiled and compared for treatment effects.

A repeatability test was performed on a subset of bone marrow cytology data. Twenty samples were randomly selected from the original samples and given a new number. After a 1000 cell differential count was performed, slides were decoded and the results compared with the original differential cell count.

5. Bone Marrow Histology

Bone marrow core biopsies were fixed in B5 (Appendix 5) and 10% buffered formalin, decalcified and embedded in paraffin. Sections were cut at 3 microns, stained with Harris' hematoxylin and eosin (H&E) and coverslipped with a xylene-based mounting medium. Any bone marrow samples remaining after preparing slides for cytology and immunostaining were centrifuged and the pellet fixed in B5, embedded and paraffinized. These sections were cut at 5 microns and slides were prepared the same as the core biopsies.

Core biopsies were assessed for architecture, megakaryocyte numbers, cellularity and any abnormalities. Megakaryocyte numbers were counted in an area of representative cellularity free
of bone, cartilage and fat. Counts were made at 10x magnification in a 1000 µm diameter using an ocular micrometer.

6. Bone Marrow Immunocytochemistry

The presence of viral antigen in bone marrow was assessed in acetone-fixed aspirate smears and in B5-fixed, paraffin-embedded sections for each individual sample. Ten bone marrow slides were prepared at each sampling, fixed in cold acetone for 30 seconds and stored at -70 degrees Celcius until immunocytochemistry was performed. In addition, unstained paraffin embedded core biopsies and marrow clots were affixed to Snowcoat X-tra slides and allowed to dry.

The monoclonal antibody 15C5, developed against glycoprotein 48 (gp48) in a structural region of the BVD virus, was used for immunostaining procedures. This monoclonal antibody is widely used for BVDV diagnosis and research.

Controls for the aspirate smears were prepared from cultured MDBK cells in LabTek 2-well chamber slides. Samples were either uninfected (negative control) or infected with unpurified strain 11Q, purified strain 11Q, unpurified strain 24515 or purified strain 24515 (positive controls). Preliminary staining of these samples revealed that each virus resulted in similar staining patterns. An irrelevant monoclonal antibody, mouse anti-rabies, was also used as a negative control. Negative, positive and irrelevant controls were used with each batch of slides.

Controls for B5-fixed, paraffin-embedded sections were prepared from uninfected or virus-infected MDBK cells. Cells were harvested, centrifuged and the pellet fixed and processed identically to the bone marrow core biopsy samples. Irrelevant antibody and pre-inoculation bone marrow samples were used as further negative controls.
Positive controls for formalin-fixed paraffin-embedded sections were prepared from similarly processed tissues from an animal with confirmed ncp type II BVDV infection. Infection was confirmed by virus isolation and a type-specific antibody assay. Again, irrelevant antibody and pre-inoculation samples were used as negative controls.

Paraffin-embedded sections were deparaffinized and rehydrated through graded xylene and alcohol solutions prior to staining. BS-fixed sections were additionally immersed in Gram’s iodine for 10 minutes and dipped in sodium thiosulfate three times for removal of mercuric chloride and decolorization, respectively. Slides were immersed in an endogenous peroxidase inactivating solution of 4ml 30% H2O2 in 100ml methanol for 12 minutes and rinsed three times in automation buffer solution. Working automation buffer solution was prepared by adding 100ml of the 10X concentrate and 10ml 15% Brij-35 to 800ml of sterile water. This solution was thoroughly mixed, 100ml of acetone added, and the solution mixed again.

The sections were digested in a solution of 50mg protease XIV per 100ml of phosphate buffered saline (PBS) at 37°C for 20 minutes and rinsed three times in automation buffer solution. Sections were outlined with a PAP pen to prevent subsequent solutions from running off the slides while incubating.

Three or four drops of a blocking solution made of PBS supplemented with 4% normal horse serum and 2% normal bovine serum (BVDV antibody-free) was applied to the slides to decrease any non-specific binding. Slides were incubated in a humidified chamber for 10 minutes at room temperature before removing excess blocking solution and applying the primary antibody, mouse anti-BVDV 15C5, or the irrelevant antibody, mouse anti-rabies. All antibodies were diluted in blocking solution. An optimal dilution of 1:3200 was determined for the primary antibodies in preliminary studies. Slides were incubated in a humidified chamber at room temperature for 2 hours or overnight at 4 °C.

After rinsing three times with automation buffer solution, the secondary antibody, biotinylated horse anti-mouse, at a 1:800 dilution in blocking solution, was applied and incubated
for 30 minutes at room temperature in a humidified chamber. After rinsing three times with automation buffer solution, an avidin-biotin peroxidase solution made in PBS was then applied for 45 minutes. After rinsing three times with automation buffer solution, three to four drops of a solution of 100 μl of the chromogen 3,3-diaminobenzidine (DAB) in 10ml PBS was applied and the slides incubated for 5 minutes. Just before the DAB solution was applied, 3.5μl of 30% H₂O₂ was added to develop the chromogen. After rinsing with deionized water, sections were stained with Harris' hematoxylin and dehydrated through graded alcohol and xylene solutions before coverslipping with xylene-based mounting medium.

Acetone-fixed aspirate smears were processed in a similar manner with the following exceptions. On removal from the −70°C freezer, slides were immediately immersed in cold acetone for 5 minutes. Rehydration through alcohols and xylenes and protease digestion were unnecessary (personal communication, D. Haines, 1998). The inactivator solution contained 1ml 10% sodium azide and 2ml 30% H₂O₂ in 100ml methanol. Slides were stained in Mayer's hematoxylin solution for 20 seconds, rinsed in water and air-dried prior to coverslipping.

Slides were coded in a manner similar to those for cytological analysis. 1000 cells were examined and classified as to cell type and staining. A positive staining cell was identified by the presence of prominent diffuse, red-brown cytoplasmic granular stippling (Figure 14).

7. Virus Isolation and Serology

Virus isolation was performed by standard methods. Briefly, buffy coat cell preparations were frozen and inoculated onto embryonic bovine spleen cells. Noncytopathic isolates were detected by immunofluorescence using the monoclonal antibody 15C5 and were typed as type I or II, using type-specific monoclonal antibodies.

For serology, anti-BVDV antibodies in serum were titrated by virus neutralization using BVDV type II strain 125 according to standardized methods by a centralized laboratory.
8. Statistical Analysis

Data were analyzed using the SAS System for Windows version 6.12 and Analyse-It. Differences with a p-value ≤ 0.05 were considered statistically significant.

Peripheral blood total leukocyte, neutrophil, lymphocyte and platelet counts were assessed for significant decreases (cytopenia) by comparing mean absolute values before and after inoculation with virus. Bone marrow data were similarly analyzed. Results were examined for differences among virus treatments and among groups of animals. The difference of the absolute mean value before inoculation and the post-inoculation nadir were also compared between viruses and groups of animals for hematology and bone marrow data. One-way ANOVA analyses were performed on these data. Rectal temperatures were also compared between viruses and groups by one-way ANOVA.

Comparison of day of onset and duration of cytopenia or changes in the proliferation and maturation pools in bone marrow between viruses was evaluated by an unpaired Student’s t-test.

Three parameters (proliferation pool, maturation pool and M:E ratio) were examined for differences between the first and second determination using the Wilcoxon signed-ranks test and a Spearman rank correlation.
RESULTS

1. Animals

All six calves (100%) infected with purified (n=3) or unpurified (n=3) 11Q virus exhibited no overt clinical signs. Mild fever (39.5 - 40°C) developed in 3/6 (50%) of these animals on day 3 or 4 but quickly subsided. All six calves (100%) again had fevers of 2 to 3 days duration by day 7 or 8 post-inoculation (pi). During this latter febrile period, rectal temperatures ranged from 40-41°C. By day 14 pi (day of euthanasia), all six calves had normal temperatures.

Calves inoculated with 24515 strains also developed pyrexia. Mild fever (39.5-40 °C) occurred initially by day 3 or 4 pi but subsided to some degree after 1 to 2 days. Fever returned by day 7 or 8 pi and persisted for 4 to 6 days. During the second febrile period, temperatures were 41-42 °C. However, 7/12 (58%) calves had normal temperatures by the end of the study. The second fever peaks in calves inoculated with 24515 strains were significantly higher (p<0.05) than in calves inoculated with the 11Q strains.

All twelve calves infected with purified or unpurified 24515 virus developed clinical signs commonly observed with acute BVDV infection. Diarrhea was observed in all calves with an onset at days 6 to 8 pi. The character of the diarrhea varied from profuse and watery to scant with hematochezia in two calves in group 6. Melena was observed in a calf in group 7. Respiratory signs, including coughing, increased respiratory rate and serous nasal discharge, developed in 9/12 (75%) calves. Calves in groups 6 and 7 tended to have more severe respiratory signs, including dyspnea and mucopurulent nasal discharge. Most calves developed partial inappetence, and 4/6 (67%) infected with up24515 were completely anorexic from day 12 pi until termination. These four calves also became lethargic and responded minimally to external stimulation. Subjective evaluation of hydration status suggested mild dehydration in several calves by days 12-14 pi. Of calves in groups 6 and 7, 2/6 (33%) developed rare multifocal erythematous erosions of the nasal mucosa by day 12 pi. All three calves in group 7 fulfilled the
criteria for euthanasia by day 13 and were immediately euthanized. One calf in group 6 fulfilled the criteria for euthanasia at day 14 pi. Other calves were not deteriorating or were actually showing some clinical improvement by day 14 pi.

2. Virology and Serology

Type II non-cytopathic BVDV was isolated from buffy coats of 17/18 (94%) virus-inoculated calves. The one calf in group 4 that did not develop detectable viremia did develop anti-BVDV antibodies on day 12 pi. In all virus-inoculated calves, detectable viremia was first demonstrated on day 4 or 6 pi and was undetectable by day 12 pi (Table 3).

All virus-inoculated calves developed neutralizing antibodies to BVDV type II strain 125 on day 12 or 14 pi.

3. Gross and Histological Findings

Gross lesions were not common with either virus, but were more pronounced in calves inoculated with strain 24515. In groups 2 and 3, inoculated with strain 11Q, lesions were limited to mild to moderate lymph node enlargement with occasional petechial hemorrhages, and infrequent patchy mucosal congestion in the small intestine.

Calves inoculated with strain 24515 had mildly enlarged internal and peripheral lymph nodes, with frequent petechial hemorrhages. Retropharyngeal lymph nodes were markedly enlarged. The serosal surfaces of Peyer's patches were occasionally raised and hemorrhagic. Intestinal lesions were generally limited to patchy mucosal congestion with occasional petechial hemorrhages; however, intestinal congestion and petechial hemorrhage were severe in one calf in group 6. Rarely, 3-8mm diameter mucosal erosions were observed on the ventral surface of the tongue and in the esophagus. Mild to moderate cranioventral bronchopenumonia was observed in
most calves inoculated with strain 24515 and one calf in group 6 developed marked fibrinous bronchopneumonia. This same calf had multifocal petechial to ecchymotic hemorrhages in most tissues.

Histological lesions associated with both strains were primarily limited to the intestinal tract, lungs and lymphatic tissues. They also were more prominent in calves inoculated with strain 24515.

Calves inoculated with strain 11Q had no substantial intestinal lesions. However, 24515-inoculated calves had mild to severe multifocal mucosal ulceration with crypt necrosis. Peyer’s patches were frequently completely depleted or there was evidence of ongoing lymphocyte necrosis. Follicular remnants were present in many sections. Congestion was present in most lymph nodes. Internal and peripheral lymph nodes were similarly affected and were often completely depleted of lymphocytes. Follicular necrosis and neutrophilic lymphadenitis were occasionally observed. Similar but milder lymph node lesions were occasionally observed in calves inoculated with strain 11Q.

No substantial lesions were noted in the lungs of 11Q-inoculated calves, although there was mild microscopic congestion and interstitial thickening in several calves. Calves inoculated with strain 24515 had more severe but similar lesions as well as mild to severe cranioventral fibrinopurulent bronchopneumonia.

Histology of bone marrow samples revealed that architecture was uniformly retained after inoculation in all groups. Cellularity ranged from 20-70% in pre-inoculation samples and did not vary significantly after infection, although there was a tendency for cellularity to increase mildly in calves inoculated with 11Q and to decrease mildly in calves inoculated with 24515(Fig. 1). One animal in group 6 developed a marked decrease in bone marrow cellularity towards the end of the study period.
Megakaryocyte numbers were significantly higher post-inoculation for groups 2 through 5. All three calves in group 6 had significantly lower megakaryocyte numbers post-inoculation. Rare focal bone marrow necrosis was observed in group 6 calves.

4. Hematology

Data collected from calves in groups 2 and 3 (11Q strains) were never statistically different so results are based on the combined six animals. In some instances, there were differences between purified and unpurified 24515 strains, and these differences are indicated when relevant. Therefore, results may be based on six or twelve calves. There were never significant differences when comparing groups given different titers of virus, so results were pooled and therefore minimum group size is six animals.

Mean leukocyte counts were significantly decreased after viral inoculation compared to the pre-inoculation mean values in 17/18 (94%) animals (Fig. 2). Only one calf in group 2 did not develop significant leukopenia. Calves inoculated with strain 24515 developed significantly lower post-inoculation mean leukocyte counts than calves inoculated with strain 11Q.

Leukopenia was characterized by moderate to severely decreased neutrophil numbers and mild to moderately decreased numbers of lymphocytes. Mean neutrophil counts post-inoculation were significantly decreased compared to pre-inoculation mean values in 18/18 (100%) infected calves (Fig. 3). There were no differences when comparing neutropenia between 11Q infected animals and 24515 infected animals except for days 8 and 14.

All groups of calves developed a significant decrease in the mean lymphocyte counts post-inoculation (Fig. 4). Calves inoculated with strain 24515 developed significantly lower post-inoculation lymphocyte counts than strain 11Q infected calves, and up24515 calves had significantly lower counts than p24515 calves on days 10 to 14.
Mean platelet counts also decreased significantly post-inoculation in all infected calves (Fig. 5). Calves infected with strain 24515 developed significantly greater decreases in platelet counts than strain 11Q inoculated calves and calves given unpurified 24515 had significantly lower platelet counts than other groups from days 10 to 14.

For each animal, the difference of the pre-inoculation mean absolute cell count and the post-inoculation nadir value were calculated for leukocytes, neutrophils and lymphocytes for each group of animals (Fig. 6). The mean leukocyte nadir of 11Q inoculated calves was $4.8 \times 10^9/L$ (range 3.5-6.4) and of 24515 inoculated calves was $3.4 \times 10^9/L$ (range 0.9-4.8). Significant decreases in leukocyte count post-inoculation were observed in all infected groups compared to control animals. The decrease was significantly greater in 24515-inoculated calves ($n=12$) compared to 11Q infected animals ($n=6$).

Reductions in neutrophil count were significantly different post-inoculation compared to control animals in 3/6 (50%) groups (3,6,7). The mean neutrophil nadir of 11Q inoculated calves was $0.9 \times 10^9/L$ (range 0.6-1.1) and that of 24515 inoculated calves was $0.4 \times 10^9/L$ (range 0.03-1.1). There were no differences in the magnitude of the decrease in neutrophil count between all 11Q infected calves and all 24515 infected calves. There was a significant difference however, between animals inoculated with purified or unpurified 24515 virus.

Decreases in lymphocyte count post-inoculation were significantly different from control animals in all groups except group 3. The mean lymphocyte nadir of 11Q inoculated calves was $3.4 \times 10^9/L$ (range 2.4-4.3) and that of 24515-inoculated calves was $2.5 \times 10^9/L$ (range 0.8-4.2). Calves inoculated with strain 24515 had significantly greater decreases than did calves inoculated with strain 11Q.

Differences in the day of onset and duration of cytopenia up to euthanasia (day 14) were studied. Results are shown in Figure 7 and significant differences are summarized in Table 4.

There were no significant changes in red blood cell parameters after virus inoculation in any group (data not shown).
5. Bone Marrow Cytology

Differential cell counts on sequential bone marrow myeloid cells are shown in Figures 8 and 9. The percentage of cells in the proliferation pool before and after virus inoculation was significantly greater in animals given strain 24515 (Fig. 9) but not with animals given strain 11Q (Fig. 8). However, there were no significant differences when comparing the severity of change between 11Q and 24515 viruses.

Similar findings were present in the myeloid maturation pool. The percentage of cells after virus inoculation was significantly decreased in animals given strain 24515 but not with animals given strain 11Q. Furthermore, when comparing strains 11Q and 24515, there was a significant difference in the degree of change in the means after inoculation.

Animals infected with the 11Q strains developed a maximal decrease in the maturation pool at a mean of day 8 pi (range day 6 – day 12 pi) and a maximal increase in the proliferation pool at a mean of day 8 pi (range day 8 – day 12 pi). Both pools were not different from pre-inoculation values by the end of the study period.

Conversely, animals infected with strain 24515 developed a maximal decrease in the maturation pool at a mean of day 6 pi (range day 2 – day 14 pi) and a maximal increase in the proliferative pool at a mean of day 10 pi (range day 6 – day 12 pi).

Myeloid to erythroid (M:E) ratios were calculated for each cytology sample in groups 2,3,4,5, and 6 (Fig. 10). Mean M:E ratios were calculated for calves inoculated with 11Q and with 24515 strains. There was no significant difference in the mean M:E ratios before and after virus inoculation except for day 6 pi for 24515 inoculated calves.

An assessment of marrow granulocyte reserve (MGR) was performed on each sample. 11Q-inoculated animals tended to have adequate MGR until day 8 pi when there was a decrease
which persisted until day 10 pi. Animals inoculated with 24515 tended to have decreased MGR by day 4 pi, and it persisted until day 10 pi (Fig. 11). Two animals in group 6 did not have a rebound to normal MGR by the end of the study period.

Bone marrow lymphocyte percentages were evaluated but there were no trends or abnormalities noted in experiments with either virus.

Results of a repeatability study on bone marrow cytology are shown in Table 5 and Figure 12.

6. Immunocytochemistry

Immunostaining was performed on bone marrow aspirates and fixed core biopsies. Controls used for staining procedures are depicted in Figures 13 to 16. Results were similar for aspirate and core samples. No positive staining was observed in any samples from animals infected with strain 11Q. In animals inoculated with strain 24515, positive staining was very dispersed and transient. However, 6/9 (67%) animals exhibited at least some positive staining, including all three animals in group 5, two animals in group 4 and one animal in group 6. Viral antigen was detected in bone marrow cells primarily from day 6 to day 10 pi (Table 6). Megakaryocytes were the most common cell type with virus (69%) followed by early myeloid cells (28%) (Figures 17 to 19). The other 3% of cells were not identifiable morphologically. Of the six animals that exhibited positive staining, two had positive staining cells on only one day. One animal had positive staining on two consecutive sampling days and three had positive staining on three sampling days. The percentage of cells staining positive ranged from 0.1% to 0.8% with a mean of 0.32% (n=9). Only 9/105 (8.5%) of post-inoculation samples (all 11Q and 24515) exhibited positive staining for viral antigen and only 9/63 (14%) of 24515 samples had positive staining.
Table 1. List of treatment groups with strain of BVD virus, purification status*, volume† and titer administered intranasally.

<table>
<thead>
<tr>
<th>Treatment Group‡</th>
<th>Virus</th>
<th>Volume and Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>2ml medium only</td>
</tr>
<tr>
<td>2</td>
<td>p11Q</td>
<td>2ml x 10⁷ TCID₅₀</td>
</tr>
<tr>
<td>3</td>
<td>up11Q</td>
<td>2ml x 10⁷ TCID₅₀</td>
</tr>
<tr>
<td>4</td>
<td>p24515</td>
<td>2ml x 10⁵ TCID₅₀</td>
</tr>
<tr>
<td>5</td>
<td>p24515</td>
<td>2ml x 10⁷ TCID₅₀</td>
</tr>
<tr>
<td>6</td>
<td>up24515</td>
<td>2ml x 10⁵ TCID₅₀</td>
</tr>
<tr>
<td>7</td>
<td>up24515</td>
<td>2ml x 10⁷ TCID₅₀</td>
</tr>
</tbody>
</table>

* p = purified; up = unpurified
† 1ml per nostril
‡ n=3 in all groups
Table 2. Upper and lower threshold values for hematologic parameters calculated from pre-inoculation samples*.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Threshold Values†</th>
<th>Units</th>
<th>Mean</th>
<th>SD‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes</td>
<td>7.1-15.1</td>
<td>$10^9/L$</td>
<td>11.1</td>
<td>2.0</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>2.3-5.3</td>
<td>$10^9/L$</td>
<td>3.8</td>
<td>0.77</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>3.5-9.9</td>
<td>$10^9/L$</td>
<td>6.7</td>
<td>1.6</td>
</tr>
<tr>
<td>Platelets</td>
<td>406-678</td>
<td>$10^9/L$</td>
<td>542</td>
<td>68</td>
</tr>
<tr>
<td>RBC</td>
<td>7.0-10.4</td>
<td>$10^{12}/L$</td>
<td>8.7</td>
<td>0.85</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>0.25-0.33</td>
<td>L/L</td>
<td>0.29</td>
<td>0.02</td>
</tr>
</tbody>
</table>

* n = 21, six to eight month old steers  
† Range of mean +/- 2SD  
‡ SD = standard deviation
Table 3. Isolation of non-cytopathic type II BVDV from buffy coat cells from blood samples collected on alternate days.

<table>
<thead>
<tr>
<th>Group</th>
<th>Calf</th>
<th>Day post-inoculation*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>A7-18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A7-20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A8-23</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>B2-19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B3-27</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B3-28</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>B2-22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B3-23</td>
<td></td>
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<tr>
<td></td>
<td>B3-26</td>
<td></td>
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<tr>
<td>5</td>
<td>A8-25</td>
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<td>A9-26</td>
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<td></td>
<td>B5-34</td>
<td></td>
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<tr>
<td>7</td>
<td>A1-02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A1-03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A1-04</td>
<td></td>
</tr>
</tbody>
</table>

* - = no virus isolated; + = ncp type II BVDV isolated.
† The last blood sample for animals in group 7 was day 13 post-inoculation.
Table 4. Prevalence of blood cytopenia, mean day of onset of cytopenia post-inoculation (pi) and mean duration of cytopenia among groups of calves inoculated with 11Q and 24515 strains of ncp type II BVDV.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Prevalence of cytopenia</th>
<th>Mean day of onset (pi)</th>
<th>Mean duration (days) of cytopenia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>11Q</td>
<td>24515</td>
<td>11Q</td>
</tr>
<tr>
<td>Total leukocytes</td>
<td>5/6</td>
<td>12/12*</td>
<td>4</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>6/6</td>
<td>12/12</td>
<td>5</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>3/6</td>
<td>11/12*</td>
<td>4</td>
</tr>
<tr>
<td>Platelets</td>
<td>5/6</td>
<td>11/12*</td>
<td>6</td>
</tr>
</tbody>
</table>

Significant differences (p<0.05) between 11Q and 24515 inoculated calves are indicated by *. 

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Table 5. Repeatability study for bone marrow cytology. Twenty samples were randomly re-evaluated and the data for three variables are presented.

<table>
<thead>
<tr>
<th>Statistic</th>
<th>Proliferation Pool</th>
<th>Maturation Pool</th>
<th>M:E Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean*</td>
<td>4.6</td>
<td>24.6</td>
<td>0.735</td>
</tr>
<tr>
<td>SD†</td>
<td>1.7</td>
<td>6.9</td>
<td>0.264</td>
</tr>
<tr>
<td>CV‡</td>
<td>37.7</td>
<td>28.4</td>
<td>35.9</td>
</tr>
<tr>
<td>Mean delta§</td>
<td>1.6</td>
<td>5.4</td>
<td>15.9</td>
</tr>
<tr>
<td>Wilcoxon signed ranks test</td>
<td></td>
<td>0.473</td>
<td>0.812</td>
</tr>
<tr>
<td>Spearman rank correlation¶</td>
<td>0.009</td>
<td>0.036</td>
<td>0.002</td>
</tr>
</tbody>
</table>

* mean of the 40 values derived from original and repeat of 20 samples; (%) for maturation and proliferation pools
† standard deviation
‡ coefficient of variation (%)
§ mean difference between repeat values of 20 samples (%)
¶ p value, repeat samples not significantly different if >0.05
¶ p value, repeat samples significantly correlated if <0.05
Table 6. Immunostaining of sequential bone marrow samples. BVDV-positive staining in cells of the megakaryocyte series (M) and myeloid series (N) are indicated.

<table>
<thead>
<tr>
<th>Group*</th>
<th>Animal</th>
<th>Day Post-Inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>A7-18</td>
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<td>A7-20</td>
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<tr>
<td></td>
<td>A8-23</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>B2-19</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>B3-27</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>B3-28</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>B2-22</td>
<td>-</td>
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<tr>
<td></td>
<td>B3-23</td>
<td>-</td>
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<tr>
<td></td>
<td>B3-26</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>A8-25</td>
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<td>A9-26</td>
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<td>B5-32</td>
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<td>-</td>
</tr>
<tr>
<td></td>
<td>B5-34</td>
<td>-</td>
</tr>
</tbody>
</table>

* Groups 2 and 3 inoculated with 11Q, groups 4 to 6 inoculated with 24515. For further group identification see Table 1.
Figure 1. Bone marrow cellularity assessed from core biopsy samples for strain 11Q (◆) (n=6) and strain 245 (■) (n=9) inoculated calves pre- and post-inoculation with virus. Open symbols represent significant differences compared to pre-inoculation values. Asterisks indicate differences between viruses.
Figure 2. Mean blood leukocyte counts for control (◆, n=3), 11Q (■, n=6), p24515 (▲, n=6) and up24515 (●, n=6) inoculated calves. Day 0 is the day of inoculation. Standard error bars are shown. Dotted lines indicate the threshold values (mean +/- 2 SD). Significant differences between pre- and post-inoculation values are indicated by open symbols. Significant differences between groups are indicated by an asterisk.
Figure 3. Mean blood neutrophil counts for control (●, n=3), 11Q (■, n=6), p24515 (▲, n=6) and up24515 (●, n=6) inoculated calves. Day 0 is the day of inoculation. Standard error bars are shown. Dotted lines indicate the threshold values (mean +/− 2 SD). Significant differences between pre- and post-inoculation values are indicated by open symbols. Significant differences between groups are indicated by an asterisk.
Days (pre- and post-inoculation)

Figure 4. Mean blood lymphocyte counts for control (●, n=3), 11Q (■, n=6), p24515 (▲, n=6) and up24515 (○, n=6) inoculated calves. Day 0 is the day of inoculation. Standard error bars are shown. Dotted lines indicate the threshold values (mean +/- 2 SD). Significant differences between pre- and post-inoculation values are indicated by open symbols. Significant differences between groups are indicated by an asterisk.
Figure 5. Mean blood platelet counts for control (♦, n=3), 11Q (■, n=6), p24515 (▲, n=6) and up24515 (○, n=6) inoculated calves. Day 0 is the day of inoculation. Standard error bars are shown. Dotted lines indicate the threshold values (mean +/- 2 SD). Significant differences between pre- and post-inoculation means are indicated by open symbols. Significant differences between groups are indicated by an asterisk.
Figure 6. Comparison of differences between the mean pre-inoculation value and the post-inoculation nadir for leukocytes (A), neutrophils (B) and lymphocytes (C) for 11Q- and 24515-inoculated calves. Each bar represents the mean of three animals. For group details see Table 1. Results were compared to the control group (group 1) to assess for significant differences which are indicated by an asterisk. Inter-group differences are indicated by different column shading.
Figure 7. Comparison of day of onset and duration of leukopenia, neutropenia, lymphopenia and thrombocytopenia for 11Q- and 24515-inoculated calves. Cytopenia was defined as falling below the lower threshold value calculated from pre-inoculation samples (see Table 2, page 45)
Figure 8. Percentage myeloid proliferation (●) and maturation (■) pools from serial bone marrow cytology samples from calves inoculated with strain 11Q. Each data point represents the mean of six animals. Day 0 is the day of inoculation. Standard error bars are shown. Significant differences between pre- and post-inoculation means are indicated by open symbols.
Figure 9. Percentage myeloid proliferation (∗) and maturation (■) pools from serial bone marrow cytology samples of calves inoculated with strain 24515. Each data point represents the mean of nine animals. Day 0 is the day of inoculation. Standard error bars are shown. Significant differences between pre- and post-inoculation means are indicated by open symbols.
Figure 10. M:E ratios from bone marrow cytology pre- and post-inoculation for 11Q- (♦, n=6) and 24515- (▪, n=9) inoculated calves. Dotted lines represent the mean +/- 2 SD of the mean pre-inoculation M:E ratios of 15 animals. Standard error bars are shown. Significant differences from the mean pre-inoculation values are indicated by open symbols. Significant differences between groups are indicated by an asterisk.
Figure 11. Bone marrow cytology from an animal inoculated with virulent 24515 non-cytopathic type II bovine viral diarrhea virus on day 8 post-inoculation. Marrow granulocyte reserve is depleted, there is a shift to early myeloid precursors and the M:E ratio is increased favoring myelopoiesis. Wright’s stain. 200x
Figure 12. Scatterplot of the mean of the difference (delta) between repeat values from 20 samples and the mean of 40 values collected from the 20 repeat samples for the proliferation pool. As the mean value increased, delta values also increased.
Figure 13. Acetone-fixed cultured Madin-Darby bovine kidney (MDBK) cells used as controls for immunocytochemistry of frozen bone marrow aspirates. This image is from an uninfected sample used as a negative control. Stained with Mayer's hematoxylin. 200x

Figure 14. Acetone-fixed cultured Madin-Darby bovine kidney (MDBK) cells used as controls for immunocytochemistry of frozen bone marrow aspirates. This image is from a sample infected with non-cytopathic type II bovine viral diarrhea virus. Positive staining for BVDV antigen is identified as red-brown cytoplasmic stippling. Stained with Mayer's hematoxylin. 200x
Figure 15. B5-fixed cultured Madin-Darby bovine kidney (MDBK) cells used as controls for immunohistochemistry of paraffin-embedded bone marrow sections. This image is from an uninfected sample used as a negative control. Stained with Harris' hematoxylin. 200x

Figure 16. B5-fixed cultured Madin-Darby bovine kidney (MDBK) cells used as controls for immunohistochemistry of paraffin-embedded bone marrow sections. This image is from a sample infected with non-cytopathic type II bovine viral diarrhea virus. Positive staining is identified as red-brown cytoplasmic stippling. Stained with Harris' hematoxylin. 200x
**Figure 17a.** Positive-staining megakaryocytes from an acetone-fixed frozen bone marrow aspirate sample from a 24515-inoculated animal. Positive staining is identified as red-brown cytoplasmic stippling. Mayer's hematoxylin. 100x

**Figure 17b.** Positive-staining megakaryocyte from a B5-fixed bone marrow sample from a 24515-inoculated animal. Positive staining is identified as red-brown cytoplasmic stippling. Harris' hematoxylin. 400x
Figure 18. Positive-staining myeloid cell from a B5-fixed bone marrow sample from a 24515-inoculated animal. Positive staining is identified as red-brown cytoplasmic stippling (arrow).
Harris' hematoxylin. 400x
Figure 19. Positive-staining myeloid cells from a B5-fixed bone marrow sample from a 24515-inoculated animal. Positive staining is identified as red-brown cytoplasmic stippling (arrow).

Harris’ hematoxylin. 400x
Discussion

Experimental infection of Holstein steers with two strains of ncp type II BVDV resulted in a varied clinical presentation. Typical clinical signs of severe acute BVDV were observed in all animals inoculated with the virulent 24515 strains. Most importantly, all animals inoculated with the lower virulence 11Q strains lacked overt clinical symptoms.

The 11Q virus has not previously been evaluated in experimental studies. It was one of several strains isolated from cattle in a feedlot in Manitoba (personal communication, Gopi Nayar). Calves in this herd had been experiencing intermittent diarrhea and pneumonia. Furthermore, abortions were being attributed to BVDV infection. This strain was fortuitously chosen as the first to evaluate for clinical signs in animals. The goal was to isolate a low virulence ncp type II BVDV for molecular evaluation. Pyrexia was the only abnormality noted during daily physical examination and observation during the fourteen day study period. Fever is common during BVDV infection but in a herd situation, the febrile period induced by 11Q would likely go unnoticed. Although bi-phasic fever has been reported with virulent BVDV strains,\textsuperscript{11} it is interesting that the animals with otherwise inapparent disease also developed pyrexia. The fever induced by this lower virulence strain, however, was significantly lower and resolved more quickly than with the more virulent strain 24515. Experimental infection with strain 11Q was characteristic of the traditional self-limiting, clinically inapparent ncp type II BVDV infections.\textsuperscript{31}

The virulent strain 24515 has been used in previous experimental infection studies of neonatal animals and the clinical signs have been documented.\textsuperscript{11} In the field situation, infection in older calves and adult animals is also important.\textsuperscript{9} However, it was unknown if older animals with mature immune systems, though naïve to BVDV, would develop severe disease when infected with this strain. These experiments prove that older calves experimentally infected with the strain 24515 develop severe disease, which closely resembles the field situation. The severity of clinical signs varied substantially among the twelve animals inoculated with 24515
strains. All calves developed diarrhea by day six or eight pi but only three calves developed hemorrhagic diarrhea. This strain can cause severe thrombocytopenia with subsequent development of hemorrhagic syndrome.\textsuperscript{11} In the present study, hemorrhagic diarrhea developed only in animals inoculated with unpurified strains and even then was found in only 50% of calves. The effects of purification are discussed below.

Respiratory signs were very common in these experimental infections and were observed only with 24515 strains. Coughing and nasal discharge often were noted, but three calves from groups 6 and 7 also developed severe fibrinous bronchopneumonia. The role of BVDV in the development of bovine pneumonia is an ongoing debate. Immunosuppression secondary to BVDV infection may play a role in predisposing animals to opportunistic infections.\textsuperscript{32-34}

The viruses differed in the range of gross lesions present at necropsy at day 14 pi and on histopathologic evaluation. Previous reports of lesions induced by virulent ncp type II BVDV infections found that in animals which develop severe clinical disease, post-mortem lesions may be minimal to quite extensive.\textsuperscript{9,11,35} Gross and histological lesions in 24515-inoculated calves 14 days after inoculation were found primarily in the lymphoid tissues, intestinal tract and lungs, similar to other reports. There is one report of minimal lesions induced by a low virulence strain, but the strain was a type I virus.\textsuperscript{36} In this study, the only abnormalities found were enlarged lymph nodes containing scattered petechial hemorrhages. Lesions at day 14 pi in animals inoculated with 11Q were also limited to lymphoid tissues and similar in character. Necropsy at an earlier time during the infection period (during pyrexia and viremia) may have resulted in a greater frequency and severity of gross and histological lesions that may have resolved by day 14 pi when animals were afebrile and clinically improving. This may also have been the case with animals inoculated with purified 24515, that were also improving by the end of the study period. Lesions induced by virulent 24515 were more extensive and severe than those caused by 11Q.

Chemotactic demand for neutrophils in tissues can contribute to the development of neutropenia. Neutrophil accumulation in tissues of the intestinal tract was minimal and limited to
occasional necrotic crypts. However, neutrophil loss into the intestinal lumen could not be
demonstrated as migration through the wall of the gut can be quite rapid. Again, analysis of
lesions during peak pyrexia and viremia may have revealed this lesion if it occurred. In animals
with severe bronchopneumonia, areas of necrosis frequently were accompanied by a neutrophilic
infiltrate.

Some animals had sinusoidal and cortical neutrophilic infiltrates of both peripheral and
internal lymph nodes. The cause of this lesion is unknown and has not previously been described
with BVDV infection. In some lymph nodes, it may have been a response to lymphoid necrosis,
but it was present in other lymph nodes that had no evidence of necrosis. It is impossible to
directly quantify the contribution of neutrophil tissue loss to the development of leukopenia in
this experiment but indirect support is provided by the sequential examination of blood and bone
marrow.

Thrombocytopenia and leukopenia characterized by neutropenia and lymphopenia have
been described in 24515-inoculated calves based upon a single blood sample collected prior to
euthanasia. Other studies have documented the changes in blood parameters during infection
with other ncp type II BVDV strains. One study evaluated two ncp BVDV strains and
showed that one induced severe clinical disease (BVDV-890) and caused marked hematological
abnormalities and that the other (BVDV-TGAN) did not result in clinically apparent disease or
cause changes in hematological parameters. This was done in 2-9 week old calves, but
immunological and hematological function differs from older animals. Furthermore, BVDV-
890 is a type II virus while BVDV-TGAN is a type I virus. Therefore, this is the first study
comparing virulence of two ncp type II BVDV strains.

The experiments performed in this study document sequential changes in CBC
parameters in ncp type II BVD viruses of differing virulence in BVDV-naïve animals with mature
immune systems. An advantage of this study is that pre- and post-inoculation samples could be
compared. These values could also be used to develop age-specific threshold values to further
characterize cytopenias. Not only did animals inoculated with virulent 24515 strains develop leukopenia and thrombocytopenia, but animals inoculated with the less virulent 11Q strain also developed similar, albeit less severe, hematological abnormalities.

Despite the fact that strain 11Q inoculation did not result in clinically apparent disease, significantly decreased post-inoculation neutrophil and lymphocyte counts occurred for most animals. Platelet counts dropped slightly but rarely below the pre-inoculation established lower threshold value. This is in contrast to an earlier study that evaluated a low virulence strain in which no hematological changes occurred during infection. However, this virus was not a type II strain making it difficult to compare the two studies.

When comparing all strain 11Q-inoculated calves to all strain 24515-inoculated calves, there was no difference in the degree of change in neutrophil counts. However, the magnitude of decreased lymphocyte numbers in 24515-inoculated animals was larger than any animal infected with an 11Q strain. This suggests that one or more of the mechanisms contributing to neutropenia in virulent BVDV also occur during subclinical disease, while this is not the case for lymphopenia. Hence, the ability of a BVDV strain to cause neutropenia alone may not be an important virulence factor.

There was no difference in the day of onset of leukopenia or lymphopenia for either virus, and neutropenia occurred only a day earlier in 24515-inoculated calves. However, decreased cell counts persisted 2-5 days longer in calves inoculated with strain 24515. This suggests that the reason for onset of decreased cell count is similar regardless of virulence, but due to other reasons, these changes persist longer with a more virulent strain. There may be a delay before production of myeloid precursors increases or there may be a larger peripheral demand beyond the capacity of the marrow granulocyte reserve.

Although platelet counts did decrease, severe thrombocytopenia was not a prominent feature of infection with 11Q strains and platelet numbers never were low enough to result in hemorrhage. In contrast, 24515 infection caused significantly greater thrombocytopenia and was
occasionally associated with hemorrhage. This suggests that the ability to cause a large decrease in platelet counts may enhance virulence.

Sequential bone marrow samples were examined to further define the reasons for hematological alterations. A single sample of bone marrow is thought to be representative of the organ and therefore there should be minimal variation due to sample site. However, there is within-sample variation depending on the area of the smear examined and there can also be variation in morphologic evaluation of cells between and within observers. To show variation in evaluation of bone marrow cytology for the observer in this study, a repeatability assay was performed. The results of this study suggest that for blinded re-evaluations, the observer was capable of producing a similar differential cell count. It was noted that as the percentage of a cell type increased in a sample, there was more variation between determinations.

In calves inoculated with 11Q strains, peripheral blood neutrophil counts were beginning to decrease on day 2 pi and were even lower by day 4 pi. Concurrently, the maturation pool in the bone marrow also decreased and reached a nadir by day 8 pi (Figure 8). This appeared to represent increased peripheral demand, as there was no delay apparent for myeloid precursor increases. In fact, the percentage of cells in the proliferation pool was increasing by day 4 or 6 pi and peaked by day 8 pi, at the same time that the peripheral neutrophil counts were lowest. Bone marrow cellularity was not decreased. Given that some time is necessary for up-regulation of stem cells to commit to the myeloid developmental pathway, this would indicate that even before there was much drain from the maturation pool, there was already stimulus to increase myeloid production. Considering the kinetics of neutrophil production, the stimulus to increase marrow production must have occurred by the first or second day pi. This is interpreted as an appropriate and timely response by the bone marrow to increased peripheral demand. By the end of the study period, the peripheral blood leukocyte counts were returning to pre-inoculation values. In addition, the maturation and proliferation pools in the bone marrow were also returning to pre-inoculation values, indicating an end to the increased peripheral demand and return toward
homeostasis. It is clear that lack of production in the bone marrow does not contribute to leukopenia in calves infected with the 11Q strain.

In calves inoculated with 24515 strains, neutrophil counts were beginning to decline by day 2 pi, and were dramatically reduced by day 4 pi. This was similar to 11Q inoculated calves. The bone marrow maturation pool was concurrently diminishing and actually reached its low point by day 4 to 6 pi in many animals and continued to remain low until day 10 pi if recovery did occur (group 6) (Figure 9). Therefore, there appeared to be an abrupt drain of granulocyte reserves that did not rebound as quickly as with inoculation with the 11Q strains. This was primarily because of a slow increase in the proliferation pool. In most animals, it was increasing by day 8 or 10 pi, but did not peak until day 12 pi. At this point, it was also difficult to project whether a few animals were going to effectively replenish the maturation pool and restore the neutrophil count.

Bone marrow cellularity was also significantly lower in 24515 inoculated animals at the end of the study period (Figure 1). It could be hypothesized that because this was a more virulent strain there was a larger peripheral demand and therefore the bone marrow maturation pool was more quickly and extensively exhausted. When demands are large, because of tissue necrosis or inflammation, there should be a very prompt increase in bone marrow precursor cells and subsequently in the pool of mature cells. If a large demand persisted for some time however, cells would continue to leave the bone marrow as soon as they mature or even sooner (seen as a left shift in peripheral blood).

Most studies on the kinetics of cells of the myeloid series in the bone marrow and neutrophils in the peripheral blood and tissues occurred in the 1960s and 1970s. In the bovine, it takes about seven days for a segmented neutrophil to develop from a myeloblast. In other species, this maturation interval may be shorter when production is accelerated due to acute peripheral demand. If this was the case in mature cattle, it might be possible to recruit segmented neutrophils more quickly to meet the chemotactic demand, whereas a neonatal calf
may be unable to mount such a response. However, it is suspected that cattle cannot mount a response to peripheral demand as quickly as some other species.44

Regardless, if the bone marrow was responding appropriately, there should have been at least a similar onset in increased proliferation pool numbers in the 24515-inoculated calves as in the 11Q-inoculated calves. Furthermore, if increased peripheral demand was the only mechanism, there should have been an early and persistent shift to immature myeloid cells and a subsequent equal increase in both bone marrow pools. However, this was not the case. Instead, production was significantly delayed by about 4 days. Furthermore, in one group (group 5) there was a decrease in numbers in the proliferation pool percentage cells before they increased.

Therefore, virulent ncp type II BVDV infection, like other Flaviviridae, does appear to cause a decrease or delay in bone marrow production, as opposed to less virulent strains. Still, in immunocompetent animals this bone marrow suppression usually is transient and clinical recovery corresponds with elimination of virus and virus-infected cells and an increase in bone marrow cellularity. The onset of bone marrow suppression occurred at day 2 to 4 pi and persisted for 5 to 6 days. Bone marrow suppression with dengue fever has been well described and similarly occurs at 3 to 4 days pi.12

Delayed production and larger peripheral demand contribute to longer duration of leukopenia in calves inoculated with strain 24515, which provides a window of opportunity for secondary pathogens to establish infection, further increasing animal morbidity and risk of mortality.

In all instances, there were no significant differences in hematologic parameters between groups 2 and 3 (11Q strains), indicating that there was no effect of purification or lower viral titer. However, since changes in blood parameters were not as severe as with the virulent 24515 strain, a reduction in virulence may have been more difficult to appreciate.

It was interesting that 4/6 (67%) calves inoculated with the unpurified 24515 strains (groups 4 and 5) required euthanasia towards the end of the study period, but all six calves
inoculated with purified 24515 virus were showing clinical improvement by day 14 pi. There was also considerable variability of clinical signs among those inoculated with purified strains. The reasons for this apparent difference are probably multifactorial. Firstly, virulence may be attenuated during the purification process. Multiple passaging of virus has been shown to result in decreased virulence compared to the unpurified state.\textsuperscript{45-47} During the purification process, a single viral clone is isolated. This isolate may not represent the sum virulence of the multiple clones present in an unpurified stock. It is possible that if unpurified virus stock were purified again, a more virulent clone might be isolated due to random selection. Secondly, individual virus-host interactions are very important. To a certain extent, each animal may respond differently to an infecting strain due to biological variation.

The titer of virus inoculated did not appear to influence disease severity. Calves in group 6 developed similar and occasionally more severe signs than the three calves in group 7 which were exposed to the higher titer. A difference in titer of 10\textsuperscript{2} TCID\textsubscript{50} in the inoculum, within the range of titers used, does not appear to affect ability to cause disease. This is not surprising, considering that initial viral replication occurs in lymphoid tissues of the oropharynx. Afterwards, viral amplification occurs throughout the body. Therefore, the number of infecting particles may be greatly amplified regardless of the infecting dose.

Viral infection may alter cell function or shorten life-span. Therefore, bone marrow stem cells harboring viral antigen may not mature into differentiated cells. No BVDV-positive staining was demonstrated in any bone marrow samples collected from 11Q-inoculated calves, suggesting that infection of bone marrow precursor cells is not an important contributor to the transient leukopenia that developed during infection.

Calves inoculated with 24515 strains did have demonstrable viral antigen in hematopoietic cells, however, the number of cells involved was very low. Still, it is possible that virus-induced interference with progenitor cell maturation is a major contributory mechanism to the development of marked leukopenia (and thrombocytopenia) in animals infected with virulent
ncp type II BVDV strains. To further characterize the cell types infected, virus isolation could be attempted from subsets of hematopoietic cells.

Other factors may contribute to bone marrow suppression, including production of cytokines that interfere with normal granulopoiesis and thrombopoiesis, inhibition or cytolysis of stromal cells necessary for hematopoiesis, or 'apparent' suppression because of large peripheral demand from intestinal and lung lesions. No viral antigen was definitively demonstrated in marrow stromal cells in these experiments. But, as with hematopoietic cells, only a few stromal cells may need to be affected to produce a major change in bone marrow dynamics. In support of this theory, an in vitro study of dengue-infected bone marrow progenitors did not show direct cytotoxicity, but proliferation of bone marrow cells was decreased.\textsuperscript{12} Other experiments by the same authors showed that the earliest progenitor cells did not become infected until they had differentiated for at least 7 days, and even then positive staining was rare. This could be a protective mechanism since non-dividing hematopoietic cells would be less susceptible to infection.\textsuperscript{12} Infection of marrow stromal cells, although few in number, predominated.\textsuperscript{12} Therefore, since strain 24515 was present in bone marrow cells but 11Q was not, tropism for hematopoietic cells may be an important virulence factor.

As with most biological systems, one mechanism rarely is the sole reason for production of an alteration in a body system during disease. However, if one mechanism predominates, altering or eliminating this pathway might largely change the outcome. This scenario may apply to the reasons for alterations in hematologic parameters during BVDV infection. Although decreased bone marrow production probably contributes to the development of leukopenia, as may direct cytolysis of infected precursor cells, increased peripheral demand is probably very important. The role of endotoxin as a contributor to neutropenia early in infection was not evaluated in this study. In calves inoculated with 11Q, there was less peripheral demand, no viral antigen in the bone marrow and no delay in bone marrow response. Calves inoculated with 24515 however, had a larger peripheral demand, viral antigen in hematopoietic cells and a
delayed bone marrow response. Tissue demand was not easily demonstrable in histology sections obtained at post-mortem, but many animals were actually recovering and therefore the most severe lesions including neutrophilic infiltrate in the intestinal mucosa, might have been resolved.

In conclusion, it appears that ncp type II BVDV strains of differing virulence induce cytopenias proportional to severity of infection. Severity of neutropenia does not appear to be an important virulence factor, but neutropenia persisted significantly longer with the more virulent strain providing potential increased risk of opportunistic infections. Bone marrow response during infection with a virulent strain was delayed. The delay could be mediated by an altered hematopoietic inductive microenvironment or direct infection of hematopoietic myeloid cells. Infection of megakaryocytes also appears to be an important virulence factor. Future work should explore alterations in local bone marrow cytokines, virus isolation from subsets of hematopoietic cells, and demonstration of histological lesions at peak fever and viremia. Radiolabelling and tracking of neutrophils could be performed in the live animal, to further characterize their kinetics and distribution during infection.
Sources and Manufacturers

a. Gil Technologies, Orangeville, ON, Canada.
b. Animal Health Laboratory, University of Guelph, ON, Canada.
c. Xylamax 100mg/ml, Bimeda-MTC Animal Health Inc., Cambridge, ON, Canada.
d. Steri-Stat, Ingram & Bell, Don Mills, ON, Canada.
e. Gibco-BRL, Life Technologies, Burlington, ON, Canada.
f. Penicillin-Streptomycin, Life Technologies, Burlington, ON, Canada.
g. H*1 Technicon, Tarrytown, PA.
h. Hema-Tek Slide Stainer, Ames Co., Elkhart, IN
i. Fisher Scientific, Fairlawn, NJ.
j. AFIP method.
k. CytoSeal 60, Stephens Scientific, Kalamazoo, MI.
l. Surgipath, Winnipeg, MB, Canada.
m. Nalge Nunc International, Naperville, IL.
n. Cedarlane Laboratories Ltd., Hornby, ON, Canada.
o. Biomeda Corp., Foster City, CA.
p. ICN Biomedicals Inc., Aurora, OH.
q. Sigma Chemical Co., St. Louis, MO.
r. Vector Horse Biotinylated Anti-Mouse IgG, Vector Laboratories Inc., Burlingame, CA.
s. ABC Vectastain Elite, Vector Laboratories Inc, Burlingame, CA.
t. SAS Institute, Cary, NC.
u. Analyse-It Software Ltd., UK.
References


## APPENDIX 1

### Daily Physical Examination Sheet

<table>
<thead>
<tr>
<th>Date into Isolation</th>
<th>Date Inoculated</th>
<th>Strain</th>
<th>Girth at Inoculation</th>
<th>Girth at Euthanasia</th>
<th>Date Euthanized</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>From a Distance</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inquisitive to Visual/Noise</td>
<td>Yes=0 Mildly=1 No=2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standing or Stands Up on Entry</td>
<td>Yes=0 Eventually=1 No=2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breathing</td>
<td>Norm=0 Sl. Labored=1 V.Labored=2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Appetite</td>
<td>Yes=0 Some food left=1 No=2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oculo-Nasal Discharge</td>
<td>No=0 Mild=1 Severe=2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Physical Examination</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Responds to Physical Stimuli</td>
<td>Yes=0 Mildly=1 No=2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart Rate / minute</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiratory Rate / minute</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dehydration</td>
<td>No=0 5-7%=1 7-10%=2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formed Feces</td>
<td>Yes=0 Some Animals=1 No=2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Score</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Blood Drawn</strong></td>
<td>3x10ml EDTA, 3x10ml Red Top</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Comments</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\[5\% = \text{doughy skin, conjunctival congestion}; 7\% = \text{prolonged skin tent, sunken eyes}; 10\% = \text{circulatory signs, muscle tremors}\]
## APPENDIX 2

List of tissues examined by histopathology

<table>
<thead>
<tr>
<th>Oral mucosa</th>
<th>Retropharyngeal lymph node</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pharynx</td>
<td>Hilar lymph node</td>
</tr>
<tr>
<td>Esophagus</td>
<td>Superficial cervical lymph node</td>
</tr>
<tr>
<td>Rumen</td>
<td>Mesenteric lymph node</td>
</tr>
<tr>
<td>Reticulum</td>
<td>Prefemoral lymph node</td>
</tr>
<tr>
<td>Omasum</td>
<td>Thymus</td>
</tr>
<tr>
<td>Abomasum</td>
<td>Liver</td>
</tr>
<tr>
<td>Duodenum</td>
<td>Spleen</td>
</tr>
<tr>
<td>Jejunum</td>
<td>Pancreas</td>
</tr>
<tr>
<td>Ileum</td>
<td>Adrenal gland</td>
</tr>
<tr>
<td>Cecum</td>
<td>Kidney</td>
</tr>
<tr>
<td>Spiral colon</td>
<td>Bladder</td>
</tr>
<tr>
<td>Rectum</td>
<td>Urethra</td>
</tr>
<tr>
<td>Trachea</td>
<td>Skin</td>
</tr>
<tr>
<td>Cranioventral lung</td>
<td>Eye</td>
</tr>
<tr>
<td>Middle lung</td>
<td>Cerebrum</td>
</tr>
<tr>
<td>Caudodorsal lung</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>Right auricle</td>
<td></td>
</tr>
<tr>
<td>Left auricle</td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX 3

CBC Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocyte count</td>
<td>x $10^9$/L</td>
</tr>
<tr>
<td>Red blood cell count</td>
<td>x $10^{12}$/L</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>g/L</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>L/L</td>
</tr>
<tr>
<td>Mean corpuscular volume</td>
<td>fl</td>
</tr>
<tr>
<td>Mean corpuscular hemoglobin</td>
<td>pg</td>
</tr>
<tr>
<td>Mean corpuscular hemoglobin concentration</td>
<td>g/L</td>
</tr>
<tr>
<td>Red cell distribution width</td>
<td>%</td>
</tr>
<tr>
<td>Platelet count</td>
<td>x $10^9$/L</td>
</tr>
<tr>
<td>Mean platelet volume</td>
<td>fl</td>
</tr>
<tr>
<td>Neutrophil count</td>
<td>x $10^9$/L</td>
</tr>
<tr>
<td>Lymphocyte count</td>
<td>x $10^9$/L</td>
</tr>
<tr>
<td>Monocyte count</td>
<td>x $10^9$/L</td>
</tr>
<tr>
<td>Eosinophil count</td>
<td>x $10^9$/L</td>
</tr>
<tr>
<td>Basophil count</td>
<td>x $10^9$/L</td>
</tr>
</tbody>
</table>
# APPENDIX 4

Bone Marrow Cytology Differential Sheet

<table>
<thead>
<tr>
<th>Code:</th>
<th>Animal:</th>
<th>Date:</th>
<th>Virus:</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rubriblast</td>
<td>Myeloblast</td>
</tr>
<tr>
<td>Prorubricyte</td>
<td>Progranulocyte</td>
</tr>
<tr>
<td>Basophilic rubricyte</td>
<td>Neutrophilic myelocyte</td>
</tr>
<tr>
<td>Polychromatophilic rubricyte</td>
<td>Eosinophilic myelocyte</td>
</tr>
<tr>
<td>Normochromic rubricyte</td>
<td>Basophilic myelocyte</td>
</tr>
<tr>
<td>Metarubricyte</td>
<td>Neutrophilic metamyelocyte</td>
</tr>
<tr>
<td>Mitotic cells</td>
<td>Eosinophilic metamyelocyte</td>
</tr>
<tr>
<td><strong>TOTAL ERYTHROCYTIC CELLS</strong> =</td>
<td>Basophilic metamyelocyte</td>
</tr>
<tr>
<td>Hematogones</td>
<td>Neutrophilic band</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>Eosinophilic band</td>
</tr>
<tr>
<td>Plasma cells</td>
<td>Basophilic band</td>
</tr>
<tr>
<td>Monocytes</td>
<td>Segmented neutrophil</td>
</tr>
<tr>
<td>Mitotic cells</td>
<td>Eosinophil</td>
</tr>
<tr>
<td>Megakaryocytes</td>
<td>Basophil</td>
</tr>
<tr>
<td>Osteoclasts</td>
<td><strong>TOTAL GRANULOCYTIC CELLS</strong> =</td>
</tr>
<tr>
<td>Macrophages</td>
<td></td>
</tr>
<tr>
<td>Unclassified cells</td>
<td>M:E Ratio</td>
</tr>
<tr>
<td>Degenerated cells</td>
<td>Granule cellularity</td>
</tr>
<tr>
<td><strong>TOTAL OTHER CELLS</strong> =</td>
<td>MGR (- N +)</td>
</tr>
<tr>
<td>Other comments</td>
<td>Mega/granule/10x</td>
</tr>
</tbody>
</table>

- MGR: Mega/granule 
- M:E Ratio: Myeloid:erythroid ratio
APPENDIX 5

B5 Fixative

Ingredients:

60 grams mercuric chloride
12.5 grams anhydrous sodium acetate
900 ml hot distilled water

Mix and refrigerate until use.

Before use mix:

9 parts B5 solution
1 part 37% formaldehyde
APPENDIX 6
Individual Animal Hematology Data

Group 1 - Control

A3-7

A3-8

A3-9

Days (Pre- and Post-Inoculation)

- Leukocytes
- Lymphocytes
- Neutrophils
Group 2 - p11Q

A7-18

A7-20

A8-23

Days (Pre- and Post-Inoculation)

- Leukocytes
- Lymphocytes
- Neutrophils

Cells x 10^9/L
Group 4 - p24515x5

B2-22

B3-23

B3-26

Days (Pre- and Post-Inoculation)

Cells x 10^6/L

- Leukocytes - Lymphocytes - Neutrophils
Group 7 - up24515x7

A1-02

A1-03

A1-04

Cells x 10^9/L

Days (Pre- and Post-Inoculation)

- Leukocytes
- Lymphocytes
- Neutrophils
Appendix 7
Individual Animal Bone Marrow Cytology

Group 2 - p11Q

Days (Pre- and Post-Inoculation)

- Proliferation Pool
- Maturation Pool
APPENDIX 7 (con't)
Individual Animal Bone Marrow Cytology Data

Group 3 - up11Q

B2-19

B3-27

B3-28

Days (Pre- and Post-Inoculation)

Proliferation Pool

Maturation Pool

% bone marrow cells

Days

-10 -8 -6 -4 -2 2 4 6 8 10 12 14

-10 -8 -6 -4 -2 2 4 6 8 10 12 14

-10 -8 -6 -4 -2 2 4 6 8 10 12 14

0 0.1 0.2 0.3 0.4 0.5
APPENDIX 7 (con't)
Individual Animal Bone Marrow Cytology Data

Group 4 - p24515

B2-22

% bone marrow cells

-11 -9 -7 -5 -2 2 4 6 8 10 12 14

B3-23

% bone marrow cells

-11 -9 -7 -5 -2 2 4 6 8 10 12 14

B3-26

% bone marrow cells

-11 -9 -7 -5 -2 2 4 6 8 10 12 14

Days (Pre- and Post-Inoculation)

- Proliferation Pool
- Maturation Pool
Appendix 7 (con’t)
Individual Animal Bone Marrow Cytology

Group 5 - p24515x7

A8-25

% bone marrow cells

-15 -13 -11 -9 -7 -2 2 4 6 8 10 12 14

A9-26

% bone marrow cells

-15 -13 -11 -9 -7 -2 2 4 6 8 10 12 14

A9-27

% bone marrow cells

-15 -13 -11 -9 -7 -2 2 4 6 8 10 12 14

Days (Pre- and Post-Inoculation)

- Proliferation Pool - Maturation Pool
APPENDIX 7 (con't)
Individual Animal Bone Marrow Cytology Data

Group 6 - up24515x5

- B5-32
- B5-33
- B5-34

% bone marrow cells vs Days (Pre- and Post-Inoculation)

- Proliferation Pool
- Maturation Pool