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PATHOGENESIS OF THE BOVINE VIRAL DIARRHEA VIRUS-INDUCED HEMORRHAGIC DISORDER

presented by

Paul Harold Walz

has been accepted towards fulfillment
of the requirements for

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John C. Baker
Major professor

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PATHOGENESIS OF THE BOVINE VIRAL DIARRHEA VIRUS-INDUCED HEMORRHAGIC DISORDER

By

Paul Harold Walz

A DISSERTATION

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2000

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ABSTRACT

PATHOGENESIS OF THE BOVINE VIRAL DIARRHEA VIRUS-INDUCED HEMORRHAGIC DISORDER

By

Paul Harold Walz

Type II bovine viral diarrhea virus (BVDV) has been associated with severe clinical disease and a hemorrhagic syndrome characterized by thrombocytopenia, bleeding, and death. The mechanism by which type II BVDV causes severe disease and the hemorrhagic syndrome is unknown, but the induction of thrombocytopenia by type II BVDV may be an important factor. Previous experimental studies on thrombocytopenia associated with BVDV infection have been descriptive, but difficult to reproduce, and the mechanism of BVDV-induced thrombocytopenia remains unknown. Moreover, clinical signs of spontaneous hemorrhage have been observed in type II BVDV-infected cattle whose platelet count was not depressed enough to fully account for the hemorrhage, suggesting that altered platelet function may additionally be involved in the pathogenesis. The focus of our first experimental study was to address the need for a reproducible model of BVDV-induced thrombocytopenia and to investigate the discrepancy between development of hemorrhage and platelet count. Calves (n=5) were inoculated on day 3 of age with 10^7 $TCID_{50}$ of BVDV 890 (type II), while sham-inoculated calves served as controls (n=4). Blood cell counts and virus isolation from blood components were performed daily and platelet aggregation tests were performed every other day throughout the study. Calves were euthanatized on day 12 after inoculation, and pathologic, virologic, and immunohistochemical examinations were performed. The

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platelet counts decreased and platelet function was depressed in type II BVDV-infected calves. Bone marrow examination from infected calves revealed megakaryocyte infection and evidence of concurrent megakaryocyte necrosis and hyperplasia. A model of BVDV-induced thrombocytopenia was characterized, and it was concluded that altered platelet function may contribute to clinical hemorrhage in cattle infected with type II BVDV.

The hemorrhagic syndrome and outbreaks of severe disease have been most often associated with type II BVDV, and not type I BVDV. The focus of our second study was to compare platelet function and levels of viremia between a type I BVDV infection, a non-thrombocytopenic type II BVDV infection, and thrombocytopenic type II BVDV infection. Control calves (n=4) were sham inoculated, while calves in infected groups (n=4 for each group) were inoculated with 10^7 $TCID_{50}$ of either BVDV 890 (type II), BVDV 7937 (type II), or BVDV TGAN (type I). Platelet function and levels of viremia were evaluated prior to inoculation (day 0) and on days 4, 6, 8, 10, and 12 after inoculation. Level of viremia was determined using the serum viral titers and the percentage association of BVDV with lymphocytes and platelets. Depressed platelet function was only observed in type II BVDV-infected calves. A parallel relationship between level of viremia and rectal temperatures, and an inverse relationship between level of viremia and blood cell counts was observed; that is, the greatest elevations in rectal temperature and greatest depressions in blood counts were observed in BVDV 890-infected calves. Isolates of BVDV that induce higher levels of viremia may be more capable of inducing disease. Severe BVDV infections and the hemorrhagic syndrome may occur during infection with isolates of BVDV capable of inducing high levels of viremia.

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It gives me great pleasure to dedicate this dissertation to my parents, Lloyd and Marion Walz. In addition to their unfailing love, support, and encouragement, they have instilled in me the virtues of hard work, perseverance, loyalty, and faith. To them, I shall be forever grateful.

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sincere and heartfelt gratit
his guidance, friendship, a
dissertation.

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Harold Tvedten for provi
the project. I am especia
points regarding platelet a
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in this project.

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INTRODUCTION

The disease of cattle that would become known as bovine viral diarrhea was first described in 1946 (Childs, 1946; Olafson *et al.*, 1946). The virus, which would be redundantly named bovine viral diarrhea virus (BVDV), was discovered seven years later (Baker *et al.*, 1954). The 50 years following the first report brought into view other clinical and pathologic features associated with the virus such as persistent infection, mucosal disease, immunosuppression, and reproductive inefficiency (Baker, 1995). In the past 15 years, there has been a vast increase in the knowledge of the molecular biology of this virus. The pathogenesis of mucosal disease has been determined (Bolin *et al.*, 1985; Brownlie *et al.*, 1984), and the complete genomes for several BVDV isolates have been sequenced (Colett *et al.*, 1988; De Moerlooze *et al.*, 1993; Deng & Brock, 1992). In spite of the advances that have been made in our understanding of this complex virus and its associated diseases, BVDV remains an important pathogen of cattle worldwide and control has not been achieved. Economic losses created by infection with BVDV continue to be substantial.

Outbreaks of severe clinical disease associated with BVDV infection in cattle immunocompetent to BVDV have been reported within the last decade (Carman *et al.*, 1998; David *et al.*, 1994; Drake *et al.*, 1994), and this has reinforced the importance of BVDV as a pathogen. These outbreaks were characterized by high morbidity and mortality rates (Carman *et al.*, 1998; David *et al.*, 1994; Drake *et al.*, 1994). Affected

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cattle developed fever and diarrhea, and pregnant cattle aborted (Carman *et al.*, 1998; Drake *et al.*, 1994). Laboratory evaluation revealed leukopenia and thrombocytopenia. On some farms, affected cattle died without demonstrating clinical signs of disease (Carman *et al.*, 1998). Prior to these outbreaks, it was generally believed that acute infections with BVDV resulted in mild disease, without considerable mortality (Baker, 1995). The BVDV isolates from these outbreaks were analyzed, and the type II genotype of BVDV was identified (Pellerin *et al.*, 1994; Ridpath *et al.*, 1994).

A hemorrhagic syndrome has also been recently reported in association with BVDV infection in adult cattle and calves (Rebhun *et al.*, 1989). Hematologic evaluation revealed thrombocytopenia as the cause of the hemorrhagic syndrome (Rebhun *et al.*, 1989). Thrombocytopenia and the resulting hemorrhagic syndrome have been most often associated with infection of cattle with type II BVDV. Thrombocytopenia associated with BVDV infection has been reported under natural and experimental conditions (Bolin & Ridpath, 1992; Corapi *et al.*, 1990; Corapi *et al.*, 1989; Rebhun *et al.*, 1989). Although previous experimental studies evaluating type II BVDV infection have characterized the hemorrhagic syndrome, they have been descriptive in nature and difficult to reproduce. For example, previous experimental studies used infection protocols that did not allow quantitation of the infectious dose of virus, making reproduction of their study impossible (Bezek *et al.*, 1994; Corapi *et al.*, 1990; Corapi *et al.*, 1989). Furthermore, several studies failed to use controls or failed to use age matched controls (Bolin & Ridpath, 1992; Corapi *et al.*, 1990; Corapi *et al.*, 1989). Control calves are critical in the evaluation of thrombocytopenia because of the variation in the normal platelet count (Nelson *et al.*, 1974; Semrad & Dubielzig, 1993); and age-matching is important as the

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platelet count varies between calves of different ages (Nelson *et al.*, 1974; Semrad & Dubielzig, 1993; Vaugher *et al.*, 1973). Finally, some investigations have used type II BVDV isolates that failed to consistently induce thrombocytopenia in all infected calves (Corapi *et al.*, 1989; Ellis *et al.*, 1998).

The emergence of type II BVDV isolates capable of inducing severe clinical disease and hemorrhage has created a renewed interest in the study of BVDV infections in immunocompetent cattle. At present, much remains unknown about BVDV infection in immunocompetent cattle and thrombocytopenia, including the mechanism of thrombocytopenia induction. The purpose of the studies described within this dissertation is to provide an understanding of the mechanism of thrombocytopenia and the pathophysiology of the hemorrhagic syndrome caused by BVDV.

CHAPTER I:

REVIEW OF THE LITERATURE

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Bovine Viral Diarrhea Virus

The History of Bovine Viral Diarrhea Virus

Bovine viral diarrhea virus (BVDV) was first recognized and reported in 1946 from the United States (Olafson *et al.*, 1946) and Canada (Childs, 1946). In the United States, the initial outbreak involved 6 dairy herds in New York state with clinical signs of gastroenteritis with severe diarrhea. Other clinical characteristics of affected cattle included elevated body temperatures, salivation, nasal discharge, depression, anorexia, dehydration, and abortions during the outbreak and extending three months beyond the initial outbreak. Interestingly, the first description of hemorrhages associated with BVDV infection appeared in this report (Olafson *et al.*, 1946), with hemorrhages being present in the gastrointestinal tract, subcutaneous tissues, epicardium, and vaginal mucosa on postmortem examination of affected cattle. Experimental reproduction of this novel disease was also reported. Oral inoculation with fecal material, subcutaneous injection of blood collected from febrile cases, and subcutaneous injection of splenic emulsions from deceased cases all proved to be infective to susceptible cattle (Olafson *et al.*, 1946). A very similar disease syndrome, which was termed "X disease," was reported from Canada during the same year (Childs, 1946). Hemorrhage, in the form of bloody diarrhea, was reported as a clinical sign, in addition to the clinical signs mentioned in an earlier report (Olafson *et al.*, 1946).

A viral etiology for this new transmissible disease of cattle was not demonstrated until seven years after the initial reports (Baker *et al.*, 1954). Early descriptions of the virus indicated that two forms existed, one causing cell vacuolation and death and the other producing no obvious pathology in cell monolayers (Gillespie *et al.*, 1960; Lee &

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Gillespie, 1957). This formed the first subclassification of BVDV, cytopathic and noncytopathic BVDV, which later was designated as the biotype of the virus.

The virus was subsequently recognized to be associated with a sporadically occurring enteric disease characterized by low morbidity and high mortality that was termed mucosal disease (Ramsey & Chivers, 1953). Bovine viral diarrhea virus isolates associated with mucosal disease were found to belong to the cytopathic biotype (Gillespie *et al.*, 1961). This would set the stage for the most widely studied aspect of BVDV infection, the pathogenesis of mucosal disease. Nearly 35 years passed before the pathogenesis of mucosal disease was determined (Bolin *et al.*, 1985; Brownlie *et al.*, 1984). Cattle immunotolerant to, and persistently infected with noncytopathic BVDV develop mucosal disease after superinfection with cytopathic BVDV. The persistent infection established in cattle was determined to be a result of transplacental infection of the fetus with the noncytopathic biotype of BVDV during the first 100 days of gestation (Liess *et al.*, 1984; McClurkin *et al.*, 1984). This results in the birth of a calf that is immunotolerant to, and persistently infected with noncytopathic BVDV.

Within the past decade, outbreaks of severe, peracute BVDV infections in immunocompetent cattle have been reported in the United States, Canada, and the United Kingdom involving beef, dairy, and veal operations (Carman *et al.*, 1998; David *et al.*, 1994; Pellerin *et al.*, 1994). Prior to these outbreaks, the ability of BVDV to cause severe clinical disease following primary acute infections in cattle was debatable (Baker, 1995). Analysis of BVDV isolates from outbreaks in Canada and the United States revealed a distinct group of BVDV (Ridpath *et al.*, 1994). This formed the second subclassification of the virus, termed the genotype. Two distinct genotypes of BVDV, type I and type II

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BVDV, are currently recognized. The BVDV isolates obtained from these outbreaks were primarily of the type II genotype of BVDV (Pellerin *et al.*, 1994; Ridpath *et al.*, 1994). In addition, infection of nonimmune, immunocompetent cattle with some noncytopathic isolates of type II BVDV has been associated with a hemorrhagic disorder characterized by thrombocytopenia and hemorrhage (Bezek *et al.*, 1994; Bolin & Ridpath, 1992; Corapi *et al.*, 1990; Corapi *et al.*, 1989; Rebhun *et al.*, 1989). The pathogenesis of the hemorrhagic syndrome and the mechanism of increased virulence with some type II BVDV isolates are unknown, and are the focus of this dissertation.

The Clinical Manifestations of Bovine Viral Diarrhea Virus: Severe, Peracute Bovine Viral Diarrhea

The clinical manifestations of BVDV infection in cattle have been reviewed (Baker, 1995). Multiple clinical forms of BVDV infection in immunocompetent cattle exist, including subclinical, clinical bovine viral diarrhea (BVD), severe BVD, and a hemorrhagic syndrome. The majority of BVDV infections in immunocompetent and seronegative cattle are subclinical (Baker, 1995); however, outbreaks of severe, peracute BVDV infections have been reported in immunocompetent cattle (Carman *et al.*, 1998; David *et al.*, 1994; Drake *et al.*, 1994; Pellerin *et al.*, 1994). These outbreaks have involved beef, dairy, and veal operations in the United States, Canada, and the United Kingdom (Carman *et al.*, 1998; David *et al.*, 1994; Drake *et al.*, 1994; Pellerin *et al.*, 1994; Swecker *et al.*, 1997). Both calves and adult cattle have been affected (Carman *et al.*, 1998; Drake *et al.*, 1994; Swecker *et al.*, 1997). In Quebec, mortality due to BVDV in veal operations was estimated at 25% of 143,000 calves for 1993 (Pellerin *et al.*,

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1994). The outbreak in Ontario involved 150 dairy, 600 beef, and 100 veal herds, with mortality of up to 50% in some herds (Carman *et al.*, 1998). Clinical manifestations of severe, peracute BVD include diarrhea, pyrexia, decreased milk production, and oral ulcerations in some cases (Carman *et al.*, 1998; Drake *et al.*, 1994). In addition, concurrent diseases such as pneumonia and abortion were frequently reported in herds experiencing outbreaks (David *et al.*, 1994; Drake *et al.*, 1994). In some herds, sudden death was observed, in which cattle either were found dead without clinical signs or had died within 24 hours of first exhibiting clinical signs (Carman *et al.*, 1998). Postmortem evaluation of affected cattle revealed mucosal-like lesions, including erosions and ulcers in the alimentary tract, necrosis of the intestinal mucosa, and Peyer's patch necrosis (Carman *et al.*, 1998; Swecker *et al.*, 1997).

Analysis of BVDV isolates from outbreaks in Canada and the United States has revealed that they were of the type II genotype of BVDV (Carman *et al.*, 1998; Pellerin *et al.*, 1994; Ridpath *et al.*, 1994). As an example, 61 of the 64 isolates recovered from the outbreaks in Ontario, Canada were type II BVDV (Carman *et al.*, 1998). Prior to these described type II BVDV outbreaks, there was a tendency to depreciate the importance of BVDV as causing clinical disease in cattle immunocompetent to BVDV. The reports of these severe BVDV infections clearly demonstrate that some isolates of BVDV can cause severe life-threatening disease in immunocompetent cattle. The outbreaks of severe BVDV infection have also demonstrated that some isolates of BVDV can induce severe disease and death losses in adult cattle.

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Type II Bovine Viral Diarrhea Virus

Bovine viral diarrhea virus isolates may be subdivided based upon differences in the nucleotide sequence, and these specific differences in the viral nucleic acid sequences are the basis for genotyping. Several different techniques are available for identifying different genotypes of BVDV. Most typing procedures involve the use of molecular biology techniques that identify sequence differences in specific genome segments, or monoclonal antibody profiles (Table 1.1, page 12). Reverse transcription and polymerase chain reaction of the 5' UTR have been the most widely used (Fulton *et al.*, 1999; Ridpath *et al.*, 1994), but reverse transcription and polymerase chain reaction of other regions of the BVDV genome are proving acceptable (Bolin & Ridpath, 1996). Two distinct genotypes of BVDV exist, which are classified as type I and type II (Ridpath *et al.*, 1994). In addition, type I BVDV can be further divided into type Ia and type Ib based upon analysis of the 5' UTR sequence (Ridpath & Bolin, 1998).

Analysis and comparison of nucleic acid sequences from type I and type II genotypes has revealed greater than 30% dissimilarity (Ridpath & Bolin, 1995). This dissimilarity is concentrated in the regions of the viral genome encoding for E2 and the 5' UTR. The differences in nucleic acid sequences between type I and type II BVDV have been reported to be as great as the differences in nucleic acid sequences between type I BVDV and hog cholera virus (Bolin & Ridpath, 1996).

Although outbreaks of severe peracute BVD and the hemorrhagic syndrome have been associated with type II isolates of BVDV, viral virulence does not equate with genotype. Type II BVDV infections have also resulted in subclinical to mild disease under natural (Bolin & Ridpath, 1996) and experimental conditions (Marshall *et al.*,

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1996). Differences in BVDV genotype may define antigenic differences, because differences between type I and type II nucleotide sequences are concentrated in the region of the viral genome encoding for E2. The E2 glycoprotein is the target for neutralizing antibody production by the humoral immune system during infection. Currently, most commercial BVDV vaccines contain isolates of BVDV that are classified as type I. Questions have been raised as to the ability of vaccines containing type I BVDV to induce protection to type II BVDV infection. An antigenically divergent type II BVDV may escape the neutralizing humoral immune response to the E2 glycoprotein elicited by vaccination with a type I BVDV-containing vaccine. This has been demonstrated when a type II BVDV isolate was recovered from a persistently infected calf born to a healthy cow that had been vaccinated with an inactivated vaccine containing type I BVDV (Bolin *et al.*, 1991).

The origin of type II BVDV is unknown, but it is likely that type II BVDV has existed prior to its association with outbreaks of severe peracute disease and the hemorrhagic syndrome. An analysis of BVDV isolates from 1981 was performed, and several type II BVDV isolates were identified (Bolin & Ridpath, 1996). In addition, the recovery of a type II isolate was just as likely in the 1981-1985 period as in the 1991-1994 period during which outbreaks of severe peracute BVDV were occurring (Carman *et al.*, 1998). An explanation for the emergence of type II BVDV and its association with severe peracute disease and the hemorrhagic syndrome cannot be provided; however, most Canadian herds involved in outbreaks of severe peracute BVDV infections had not been vaccinated against BVDV, or the vaccination protocols were incomplete (Carman *et al.*, 1998). Use of vaccines containing type I BVDV provides at least partial protection

from type II BVDV, but

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whereas unvaccinates dev

from type II BVDV, but the protections appears to be of limited duration (Bolin & Ridpath, 1996). For example, a modified-live vaccine containing type I BVDV provided a disease-sparing effect following challenge with a virulent isolate of type II BVDV, whereas unvaccinates developed severe BVDV infection (Cortese *et al.*, 1998).

Table 1.1. Methods for ty

Method
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RT-PCR of 5'UTR and s PCR product
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RT-PCR amplification a NS3 161 bp product
Antibody-based typing (Monoclonal antibody p Cross-neutralization exp

Key:

RT-PCR: Reverse tra
5'UTR: 5' untransl
E0: Region of
NS3: Region of

Table 1.1. Methods for typing isolates of bovine viral diarrhea virus

Method	Reference(s)
Nested RT-PCR of 5'UTR	(el-Kholy *et al.*, 1998; Ridpath *et al.*, 1994; Tajima *et al.*, 1995)
Nested RT-PCR of E0	(Fulton *et al.*, 1999; Sullivan & Akkina, 1995)
RT-PCR of 5'UTR and sequencing of PCR product	(Pellerin *et al.*, 1994; Vilcek *et al.*, 1999a; Vilcek *et al.*, 1999b)
RT-PCR of 5'UTR followed by restriction digestion of PCR products	(Harpin *et al.*, 1995; Vilcek *et al.*, 1994)
RT-PCR amplification and sequencing of NS3 161 bp product	(Yousif *et al.*, 1999)
Antibody-based typing methods (Monoclonal antibody panel analysis or Cross-neutralization experiments)	(Beer & Wolf, 1999; Dekker *et al.*, 1995; Deregt *et al.*, 1998; Paton *et al.*, 1995; Pellerin *et al.*, 1994)

Key:
RT-PCR: Reverse transcription-polymerase chain reaction
5'UTR: 5' untranslated region of the BVDV genome
E0: Region of BVDV genome coding for the structural glycoprotein 0 (gp48)
NS3: Region of BVDV genome coding for the nonstructural polypeptide 3 (p80)

The original report o
description (Olafson *et*
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Bovine Viral Diarrhea Virus and the Hemorrhagic Syndrome

The original report of BVDV infection in cattle included hemorrhages in its clinical description (Olafson *et al.*, 1946). In addition, the first report on mucosal disease included descriptions of hemorrhages, which were present on pathologic examination in the subepicardium, gall bladder, and gastrointestinal mucosa (Ramsey & Chivers, 1953). As clinical cases of BVDV were being reported throughout the world, hemorrhages were described. As an example, abomasal and renal hemorrhages were observed in calves naturally infected with noncytopathic BVDV in Hungary (Romvary, 1965). Although hemorrhages were described in these early reports, thrombocytopenia was not reported, or in many cases, evaluation of platelet counts was not performed.

A bleeding syndrome in veal calves, consistent with later descriptions of BVDV-induced thrombocytopenia, was first reported in 1978 (Harrison, 1978). However, the investigator did not include diagnostics for BVDV in the investigation, so it is uncertain if this was the first report describing BVDV-induced thrombocytopenia. Thrombocytopenia in association with BVDV infection was first reported in New York (Perdrizet *et al.*, 1987; Rebhun *et al.*, 1989). These first descriptions occurred in calves and adult cattle naturally infected with BVDV (Perdrizet *et al.*, 1987; Rebhun *et al.*, 1989). In a retrospective case study, 15 animals ranging in age from 1 to 8 years were diagnosed with BVDV and thrombocytopenia. In these animals, platelet counts ranged from 2,000 platelets/μl to 33,000 platelets/μl. All cattle were treated symptomatically, but only 6 animals survived the thrombocytopenic BVDV infection. In general, the cattle that survived the thrombocytopenic BVDV infection had higher platelet counts than the

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cattle that died. Of the 6 survivors, 4 survivors had platelet counts greater that 25,000 platelets/μl. Bloody diarrhea was the most common clinical sign associated with the thrombocytopenic BVDV infections in these cattle. Other clinical signs included epistaxis, petechial hemorrhages, ecchymotic hemorrhages, and bleeding from injection sites or insect bites. Cattle with platelet counts less than 25,000 platelets/μl had the most severe signs of bleeding. Coagulation testing, consisting of prothrombin time, activated partial thromboplastin time, and fibrinogen determination, was performed on 6 cattle, and the results obtained were normal. This provided the first evidence that the thrombocytopenia and hemorrhages were not likely the result of disseminated intravascular coagulation. An interesting observation was the presence of leukopenia in 14 of 15 cattle and nonregenerative anemia in 3 of the 15 cattle. All 14 leukopenic cattle had a neutropenia and 10 of the 14 leukopenic cattle had a lymphopenia. The presence of pancytopenia (thrombocytopenia, neutropenia, and a nonregenerative anemia) in these animals prompted this group of investigators to speculate that myelosuppression or marrow necrosis was the mechanism of BVDV-induced thrombocytopenia (Rebhun *et al.*, 1989).

Thrombocytopenia, anemia, and bone marrow necrosis were also demonstrated in a case report describing 2 beef calves naturally infected with BVDV (Scruggs *et al.*, 1995). The platelet count was only performed in 1 of the calves, and was 16,000 platelets/μl. A postmortem bone marrow evaluation was performed on both calves. Decreased numbers of megakaryocytes and megakaryocyte degeneration, characterized by pyknotic nuclei, vacuolated pale staining eosinophilic cytoplasm, and poorly defined cytoplasmic borders, were observed. In addition to megakaryocyte necrosis, osteopetrosis of the long bones

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was observed as an incidental finding. This case report supported the earlier hypothesis (Rebhun *et al.*, 1989), that BVDV-induced thrombocytopenia is likely due to bone marrow suppression and decreased platelet production.

Thrombocytopenic BVDV infections were experimentally reproduced shortly after the original descriptions in naturally infected cattle appeared (Bezek *et al.*, 1994; Bolin & Ridpath, 1992; Corapi *et al.*, 1990; Corapi *et al.*, 1989). Thrombocytopenia is defined as a reduction in the platelet count below the minimum level of the species (Jain, 1986). Intravenous injection of blood mononuclear cells from affected cattle was used in early experimental thrombocytopenic BVDV infections (Bezek *et al.*, 1994; Corapi *et al.*, 1990; Corapi *et al.*, 1989). Intranasal inoculation of cattle with BVDV-infected cell culture preparations was used in later experimental infections (Bezek *et al.*, 1994; Bolin & Ridpath, 1992; Ellis *et al.*, 1998). One study compared these two inoculation methods in their ability to induce thrombocytopenia, and found that intravenous inoculation of BVDV-infected mononuclear cells was more effective in inducing thrombocytopenia (Bezek *et al.*, 1994). In spite of this finding, later investigators using experimental infections chose intranasal routes of inoculation to simulate natural exposure. To date, the BVDV isolates that have been shown to induce thrombocytopenia following experimental inoculation in cattle include CD87 (Bezek *et al.*, 1994; Corapi *et al.*, 1990), CD89 (Corapi *et al.*, 1989), BVDV 890 (Bolin & Ridpath, 1992), BVDV 24515 (Ellis *et al.*, 1998), and NY-1 (Marshall *et al.*, 1998). Platelet count has also been evaluated with the type II BVDV isolates 7937 and 126 (Marshall *et al.*, 1996), and the type I BVDV isolates NADL (Bezek *et al.*, 1994) and TGAN (Bolin & Ridpath, 1992), but these isolates failed to induce thrombocytopenia in experimentally infected calves.

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Interestingly, the type I isolate NY-1 has been shown to induce thrombocytopenia in one study (Marshall *et al.*, 1998), but failed to induce it in another study (Bezek *et al.*, 1994). Other BVDV isolates may be capable of inducing thrombocytopenia, but the aforementioned BVDV isolates are the only isolates that have been examined for their ability to induce thrombocytopenia thus far. All of the aforementioned BVDV isolates, which induce thrombocytopenia, are noncytopathic. In addition, all isolates belong to the type II genotype of BVDV, with the exception of NY-1, which is a type I BVDV isolate.

With respect to the platelet count following experimental inoculation, a decline in the platelet count was observed to occur as early as 3 days after inoculation, with maximum platelet count depression occurring between days 9 and 17 following infection (Bezek *et al.*, 1994; Bolin & Ridpath, 1992; Corapi *et al.*, 1990; Corapi *et al.*, 1989). Several experiments monitored recovery of platelet counts following experimental inoculation. In calves infected with BVDV 890, a gradual rise in the platelet count would occur between days 10-15 after inoculation (Bolin & Ridpath, 1992). In calves infected with CD87 and CD89, an elevation in the platelet count from thrombocytopenic levels did not occur until at least 17 days after inoculation (Corapi *et al.*, 1990; Corapi *et al.*, 1989). In several calves infected with CD89 and CD87, the platelet count remained low until 35 days after inoculation. Interestingly, the rise in platelet count in calves recovering from CD87 or CD89 infection corresponded to the presence of serum neutralizing antibody (Corapi *et al.*, 1990). This study was unique in that a prolonged viremia following BVDV infection was observed in some of the calves (Corapi *et al.*, 1989). Virus was isolated from some calves as late as 46 days after inoculation, and subsequently, the appearance of neutralizing antibodies to BVDV was delayed to beyond day 50 after

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Clinical signs of hemorrhage have been observed in all reports of experimental BVDV infections resulting in thrombocytopenia (Bezek *et al.*, 1994; Bolin & Ridpath, 1992; Corapi *et al.*, 1990; Corapi *et al.*, 1989; Ellis *et al.*, 1998). Clinical signs of spontaneous hemorrhage occur when platelet numbers fall below 20,000 platelets/μl, and may be present if platelet numbers are between 20,000 and 50,000/μl (Jain, 1986). In studies utilizing the BVDV isolates CD87 and CD89, clinical evidence of hemorrhage was only observed in cattle whose platelet count decreased to less than 5,000 platelets/μl (Corapi *et al.*, 1990). In contrast, BVDV 890 infection in calves resulted in clinical evidence of hemorrhage in 3 out of 7 calves in which platelet counts failed to drop below 70,000 platelets/μl at the time of death. This discrepancy between the development of hemorrhage and the platelet count prompted our investigations of platelet function in calves infected with BVDV. The reader is referred to Chapters III and IV of this dissertation for discussion of our platelet function studies.

The extent and location of hemorrhages in experimentally infected calves are similar to the reports describing BVDV infection in naturally infected calves (Rebhun *et al.*, 1989; Scruggs *et al.*, 1995; Swecker *et al.*, 1997). Hemorrhages have been observed during physical examination of infected calves on the oral mucosa, ventral surface of the tongue, eye (hyphema), sclera and conjunctiva of the eye, and subcutis, especially in areas surrounding the carpus and hock (Corapi *et al.*, 1990). Prolonged bleeding from venipuncture sites was a common finding in BVDV-infected calves (Corapi *et al.*, 1990;

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Corapi *et al.*, 1989). Epistaxis was also observed (Corapi *et al.*, 1990), but less frequently than other antemortem hemorrhages. On postmortem examination, hemorrhages are most typically found in the alimentary tract, especially the rumen and ileum (Bolin & Ridpath, 1992; Corapi *et al.*, 1990; Corapi *et al.*, 1989). Extensive hemorrhages were found in lymph nodes in the calves infected with the type II isolate BVDV 890 and the type I isolate BVDV NY-1 (Bolin & Ridpath, 1992; Marshall *et al.*, 1998). Additional hemorrhages were observed in the urinary bladder, abomasum, reticulum, rumen, omasum, adrenal glands, gallbladder, heart, thymus, esophagus, brain and calvaria, diaphragm, and aorta. A suffusive, subserosal hemorrhage in the mucosa of the reticulum in one calf was the only hemorrhage reported using the isolate BVDV 24515 (Ellis *et al.*, 1998). Moreover, the platelet count was less than 10,000 platelets/μl in this calf.

Coagulation profile data from an experimental thrombocytopenic BVDV infection revealed no abnormalities (Corapi *et al.*, 1989), which supported the previous coagulation data findings from naturally infected cattle, indicating that disseminated intravascular coagulation is not likely to be the mechanism of BVDV-induced thrombocytopenia. However, coagulation profiles were only performed in a single experimental infection (Corapi *et al.*, 1989).

Neutropenia was an additional, consistent laboratory finding in the experimental type II BVDV infections (Bolin & Ridpath, 1992; Corapi *et al.*, 1990; Corapi *et al.*, 1989; Ellis *et al.*, 1998). In calves infected with BVDV 24515, bone marrow examination revealed that granulopoiesis was minimal, with immature granulocytic cells predominating (Ellis *et al.*, 1998). As similar to the thrombocytopenia, the neutropenia

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An interesting observation in previous experimental BVDV infections was the demonstration of BVDV association with platelets (Bezek *et al.*, 1994; Bolin & Ridpath, 1992; Corapi *et al.*, 1990; Corapi *et al.*, 1989). The association of BVDV with peripheral platelets was demonstrated by virus isolation from purified platelets (Bolin & Ridpath, 1992) and immunofluorescent staining of platelet preparations (Bezek *et al.*, 1994; Corapi *et al.*, 1990). This association was determined to occur between days 7 through 17 after inoculation (Bezek *et al.*, 1994) and days 12 through 15 after inoculation (Corapi *et al.*, 1989). Both studies utilized the BVDV isolate CD87. Another study evaluating CD87 and CD89 demonstrated that virus association with platelets was observed on day 11 after inoculation in 9 of 12 calves examined, but the specifics of which viral isolates were associated with platelets were not reported (Corapi *et al.*, 1990). An association of BVDV 890 with platelets was also demonstrated (Bolin & Ridpath, 1992). Virus association with platelets was demonstrated for an average of 5.9 days in 7 infected calves, but the initial and last days after inoculation in which an association could be demonstrated was not reported. Evaluation of BVDV 890 association with platelets was also performed in our experimental infections (See Chapters II, III, IV, and V). To date, virus association with platelets has not been evaluated with the thrombocytopenia-inducing BVDV isolates NY-1 and 24515. The origin and nature of the association of BVDV with platelets is unknown. It has been suggested that the platelets may serve as "carriers" of circulating virus (Corapi *et al.*, 1989). This may be important in the pathogenesis of the hemorrhagic syndrome, as platelets may allow for a more rapid and

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extensive dissemination of virus throughout the body. It is unlikely that BVDV associates with platelets by nonspecific adherence. Mixing experiments utilizing platelets from an uninfected normal calf and BVDV-positive serum were performed (Corapi *et al.*, 1989). After incubation and washing of platelets, virus was not isolated. In addition, we performed mixing experiments between platelets from an uninfected steer and BVDV-infected cell culture supernatant, and virus was not isolated from the washed platelets (unpublished observation). These findings suggest that BVDV may be internalized in the platelet. Internalized virus in platelets may occur as a result of receptor-mediated uptake of BVDV by platelets or may be the result of megakaryocyte infection with BVDV. Further study utilizing immunoelectron microscopy may clarify the nature of this BVDV-platelet association.

Pathologic examination of experimental thrombocytopenic BVDV infections has been performed for all the aforementioned thrombocytopenia-inducing BVDV isolates. Extent and location of hemorrhages have been described previously in this literature review. In general, lesions have been less pronounced in the experimental reproduction than in the reports describing the natural outbreaks of severe peracute BVDV or the hemorrhagic syndrome (Carman *et al.*, 1998; Rebhun *et al.*, 1989). For example, the only pathologic findings in calves infected with BVDV 24515 were hemorrhages and pulmonary consolidation (Ellis *et al.*, 1998). Besides hemorrhages, linear erosions in the spiral colon similar to lesions described for cases of mucosal disease, were observed in calves infected with BVDV 890 (Bolin & Ridpath, 1992). In addition, abomasal edema was observed in calves infected with BVDV 890 (Bolin & Ridpath, 1992), and this was a

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Histologic evaluation of bone marrow has also been performed in the experimental thrombocytopenic BVDV infections. In calves infected with CD87, a pronounced megakaryocyte hyperplasia was observed at day 12 after inoculation (Corapi *et al.*, 1989). The significance of this finding was unknown at the time, but this group of investigators hypothesized that decreased platelet production was not the mechanism of BVDV-induced thrombocytopenia because of the presence of megakaryocyte hyperplasia. In contrast, decreased thrombopoiesis was observed at days 10-12 after inoculation in 5 of 6 calves infected with BVDV 24515 (Ellis *et al.*, 1998). Of those 5 calves, overt bone marrow necrosis, characterized by areas of indistinct morphology, karyorrhexis, and phagocytosis of debris by large mononuclear cells, was reported in 2 calves. Megakaryocyte hyperplasia, characterized by increased numbers of immature megakaryocytes was observed in the remaining calf (Ellis *et al.*, 1998). Bone marrow evaluation was not described for BVDV 890-infected calves (Bolin & Ridpath, 1992). In calves infected with the type I isolate NY-1, bone marrow findings were unremarkable (Marshall *et al.*, 1998). Different interpretations of histologic examination of bone marrow likely stem from the fact that marrow was examined at a single, variable point in time. In addition, kinetics of infection may be different for the viral isolates examined. Histologic evaluation of bone marrow from BVDV-infected calves was performed in our experimental infections with BVDV 890 (see Chapters II and V).

Distribution of BVDV in tissues of experimentally infected calves has been evaluated using immunofluorescent antibody and immunohistochemical staining for

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BVDV antigen. The most interesting observation relevant to the discussion of thrombocytopenia has been the identification of BVDV antigen in megakaryocytes. Viral antigen in megakaryocytes has been demonstrated in CD87-infected calves by use of immunofluorescent antibody testing (Corapi *et al.*, 1989), and in 24515-infected calves by use of immunohistochemistry (Ellis *et al.*, 1998). Viral antigen in megakaryocytes has also been demonstrated in other studies utilizing isolates that did not induce thrombocytopenia (Marshall *et al.*, 1996) or in which the platelet count was not determined (Spagnuolo *et al.*, 1997; Spagnuolo-Weaver *et al.*, 1997). In calves infected with the type I isolate NY-1, immunohistochemistry was performed on tissue sections, but megakaryocyte infection, as determined by viral antigen in megakaryocytes, was not demonstrated (Marshall *et al.*, 1996). Megakaryocyte infection with BVDV has not been determined for the thrombocytopenia-inducing BVDV isolate CD-89. We are the only group of investigators that has demonstrated BVDV 890 infection of megakaryocytes (see Chapters II and V).

The mechanism of BVDV-induced thrombocytopenia is unknown. Thrombocytopenia, regardless of the underlying etiology, results from only three basic mechanisms: decreased production, accelerated destruction, or abnormal sequestration of platelets (Warkentin & Kelton, 1994). The mechanism of BVDV-induced thrombocytopenia may involve one or a combination of these mechanisms. Coating of platelets with antibodies or deposition of viral antigen-antibody complexes on platelet membranes can trigger immune-mediated destruction of platelets (Shulman & Reid, 1994). Immune-mediated destruction of platelets in the peripheral circulation as the cause of BVDV-induced thrombocytopenia is not likely, based upon a previous study

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demonstrating that there was no evidence of platelet-associated antibody and complement in CD87 and CD89-infected calves (Corapi *et al.*, 1989). Disseminated intravascular coagulation also causes thrombocytopenia due to an accelerated destruction of platelets (Shulman & Reid, 1994), but normal coagulation profiles in CD87 and CD89-infected calves suggest that disseminated intravascular coagulation is not involved in BVDV-induced thrombocytopenia (Corapi *et al.*, 1989). Abnormal sequestration of platelets is not likely the cause of BVDV-induced thrombocytopenia because hepatosplenomegaly was not observed on postmortem examination in BVDV 890-infected calves (Bolin & Ridpath, 1992), 24515-infected calves (Ellis *et al.*, 1998), and calves infected with CD87 or CD89 (Corapi *et al.*, 1990). In addition, postmortem examination of the spleen in BVDV 890-infected calves revealed them to be smaller than normal in size (Bolin & Ridpath, 1992). The lack of evidence that accelerated destruction and abnormal sequestration are involved in BVDV-induced thrombocytopenia and histologic bone marrow findings of marrow necrosis and viral antigen in megakaryocytes support the hypothesis that decreased production of platelets is the primary mechanism of BVDV-induced thrombocytopenia.

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The Bovine Platelet

Platelets are the smallest and most numerous elements in blood (Gentry, 1992; Johnstone, 1988). Platelets circulate as anucleate, discoid cells. Although small and anucleate, platelets participate in numerous physiologic and pathologic processes including maintenance of vascular integrity, coagulation, fibrinolysis, inflammation, thrombosis, tumor metastasis, neovascularization, and repair (Gentry, 1992). Platelets play a pivotal role in the maintenance of normal hemostasis, via their close interaction with endothelial cells. Injury to blood vessels triggers the hemostatic process. Platelets adhere to exposed subendothelial collagen or damaged endothelial cells and become activated. By adhering to injured blood vessels, and then each other (aggregation), platelets form hemostatic aggregates that contribute to the cessation of hemorrhage. Platelets contain intracytoplasmic granules that contain adenosine diphosphate, calcium, and serotonin (Gentry, 1992). When secreted, these substances promote platelet aggregation and vasoconstriction, which further enhances hemostasis. In addition to their function during blood vessel injury, the interaction between platelets and endothelial cells promotes wound healing after tissue damage, as a result of soluble substances released from platelets, including platelet-derived growth factor, epidermal growth factor, and transforming growth factor-ß (Gentry, 1992). Platelets also modulate the inflammatory response through cellular interactions with other cellular components such as neutrophils.

Platelets are derived from their hematopoietic precursor cell, the megakaryocytes (Wright, 1906). Although megakaryocytes comprise only 0.05% of the nucleated cells in the bone marrow (Harker, 1968), they are responsible for maintaining the large

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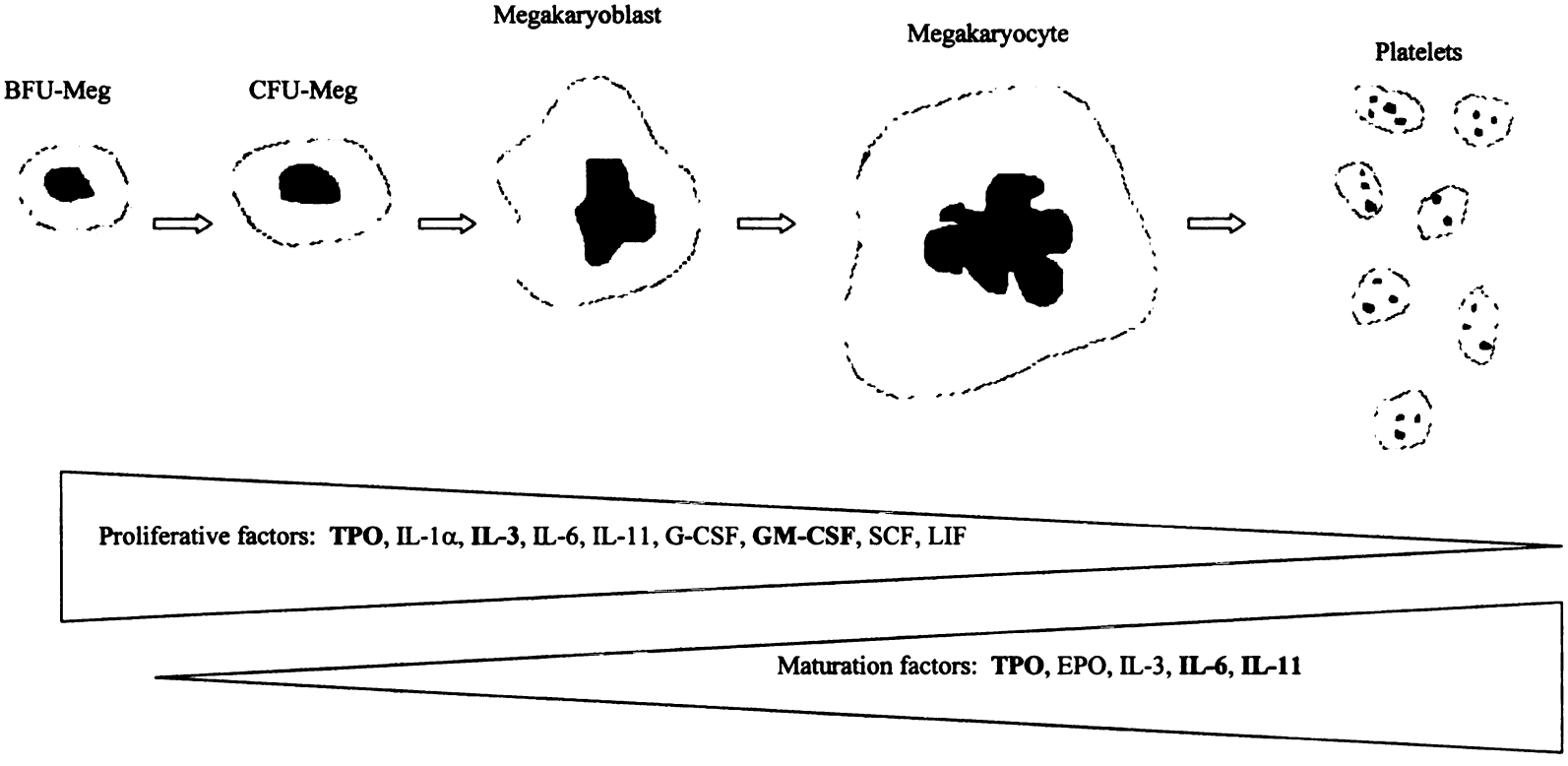
circulating platelet population. The megakaryocyte is derived from a pluripotent stem cell that is also common to the myeloid and erythroid cell lines (Zauli & Catani, 1995). Megakaryocytes undergo endoreduplication, which is a complex process involving a progressive increase in nuclear ploidy (from 2N to 64N) during proliferation (Zauli & Catani, 1995). Proliferation and maturation of megakaryocytes are regulated by numerous soluble cytokines (Figure 1.1, page 26), of which thrombopoietin has received considerable attention in recent years. Thrombopoietin is considered the most potent known stimulator of endoreduplication and polyploidy in megakaryocytes (Broudy & Kaushansky, 1995; Kaushansky, 1998). In addition, thrombopoietin is a potent megakaryocyte colony stimulating factor, capable of inducing colony formation from as many as two thirds of all megakaryocyte progenitors (Kaushansky, 1998). Many cytokines act synergistically in megakaryocytopoiesis. Thrombopoietin and the combination of interleukin-3, interleukin-11, and steel factor (stem cell factor) influence megakaryocyte growth (Broudy *et al.*, 1995). Platelets are produced by the fragmentation of the cytoplasm of mature megakaryocytes (Breton-Gorius & Reyes, 1976). Initially, the maturation of megakaryocytes is associated with the appearance of the demarcation membrane system, which is contiguous with the surface membrane of the megakaryocyte (Breton-Gorius & Reyes, 1976). Platelets and proplatelets are fragmented from individual areas in the demarcation membrane system.

Figure 1.1
Scheme of megakaryocytopoiesis (adapted from Zauli & Catani, 1995)

Figure 1.1
Scheme of megakaryocytopoiesis (adapted from Zauli & Catani, 1995)



Key: BFU-Meg: Burst forming unit-Megakaryocyte
CFU-Meg: Colony forming unit-Megakaryocyte
GM-CSF: Granulocyte/Macrophage colony stimulating factor
G-CSF: Granulocyte colony stimulating factor
SCF: Stem cell factor (Steel factor)
TPO: Thrombopoietin
IL: Interleukin
EPO: Erythropoietin
LIF: Leukemia inhibitory factor

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The primary function of platelets is maintenance of vascular integrity. Upon activation, platelets undergo a complex series of events described as adherence, shape change, secretion, and aggregation. The mechanisms of platelet activation, including the receptors, signal transduction pathways, the soluble substances released during platelet degranulation, and the interactions between platelets and the coagulation system and other cellular elements, have been reviewed (Gentry, 1992). In general, the structure and function of bovine platelets has not been characterized to the extent of human, canine, and feline platelets. The focus of the remainder of this section will be on differences between bovine platelets and those of other species, and platelet disorders of cattle.

Platelet structure has been reviewed (White & Gerrard, 1994). The platelet can be divided into 4 compartments or structural domains: the membrane system, the sol-gel zone, the organelle zone, and the peripheral zone. As part of the membrane system, the surface-connected open canalicular system is a series of invaginations in the plasma membrane whose function is to increase surface area for the presence of receptors and exchange of signals (White & Gerrard, 1994). Bovine platelets do not possess an open canalicular system, which is found in all other mammalian platelets (Zucker-Franklin *et al.*, 1985). In human platelets, the release reaction is accompanied by a centralization of granules with release of granule contents into the open canalicular system (Zucker-Franklin, 1969). In contrast, the granules remain distributed peripherally in bovine platelets, and the release of granule contents occurs on the cell surface of bovine platelets. The significance of this observation in terms of function is unknown, as bovine platelets function similarly in hemostasis as human platelets (Zucker-Franklin *et al.*, 1985).

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Following activation, platelets change shape. Platelets become irregular and extend long, pointed pseudopods, which provide surface receptors for the adhesive platelet-endothelial cell and aggregative platelet-platelet reactions. Continuous stimulation causes the platelets to develop into spread forms in which cytoplasm extends into the pseudopods. The bovine platelet is also able to change shape, but unlike platelets from other species, the development of the cytoplasm-filled pseudopods does not occur and bovine platelets do not possess the ability to spread over an exposed surface (Grouse *et al.*, 1990). In addition, once shape change has occurred, bovine platelets are more resistant to deformation than human platelets (Grouse *et al.*, 1990). The significance of these findings are unknown (Gentry, 1992; Zucker-Franklin *et al.*, 1985).

Differences exist between bovine platelets and platelets from other species in the ability of agonists to induce platelet aggregation (Gentry, 1992; Marzec *et al.*, 1975). Bovine platelets, in general, are less receptive to stimulation by adenosine diphosphate than platelets of most other mammalian species (Marzec *et al.*, 1975). Bovine platelets require 5 to 10 times more adenosine diphosphate to elicit maximal aggregation than platelets from sheep, horses, humans, and primates (Bondy & Gentry, 1989). This decreased sensitivity to adenosine diphosphate observed *in vitro* may be due to impaired secretion because bovine platelets do not possess an open canalicular system (Bondy & Gentry, 1989). In addition, bovine platelets are not responsive to either arachidonic acid or thromboxane A_2, in spite of the fact that large amounts of thromboxane A_2 are released from these platelets in response to agonists such as collagen and adenosine diphosphate (Meyers *et al.*, 1980). In fact, there is no correlation between thromboxane A_2 production and release and the extent of platelet aggregation in bovine platelets (Bondy & Gentry,

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1989). The significance of this finding is that bovine platelets have a poorly developed arachidonic acid pathway, and platelet aggregation in cattle is dependent upon other pathways such as a phospholipid pathway and calcium mobilization (Bondy & Gentry, 1989; Gentry *et al.*, 1989). This observation is supported by the inability of cyclooxygenase inhibitors such as aspirin to have an effect on platelet aggregation in cattle (Gentry *et al.*, 1989).

Variation exists in the normal platelet count in cattle (Nelson *et al.*, 1974; Semrad & Dubielzig, 1993; Vaugher *et al.*, 1973). Platelet counts on 32 normal male Holstein calves ranging in age from 9 to 15 weeks were 192,000 to 892,000 platelets/μl (Nelson *et al.*, 1974). In their study, the mean ± 2 standard deviations of the mean was 542,000 ± 175,000; however, the actual observed minimum and maximum platelet counts observed in those Holstein calves was 274,000 and 836,000 platelets/μl (Nelson *et al.*, 1974). Another study reported the mean platelet count in Holstein calves less than 2 months of age to be 841,000 platelets/μl with a range of 480,000 to 1,200,000 platelets/μl (Vaugher *et al.*, 1973). In another 4 calves less than 2 months of age, the average platelet count was 841,000 platelets/μl (Winqvist, 1954). The mean platelet count in 14 Holstein calves weighing between 115 to 300 pounds was 632,000 platelets/μl (Marzec *et al.*, 1975). Only one study has evaluated the platelet count in neonatal calves (Semrad & Dubielzig, 1993). At birth, the mean platelet count ± standard error of the mean for 5 Holstein calves was 212,050 ± 73,945 platelets/μl. The platelet count increased progressively as the calves aged with a mean platelet count of 325,500 ± 55,672 on day 1 of age, 447,750 ± 48,120 on day 2 of age, 502,000 ± 75,182 on day 3 of age, and 645,750 ± 54,571 on day 4 of age (Semrad & Dubielzig, 1993). Platelet counts were not continued beyond

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Platelet life span in cattle has also been determined (Baker *et al.*, 1998; Marzec *et al.*, 1975). Platelet life span ranges from 4.9 to 10 days. The most recent study assessed the technique of *ex vivo* whole blood biotinylation for evaluating platelet life span in calves implanted with an intraventricular assist device (Baker *et al.*, 1998). Platelet life span in 1 normal male Jersey calf was calculated to be 4.9 days, compared to a platelet life span of 6.1 days for a male Jersey calf with an intraventricular assist device (Baker *et al.*, 1998). Another study utilized disappearance of radioactivity as a method for determining platelet life span in Holstein calves (Marzec *et al.*, 1975). Platelet life span was determined for 5 calves and averaged 6.22 days with a range of 5 to 10 days (Marzec *et al.*, 1975). In the earliest study on platelet life in calves, platelet life span was determined to be 9.7 days in female Holstein calves using platelets radiolabeled *in vitro,* and then transfused back into the autologous calf. In summary, platelet life span ranged from 4.9 to 9.7 days in cattle.

Platelet Disorders of Cattle

Platelet disorders can be broadly categorized as qualitative or quantitative, and inherited or acquired. Qualitative platelet disorders are also known as thrombocytopathies and involve impairment of platelet function. Quantitative platelet disorders may involve a decrease in the number of circulating platelets

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(thormobocytopenia) or may involve an increase in the number of circulating platelets (thrombocytosis). Thrombocytosis has not been reported in cattle, so the discussion on quantitative platelet disorders will be limited to thrombocytopenia.

In general, platelet abnormalities often present with a characteristic group of clinical features, as compared to other abnormalities of the coagulation system (Johnstone, 1988). Petechiae are common clinical signs and hematomas are rare clinical signs associated with platelet disorders. In addition, bleeding at multiple sites and often involving mucous membranes, and prolonged bleeding from cuts are typical of platelet disorders. In contrast, abnormalities in the coagulation system often present with hematomas, localized bleeding, and bleeding into body cavities, muscles, and joints. Moreover, petechiae are rare with coagulation disorders, and the bleeding may be delayed at onset, then profuse, or the bleeding may stop and start again (Johnstone, 1988).

Qualitative platelet disorders of cattle

A summary of qualitative disorders of cattle is presented in Table 1.2 (page 35). Inherited disorders have been the best characterized and include Simmental hereditary thrombopathy and the Chédiak-Higashi syndrome. Acquired qualitative platelet disorders are less characterized in cattle. The most common acquired qualitative platelet disorders in dogs and cats are drug-induced (Ruiz de Gopegui & Feldman, 1998); however, drug-induced platelet disorders have not been described in cattle. Qualitative platelet disorders are characterized by an increased tendency to bleed or a prolonged bleeding time in the presence of a normal or increased platelet count (Jain, 1986). Diagnosis of qualitative bleeding disorders involves evaluation of the bleeding time and

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platelet function studies, specifically platelet aggregation testing. In addition, evaluation of the platelet count to rule out quantitative platelet disorders and evaluation of coagulation profiles to rule out disseminated intravascular coagulation are necessary.

Chédiak-Higashi syndrome is an inherited disease of platelet function that has been reported in Hereford (Bell *et al.*, 1976; Burns *et al.*, 1984; Padgett *et al.*, 1967), Brangus (Ayers *et al.*, 1988), and Japanese Black cattle (Akuzawa *et al.*, 1991; Fushuku *et al.*, 1999; Shiraishi *et al.*, 1998). Chédiak-Higashi syndrome has also been reported in humans, cats, rats, mice, foxes, mink, and killer whales (Prieur & Collier, 1978; Ruiz de Gopegui & Feldman, 1998). Chédiak-Higashi syndrome has an autosomal recessive mode of inheritance (Padgett *et al.*, 1964). In general, affected animals are incomplete albinos with generalized oculocutaneous hypopigmentation, with the exception of Japanese Black cattle in which cutaneous ablinism is an infrequent finding (Ogawa *et al.*, 1997; Ruiz de Gopegui & Feldman, 1998). Bleeding times are prolonged in affected cattle (Bell *et al.*, 1976; Ogawa *et al.*, 1997). In studies evaluating *in vitro* platelet aggregation, platelets from affected cattle fail to aggregate in response to collagen (Bell *et al.*, 1976; Ogawa *et al.*, 1997). Ultrastructurally, platelets from cattle affected with Chédiak-Higashi syndrome are characterized by a deficiency of platelet-dense granules (Bell *et al.*, 1976; Ogawa *et al.*, 1997). Decreased granule contents and decreased secretion of ATP, ADP, and serotonin are characteristic of platelets from cattle with Chédiak-Higashi syndrome. These findings have characterized Chédiak-Higashi syndrome as a storage pool disease (Bell *et al.*, 1976; Ogawa *et al.*, 1997; Shiraishi *et al.*, 1998). Recent evidence suggests that decreased granule content is not the only mechanism of decreased platelet function, and impaired cytosolic calcium mobilization

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may be involved (Shiraishi *et al.*, 1998). Impairment of calcium mobilization has now been hypothesized to be the major mechanism of decreased platelet aggregation in Japanese Black cattle affected with Chédiak-Higashi syndrome, rather than a decreased release of endogenous substances such as ADP, ATP, and serotonin (Shiraishi *et al.*, 1998).

Simmental hereditary thrombopathy has been described in Canada (Gentry *et al.*, 1997; Searcy *et al.*, 1990) and the United States (Steficek *et al.*, 1993a; Steficek *et al.*, 1993b). Mode of inheritance is unknown, but the distribution in affected cattle supports an autosomal recessive mode of inheritance (Steficek, 1994). Affected cattle commonly exhibit recurrent episodes of epistaxis and hematuria, and they bleed excessively as a result of minor trauma, during surgery, and at parturition. Laboratory evaluation, including hematologic assessment and platelet counts, coagulation profiles, and biochemistry profiles has been performed, and all values were comparable to normal reference ranges and normal matched Simmental cattle (Searcy & Petrie, 1990; Steficek *et al.*, 1993a). In platelet function studies, platelets from affected cattle had no aggregation response to adenosine diphosphate or the calcium ionophore A23187. In response to platelet activating factor and thrombin, platelet aggregation, although present, was decreased or took longer to complete when compared to control bovine platelets (Steficek *et al.*, 1993a). Platelet shape change was demonstrated with all agonists examined, which indicated the presence of functional receptors (Steficek *et al.*, 1993a). Ultrastructurally, platelets from affected Simmental cattle appeared normal. At present, the impairment in platelet function observed in affected Simmental cattle appears to be altered mobilization or utilization of external calcium (Steficek, 1994).

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Infection of cattle with *Pasteurella hemolytica*, recently moved to the genus *Mannheimia*, has been associated with an acquired qualitative platelet defect (Cheryk *et al.*, 1998). Platelet function was assessed before and after intrabronchial challenge with *Mannheimia hemolytica*, and platelets became more reactive to stimulation with known platelet agonists such as adenosine diphosphate and platelet activating factor (Cheryk *et al.*, 1998). In addition, direct exposure of bovine platelets to *Mannheimia hemolytica* leukotoxin in an *in vitro* setting resulted in increased platelet reactivity (Cheryk *et al.*, 1998). The findings of this study may partially explain the respiratory tract lesions associated with *Mannheimia hemolytica* infection in cattle. Thrombosis in the lungs of cattle infected with *Mannheimia hemolytica* may be the result of the action of leukotoxin on platelets, and the subsequent increased stimulus for aggregation. Unlike *Mannheimia hemolytica*, BVDV induces decreased platelet function as discussed in Chapters III and IV.

The trichothecene mycotoxin, T-2 mycotoxin, has been demonstrated to impair both the rate and extent of platelet aggregate formation (Bondy *et al.*, 1989; Gentry *et al.*, 1987). The most dramatic change following *in vitro* exposure of the T-2 mycotoxin to bovine platelets was the relative instability of the platelet aggregates that formed in the presence of the toxin (Bondy *et al.*, 1989). The biological significance of these findings is unknown, as these studies were conducted because bovine platelets may be used as a model cell system to evaluate the effects of T-2 toxin on cell function *in vitro* (Bondy *et al.*, 1989; Gentry *et al.*, 1987).

Table 1.2. Qualitative platelet disorders of cattle

Inherited qualitative platelet disorders of cattle	
Disorder	**Reference(s)**
Simmental hereditary thrombopathy	(Gentry *et al.*, 1997; Steficek *et al.*, 1993a; Steficek *et al.*, 1993b)
Chédiak-Higashi syndrome	(Bell *et al.*, 1976; Kim *et al.*, 1985; Menard *et al.*, 1990; Meyers & Seachord, 1990; Meyers *et al.*, 1982; Prieur *et al.*, (Akuzawa *et al.*, 1991; Fushuku *et al.*, 1999; Fushuku *et al.*, 1998; Shiraishi *et al.*, 1998)
Acquired qualitative platelet disorders of cattle	
Disorder	**Reference(s)**
Bovine viral diarrhea virus	(Walz *et al.*, 1999b)
Pasteurella haemolytica A1 (*Mannheimia haemolytica*)	(Cheryk *et al.*, 1998)
Mycotoxicosis (T-2 toxin)	(Bondy *et al.*, 1989)

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Quantitative platelet disorders of cattle

A summary of quantitative platelet disorders of cattle is presented in Table 1.3 (page 39). Thrombocytopenia is defined as a reduction in the number of circulating platelets below the minimum normal level for the species (Jain, 1986). In general, clinical signs of spontaneous hemorrhage rarely occur unless the platelet count has decreased to less than 20,000 platelets/μL (Jain, 1986). However, bleeding has sometimes been observed in cattle when the platelet count is between 20,000 and 50,000 platelets/μL (Jain, 1986). Purpura, hemorrhage, prolonged bleeding times, and failure of clot retraction are commonly seen when the circulating platelet count decreases to less than 10,000 platelets/μL.

Regardless of the underlying etiology, only three basic mechanisms are involved in the induction of thrombocytopenia: decreased production, accelerated destruction, or abnormal sequestration of platelets (Warkentin & Kelton, 1994). In certain diseases, multiple mechanisms may be contributing to the thrombocytopenia. The mechanisms of thrombocytopenia can often be differentiated by examination of the bone marrow. Disorders of platelet production often have bone marrow findings consistent with aplasia or necrosis. In contrast, bone marrow findings in cases of accelerated destruction in the peripheral circulation often demonstrate increased thrombopoiesis.

Disseminated intravascular coagulation is a common cause of thrombocytopenia in cattle (Jain, 1986). Disseminated intravascular coagulation is a disease process that may have many different causes, of which thrombocytopenia is one of its clinical features. Disseminated intravascular coagulation may be initiated by a single cause or by multiple causes occurring sequentially or simultaneously (Levi & Ten Cate, 1999). Regardless of

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cause, the common thread is the activation of the coagulation pathways leading to a hypercoagulable state (Levi & Ten Cate, 1999). Disseminated intravascular coagulation causes thrombocytopenia due to an increased consumption of platelets (Warkentin & Kelton, 1994). Thrombocytopenia as a component of disseminated intravascular coagulation has been observed in cattle with toxic mastitis (Buntain, 1980; Heuwieser & Kikovic, 1989; Kiper & Paulsen, 1988), metritis (Braun *et al.*, 1990; Buntain, 1980), and endotoxemia and sepsis (Bowersock *et al.*, 1990; Thomson *et al.*, 1974). Sepsis and endotoxemia are the most common causes of disseminated intravascular coagulation in cattle, and disseminated intravascular coagulation has been experimentally reproduced in calves administered endotoxin intravenously (Nagaraja *et al.*, 1979).

Several parasitic diseases of cattle have been associated with thrombocytopenia as a hematological finding. Thrombocytopenia has been observed with the blood parasites *Babesia bigemina, Babesia argentina, and Babesia bovis* (Brun-Hansen *et al.*, 1998a; Dalgliesh *et al.*, 1976; Smith *et al.*, 1980). Trypanosomes, including *Trypanosoma vivax*, *Trypanosoma congolense*, and *Trypanosoma brucei*, may also induce thrombocytopenia in cattle, with *Trypanosoma vivax* inducing the greatest depressions in the platelet count (Forsberg *et al.*, 1979; Wellde *et al.*, 1989a; Wellde *et al.*, 1989b). The mechanism of thrombocytopenia associated with these blood-borne parasites is thought to be the result of disseminated intravascular coagulation (Wellde *et al.*, 1989a). Pancytopenia is frequently observed in cattle infected with *Trypanosoma vivax* (Anosa *et al.*, 1992), and bone marrow suppression may be partly responsible for the depressions in blood counts observed (Anosa *et al.*, 1992). A recent study evaluating the bone marrow in cattle infected with *T. vivax* revealed a dyserythropoiesis and dysgranulocytopoiesis (Anosa *et*

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Decreased production of platelets has been proposed as the mechanism of thrombocytopenia in cattle with bracken fern toxicity (Evans, 1964; Hirono *et al.*, 1984). Bone marrow aplasia and pancytopenia may be observed due to suppression of the myeloid and erythroid cell lines, as well as the megakaryocyte cell line (Evans, 1964). The compound responsible for the bone marrow suppression associated with bracken fern poisoning in cattle has been determined to be ptaquiloside (Hirono *et al.*, 1984). Bone marrow aplasia and subsequent anemia, thrombocytopenia, and leukopenia (neutropenia) have also been observed in cattle fed soybean meal from which the oil had been extracted by trichlorethylene. Prolonged administration of furazolidone to calves (Buck, 1975; Hayashi *et al.*, 1976; Taylor *et al.*, 1991) and mycotoxins such as vomitoxin or citrinin (Dyson & Reed, 1977; Griffiths & Done, 1991; Jeffers & Lenghaus, 1986), have also been demonstrated to result in pancytopenia, bone marrow aplasia, and clinical signs of hemorrhage.

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Diso
Babesiosis (*Babesia* *bigemina*)
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Table 1.3. Quantitative platelet disorders of cattle

Quantitative platelet disorders of cattle	
Disorder	**Reference(s)**
Babesiosis (*Babesia bovis* and *Babesia bigemina*)	(Brun-Hansen *et al.*, 1998a; Dalgliesh *et al.*, 1976; Smith *et al.*, 1980)
Bovine viral diarrhea virus	(Bezek *et al.*, 1994; Bolin & Ridpath, 1992; Corapi *et al.*, 1990; Corapi *et al.*, 1989; Rebhun *et al.*, 1989; Walz *et al.*, 1999a)
Bracken fern toxicosis	(Evans, 1964; Hirono *et al.*, 1984)
Disseminated intravascular coagulation (sepsis, endometritis, mastitis)	(Bowersock *et al.*, 1990; Braun *et al.*, 1990; Buntain, 1980; Heuwieser & Kikovic, 1989; Kiper & Paulsen, 1988; Thomson *et al.*, 1974)
East Coast Fever (*Theileria parva* infection)	(Burridge, 1973; Kimeto, 1976)
Ehrlichia phagocytophila infection	(Brun-Hansen *et al.*, 1998b)
Furazolidone poisoning	(Buck, 1975; Hayashi *et al.*, 1976; Taylor *et al.*, 1991)
Lymphosarcoma (bone marrow infiltration by neoplastic lymphocytes)	(McLaughlin, 1989)
Mycotoxicosis	(Dyson & Reed, 1977; Griffiths & Done, 1991; Jeffers & Lenghaus, 1986; Nicholls *et al.*, 1985)
Sarcocystosis (*Sarcocystis cruzei*)	(Daugschies *et al.*, 1998; Frelier & Lewis, 1984)
Trichloroethylene-extracted soybean oil meal	(Pritchard *et al.*, 1956; Strafuss & Zimmermann, 1967)
Trypanosomiasis (*Trypanosoma vivax*, *Trypanosoma congolense*, and *Trypanosoma brucei*)	(Forsberg *et al.*, 1979; Wellde *et al.*, 1983; Wellde *et al.*, 1989a; Wellde *et al.*, 1989b)

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Some Examples of Virus-induced Thrombocytopenia in Other Species

Thrombocytopenia can accompany viral infections with potentially fatal hemorrhagic consequences (Zucker-Franklin, 1994). The remainder of this literature review will briefly examine the mechanisms of thrombocytopenia in other viral infections of humans and animals. As previously stated, thrombocytopenia, regardless of cause, can result from only three basic mechanisms: increased destruction, decreased production, or abnormal sequestration of platelets (Warkentin & Kelton, 1994). Virus-induced thrombocytopenia in both humans and animals can occur by all three mechanisms, but most viruses that induce thrombocytopenia do so by invoking more than a single mechanism. Although immune-mediated destruction of platelets is the best characterized, the mechanisms of many virus-induced thrombocytopenias are poorly understood. Since most viruses induce thrombocytopenia by multiple mechanisms, grouping viruses by their mechanism of thrombocytopenia induction is not possible.

Many viruses have been associated with thrombocytopenia in human and veterinary medicine, and to list and discuss all viruses that have been associated with thrombocytopenia is beyond the scope of this review. Therefore, 5 viruses relevant to human and veterinary medicine have been selected for a more detailed discussion. Hog cholera virus was chosen for discussion because of its relationship to BVDV as a member of the genus *Pestivirus* within the family Flaviviridae (Wengler, 1991). Like hog cholera virus, African swine fever virus also causes a hemorrhagic syndrome in pigs (Rodriguez *et al.*, 1996). The African swine fever virus was selected for review because of reports demonstrating and characterizing megakaryocyte infection with this virus (Perez *et al.*,

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1997; Rodriguez *et al.*, 1996). Human immunodeficiency virus and equine infectious anemia virus were chosen due to the vast amount of research generated in recent years due to the AIDS (Acquired Immunodeficiency Sydrome) epidemic. Both human immunodeficiency virus and equine infectious anemia viruses are RNA viruses in the family Retroviridae, and recent reports have suggested that decreased production of platelets may be involved in the induction of thrombocytopenia (Ballem *et al.*, 1992; Crawford *et al.*, 1996). Finally, dengue virus was chosen because of its relationship to BVDV in the family Flaviviridae, although dengue virus belongs in the genus *Flavivirus* (Westaway & Blok, 1997). In addition, dengue virus has long been considered the prototype of an immune-mediated hemorrhagic response to a viral infection, and thrombocytopenia is thought to occur through accelerated destruction of platelets in the peripheral circulation (Gubler, 1998).

Hog cholera virus

Hog cholera virus is related to BVDV, and is a member of the genus *Pestivirus* within the family Flaviviridae (Wengler, 1991). Infection of pigs with hog cholera virus may result in multiple clinical forms including acute, subacute, chronic, subclinical, or delayed-onset forms (Dahle & Liess, 1992; Terpstra, 1991). Like BVDV, isolates of hog cholera virus vary widely in virulence, and some isolates are associated with severe clinical disease in pigs (Dahle & Liess, 1992; Terpstra, 1991).

Clinically, the acute form of hog cholera virus infection may result in a severe and generalized disease in which hemorrhages on the skin and multiple internal tissues are a characteristic finding (Dahle & Liess, 1992). Laboratory evaluation has demonstrated

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thrombocytopenia and leukopenia in pigs infected with highly virulent isolates of hog cholera virus (Dahle & Liess, 1992). The mechanism of hog cholera virus-induced thrombocytopenia and hemorrhage is multifactorial. The multiple hemorrhages seen in acute hog cholera virus infections are caused by degeneration of endothelial cells in conjunction with severe thrombocytopenia (Dahle & Liess, 1992; Terpstra, 1991). Hog cholera virus-induced thrombocytopenia is believed to be due to combinations of platelet consumption caused by direct damage to the platelets, virus-induced endothelial lesions, disseminated intravascular coagulation, and inadequate platelet production by the megakaryocytes (Dahle & Liess, 1992; Weiss *et al.*, 1973).

Inadequate platelet production has received the least consideration as the mechanism of hog cholera virus-induced thrombocytopenia; however, megakaryocyte necrosis and degeneration have been reported (Hoffmann *et al.*, 1971). In addition, hog cholera virus infection of megakaryocytes has been recently described (Gomez-Villamandos *et al.*, 1998b). Pigs were inoculated with a highly virulent strain of hog cholera virus, and immunohistochemical and ultrastructural examination revealed hog cholera virus infection in approximately 2.5-9.0% of megakaryocytes from days 2 through 9 after inoculation (Gomez-Villamandos *et al.*, 1998b). Viral antigen was demonstrated in platelets, and megakaryocyte infection with hog cholera virus was proposed as the origin of viral antigen in platelets. In addition, it was hypothesized that virus association with platelets may represent a passive vehicle for spreading the virus in the pig. Like BVDV, decreased production of platelets as a result of megakaryocyte infection may be important in the pathogenesis of hog cholera virus-induced thrombocytopenia.

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African swine fever virus is associated with clinical signs that cannot be differentiated from acute hog cholera virus infection (Rodriguez *et al.*, 1996). Petechial hemorrhages on the skin, especially the ears and on flanks, and widespread hemorrhages in internal organs are typical findings associated with infection of pigs with African swine fever virus.

Thrombocytopenia is a characteristic finding during African swine fever virus infection, and has been experimentally reproduced (Edwards *et al.*, 1985a; Edwards *et al.*, 1985b; Rodriguez *et al.*, 1996). The mechanism of African swine fever virus-induced thrombocytopenia is multifactorial and incompletely understood. Immune-mediated destruction, accelerated platelet aggregation as a result of disseminated intravascular coagulation, and impaired thrombopoiesis have all been considered as the primary mechanism in different reports (Edwards *et al.*, 1985a; Edwards *et al.*, 1985b; Perez *et al.*, 1997; Rodriguez *et al.*, 1996). However, involvement of a primary mechanism may be dependent upon the virulence of the isolate (Rodriguez *et al.*, 1996). For example, highly virulent isolates of African swine fever virus primarily induce thrombocytopenia through the consumption of platelets in the peripheral circulation as a result of disseminated intravascular coagulation. Infection and mass destruction of cells of the mononuclear phagocyte system leads to the systemic release of active products such as enzymes, monokines, complement factors, and products of arachidonic acid metabolism (Levi & Ten Cate, 1999). These factors have a significant effect on hemostasis and endothelial cells (Levi & Ten Cate, 1999) and are important in the pathogenesis of thrombocytopenia in pigs infected with highly virulent isolates (Rodriguez *et al.*, 1996). In pigs infected with moderately virulent isolates of African swine fever virus, inhibition

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of thrombopoiesis as a result of megakaryocyte infection, megakaryocyte degeneration, and bone marrow necrosis may be of primary importance. Infection of a substantial number of megakaryocytes by African swine fever virus has been observed, and this finding coincided with considerable reductions in the platelet count (Rodriguez *et al.*, 1996). Megakaryocyte apoptosis has also been observed following experimental infection of pigs with African swine fever virus (Gomez-Villamandos *et al.*, 1998a; Perez *et al.*, 1997). The relationship between megakaryocyte apoptosis and thrombocytopenia is unknown, but was thought to occur as the result of an overwhelming stimulus for thrombopoiesis or a secondary effect of the release of cytokines including tumor necrosis factor-α and interleukins. Indirect damage to megakaryocytes and megakaryocyte apoptosis may be an additional contributing mechanism for the thrombocytopenia observed with African swine fever virus infection.

Human immunodeficiency virus

Thrombocytopenia has been reported in association with human immunodeficiency virus (HIV) infection and the acquired immunodeficiency syndrome (AIDS) (Kaplan *et al.*, 1992). Thrombocytopenia can be an early manifestation in asymptomatic patients, and is considered one of the criteria of the AIDS-related complex. The incidence of thrombocytopenia is 0-10% in asymptomatic HIV patients, and may be up to 40% in symptomatic HIV patients (Rossi *et al.*, 1990). The mechanisms leading to thrombocytopenia in HIV-infected patients are multiple. Immune-mediated destruction of platelets in the peripheral circulation has been implicated as a mechanism of HIV-induced thrombocytopenia due to the presence of platelet-associated immunoglobulins,

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specific antiplatelet autoantibodies, and circulating immune complexes adsorbing to platelet membranes (Kaplan *et al.*, 1992). Molecular mimicry between HIV and platelet antigens has also been implicated as an immune-mediated cause of HIV-induced thrombocytopenia (Oksenhendler & Seligmann, 1990). An antibody has been identified that recognizes the HIV envelope glycoprotein gp120 and the platelet membrane glycoprotein gpIIIa (Oksenhendler & Seligmann, 1990). However, there is lack of support for an immune-mediated mechanism, which includes evidence that platelet-associated antibodies have been demonstrated in both thrombocytopenic and nonthrombocytopenic HIV-infected patients (Walsh *et al.*, 1984).

Direct involvement of progenitor cells has also been considered to be important in the pathogenesis of HIV-induced thrombocytopenia (Ballem *et al.*, 1992; Zucker-Franklin, 1994). Viral RNA has been identified in megakaryocytes from HIV-infected patients (Louache *et al.*, 1991; Zucker-Franklin & Cao, 1989; Zucker-Franklin *et al.*, 1990). In addition, ultrastructurally aberrant megakaryocytes and denuded megakaryocyte nuclei can be found in bone marrow specimens of most HIV-infected patients (Zucker-Franklin *et al.*, 1989). This finding, in combination with the fact that HIV can productively infect megakaryocyte cell lines *in vitro*, supports the hypothesis that impaired thrombocytopoiesis is at least partially responsible for HIV-induced thrombocytopenia.

Mean platelet volume measurements from HIV-infected patients also support the hypothesis that HIV-induced thrombocytopenia may be due to impaired thrombopoiesis. The mean platelet volume has been used to help classify thrombocytopenia (Bessman *et al.*, 1982). In general, platelets recently released into the bloodstream from the bone

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marrow are larger, and therefore, the mean platelet volume is elevated when the thrombocytopenia is due to increased destruction of platelets. Peripheral destruction of platelets is often associated with an increased megakaryocytopoiesis and thrombopoiesis. Conversely, aged platelets are smaller and mean platelet volume is decreased when the thrombocytopenia is due to decreased marrow production (Bessman *et al.*, 1982). The low mean platelet volume observed in HIV-infected patients supports the hypothesis that decreased platelet production is involved (Koenig *et al.*, 1991).

In spite of conflicting evidence suggesting that accelerated platelet destruction or decreased platelet production is the predominant mechanism, the pathogenesis of HIV-induced thrombocytopenia involves both mechanisms. A study evaluating the survival of ^{111}Indium-labelled platelets demonstrated a moderate reduction in platelet survival (Ballem *et al.*, 1992). In addition, mean platelet production was decreased, thus indicating that both accelerated platelet destruction and decreased platelet production contribute to HIV-induced thrombocytopenia (Ballem *et al.*, 1992)

Equine infectious anemia virus

Thrombocytopenia is a common finding in horses infected with equine infectious anemia virus. The mechanism of thrombocytopenia in horses infected with equine infectious anemia virus was originally believed to be due to immune-mediated destruction of platelets, because platelet-associated immunoglobulins G and M were detectable in horses infected with equine infectious anemia virus (Clabough *et al.*, 1991). However, the development of severe anemia and thrombocytopenia in experimentally infected severe combined immunodeficient foals, which lack functional B and T

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Suppression of platelet production has been recently proposed to be the primary mechanism of thrombocytopenia associated with equine infectious anemia virus (Crawford *et al.*, 1996). Platelet production, as measured by metabolic incorporation of radioactive label, was reduced in foals experimentally infected with equine infectious anemia virus (Crawford *et al.*, 1996). Platelet life span was also reduced, but this group of investigators believed that platelet removal in the peripheral circulation was independent of the immune system, as shortened platelet life span was observed in normal foals and severe combined immunodeficient foals infected with equine infectious anemia virus (Crawford *et al.*, 1996).

Megakaryocyte infection has not been demonstrated in horses infected with equine infectious anemia virus (Clabough *et al.*, 1991; Crawford *et al.*, 1996). An indirect effect of equine infectious anemia virus on megakaryocytes has been proposed as the mechanism of suppressed thrombopoiesis (Tornquist & Crawford, 1997). Plasma from horses infected with equine infectious anemia virus suppressed megakaryocyte colony growth *in vitro* (Tornquist & Crawford, 1997). In addition, the suppression was partially reversed by the addition of neutralizing antibodies to tumor necrosis factor-α and transforming growth factor-β (Tornquist & Crawford, 1997). The levels of those cytokines, as well as the cytokine interferon-α, were demonstrated to be elevated in the serum and bone marrow of foals infected with equine infectious anemia virus (Tornquist *et al.*, 1997). Elevations in these cytokines during infection with equine infectious

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Dengue virus

Dengue virus is classified in the same family as BVDV, the family Flaviviridae, but dengue virus belongs in the genus *Flavivirus* (Westaway & Blok, 1997). Dengue virus infection may result in a wide spectrum of clinical disease ranging from inapparent or mild febrile illness to severe and fatal hemorrhagic disease (Gubler, 1998). The nomenclature describing the clinical syndromes associated with dengue virus is rather confusing, with two distinct syndromes being described: dengue fever and dengue hemorrhagic fever (Halstead, 1982). Dengue fever is considered the less severe and may occur in adults and children (Halstead, 1982). Evidence of hemorrhage, including petechiae, epistaxis, gum bleeding, gastrointestinal hemorrhage, or purpuric lesions, has been described with dengue fever (Gubler, 1998; Halstead, 1982). Thrombocytopenia occurs in approximately 30% of patients with dengue fever, but the infection is usually self-limiting and rarely fatal (Gubler, 1998)

Dengue hemorrhagic fever, which is sometimes referred to as dengue shock syndrome, occurs predominantly in children less than 15 years of age (Gubler, 1998; Halstead, 1982). Dengue hemorrhagic fever occurs in areas in which multiple dengue viruses are simultaneously endemic (Halstead, 1982). Hemorrhages are also common with dengue hemorrhagic fever, especially skin hemorrhages such as petechiae, purpuric lesions, and ecchymoses on the face and extremities (Gubler, 1998). Thrombocytopenia is a constant finding in cases of dengue hemorrhagic fever, and a platelet count of less

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than 100,000 platelets/mm^3 is usually found between days 3 and 8 of illness (Gubler, 1998).

The pathogenesis of dengue virus-induced thrombocytopenia involves multiple mechanisms. The majority of studies have dealt with the mechanism of virus-induced thrombocytopenia in patients with dengue hemorrhagic fever. The predominant mechanism in patients with dengue hemorrhagic fever is immune-mediated destruction of platelets in the peripheral circulation and disseminated intravascular coagulation (Funahara *et al.*, 1987a; Funahara *et al.*, 1987b; Gubler, 1998; Halstead, 1982). Dengue hemorrhagic fever usually occurs in people who are exposed to a heterologous dengue virus serotype after having developed an immune response to a previous and different dengue virus serotype (Gubler, 1998). Pre-existing, heterologous dengue antibody from the previous exposure recognizes the infecting virus and antigen antibody complexes form. Macrophages phagocytize the antigen antibody complexes, and because the antibody is heterologous, it is not neutralized and the virus replicates in the macrophage. This is referred to as antibody-dependent enhancement, which has been studied in great detail (Halstead, 1982). The macrophages produce vasoactive mediators, which ultimately results in disseminated intravascular coagulation and a consumptive thrombocytopenia (Gubler, 1998).

In addition to disseminated intravascular coagulation, platelet consumption also occurs as a result of antigen antibody complex deposition on the surface of platelets by antibody-enhanced binding of dengue virus to platelets (Wang *et al.*, 1995). A recent report has also demonstrated an antibody-enhanced binding of dengue virus to platelets (Wang *et al.*, 1995). Dengue virus can also bind to the surface of platelets but at very

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low levels when compared to antibody-enhanced binding (Funahara *et al.*, 1987a). Immune-mediated platelet removal subsequently occurs even without evidence of disseminated intravascular coagulation (Funahara *et al.*, 1987a; Gubler, 1998; Halstead, 1982).

Disseminated intravascular coagulation and consumptive thrombocytopenia are not characteristic of dengue fever (Gubler, 1998). The mechanism of thrombocytopenia in patients during the initial dengue infection is currently unknown. A possible role of decreased thrombopoiesis in dengue fever has recently been proposed (Rothwell *et al.*, 1996). During the initial stages of the primary dengue viremia, the bone marrow is hypocellular and frequently positive for dengue virus (Rothwell *et al.*, 1996). Evidence of bone marrow stromal cell infection and hematopoietic cell lines with dengue virus has been reported and this may be involved in decreased thrombopoiesis (Nakao *et al.*, 1989).

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CHAPTER II:

JOURNAL ARTICLE: EXPERIMENTAL MODEL OF TYPE II BOVINE VIRAL DIARRHEA VIRUS-INDUCED THROMBOCYTOPENIA IN NEONATAL CALVES

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CHAPTER II: JOURNAL ARTICLE:

EXPERIMENTAL MODEL OF TYPE II BOVINE VIRAL DIARRHEA VIRUS-INDUCED THROMBOCYTOPENIA IN NEONATAL CALVES

Paul H. Walz, Thomas G. Bell, Barbara A. Steficek,
Lana Kaiser, Roger K. Maes, and John C. Baker

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Running Title: Type II BVDV-induced thrombocytopenia

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Abstract

Thrombocytopenia has been associated with type II bovine viral diarrhea virus (BVDV) infection in immunocompetent cattle, but the mechanism is unknown. The purpose of the present study was to develop and characterize a reproducible model of type II BVDV-induced thrombocytopenia. Colostrum-deprived Holstein calves were obtained immediately after birth, given a BVDV negative and BVDV antibody negative plasma transfusion, housed in an isolation facility, and randomly assigned to either control (n=4) or infected (n=5) groups. Infected calves were inoculated by intranasal instillation on day 3 of age with 10^7 $TCID_{50}$ of the prototype type II isolate, BVDV 890, while control calves were sham inoculated. Blood counts and virus isolation from serum, white blood cells, and platelets were performed daily until day 12 after infection, at which time, all experimental calves were euthanatized and pathologic, virologic, and immunohistochemical examinations were performed. On physical examination, the control calves remained normal, but the infected calves developed pyrexia and diarrhea characteristic of type II BVDV infection. The platelet count decreased in all infected calves, and a statistically significant difference in the platelet count between control and infected calves was observed between days 7 through 12 after infection. In addition, the mean platelet volume and white blood cell counts also decreased. Examination of the bone marrow from the infected calves revealed immunohistochemical staining for BVDV antigen in megakaryocytes and evidence of concurrent megakaryocyte necrosis and hyperplasia. In conclusion, we have developed and characterized a reproducible model of type II BVDV-induced thrombocytopenia. This model can be used to elucidate the

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Introduction

Bovine viral diarrhea virus (BVDV) is an important pathogen of cattle, resulting in worldwide economic losses. Outbreaks of severe, peracute disease in adult cattle and calves were reported in 1993 in the United States and Canada (Carman *et al.*, 1998; Drake *et al.*, 1994; Pellerin *et al.*, 1994). These outbreaks, which involved beef, dairy, and veal operations, were characterized by fever, diarrhea, abortion, leukopenia, thrombocytopenia, and death. Prior to 1993, it was generally believed that acute infections with BVDV resulted in mild disease, without considerable mortality (Baker, 1995). The BVDV isolates from these outbreaks were analyzed, and the type II genotype of BVDV was identified (Carman *et al.*, 1998; Pellerin *et al.*, 1994; Ridpath *et al.*, 1994). Although type I and type II BVDV can be involved in the entire spectrum of clinical disease from subclinical to severe disease, the hemorrhagic disorder characterized by thrombocytopenia has been only observed with type II isolates of BVDV (S. R. Bolin, personal communication).

Thrombocytopenia associated with type II BVDV infection has been reported under natural (Rebhun *et al.*, 1989) and experimental conditions (Bezek *et al.*, 1994; Bolin & Ridpath, 1992; Corapi *et al.*, 1990; Corapi *et al.*, 1989; Ellis *et al.*, 1998). Although previous experimental studies on type II BVDV infection have characterized the hemorrhagic syndrome, they have been descriptive in nature, and the mechanism of the BVDV-induced thrombocytopenia remains unknown. Several of the previous studies on

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BVDV-induced thrombocytopenia used an infection protocol that did not allow quantitation of the infectious dose of virus, which makes reproduction of their model difficult (Bezek *et al.*, 1994; Corapi *et al.*, 1990; Corapi *et al.*, 1989). Furthermore, several studies failed to use controls or failed to use age matched controls (Bolin & Ridpath, 1992; Corapi *et al.*, 1990; Corapi *et al.*, 1989). Control calves are critical in the evaluation of thrombocytopenia because of the variation in the normal platelet count (Nelson *et al.*, 1974; Semrad & Dubielzig, 1993); and age-matching is important as the platelet count varies between calves of different ages (Nelson *et al.*, 1974; Semrad & Dubielzig, 1993; Vaugher *et al.*, 1973). Finally, some investigations have used type II BVDV isolates that failed to consistently induce thrombocytopenia in all infected calves (Corapi *et al.*, 1989; Ellis *et al.*, 1998). Since it is difficult to reproduce these studies, there is no model of type II BVDV available to study the thrombocytopenic aspect of type II BVDV infection.

The mechanism of increased virulence with some type II isolates of BVDV is currently unknown; however, the ability of type II BVDV to cause severe clinical disease and death loss is due, at least in part, to thrombocytopenia and the resulting hemorrhagic syndrome. The purpose of this study was to characterize the clinical, hematologic, virologic, and pathologic features of the thrombocytopenic BVDV infection in neonatal calves and to develop a model to study the pathogenesis of type II BVDV-induced thrombocytopenia.

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Materials and Methods

Animals

Colostrum-deprived newborn male Holstein calves were obtained immediately after birth from local dairy farms. Calves were moved to an isolation facility, randomly allocated to control or treatment groups, and individually housed in separate rooms. All calves were given a physical examination, and both serum and whole blood were obtained for virus isolation and serum was obtained for BVDV antibody by virus neutralization. All calves positive for BVDV by virus isolation or with serum antibody levels to BVDV greater that 16 were excluded from study. The calves were given, by intravenous administration, 400 ml of fresh frozen plasma obtained from a BVDV antibody negative and virus negative donor cow. Calves were given parenteral vitamins A and D,[a] vitamin E and selenium,[b] a commercial oral monoclonal antibody preparation against *E. coli* K99 pilus antigen,[b] and fed a nonmedicated milk replacer according to label directions at 12% of body weight twice daily. Each calf was examined twice daily, and the rectal temperature and the physical examination findings recorded. Prior to initiation of the study, criteria for euthanasia were established. Calves demonstrating any one or a combination of the following were euthanatized: complete anorexia, recumbency with inability to rise, dehydration in excess of 12% of body weight, and hemorrhagic diathesis. No calves met these criteria, and therefore, no calves were euthanatized prior to the completion of the study.

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Virus and challenge protocol

The noncytopathic type II BVDV isolate 890,[c] which has been previously shown to induce thrombocytopenia in calves (Bolin & Ridpath, 1992), was used for the experimental infection of calves. The BVDV isolate 890 was propagated in bovine turbinate cells in Eagle's minimum essential medium (EMEM),[d] containing 10% fetal equine serum (FES),[e] L-glutamine,[f] penicillin G[e] (100 units/ml), and streptomycin[e] (100μg/ml). After a three day adjustment period in the isolation facility, calves in the infected group (n=5) were inoculated by intranasal instillation with 10^7 $TCID_{50}$ of the BVDV 890 isolate. Control calves (n=4) were inoculated by intranasal instillation with a sham inoculum, which was prepared from an uninfected cell culture lysate.

Clinical pathologic examination

Manual platelet and leukocyte counts were performed daily on whole blood. The manual counts were performed using the Unopette System[g] and a hemocytometer. Automated complete blood counts, including the mean platelet volume, were performed using an electronic particle counter.[h] Automated counts were done at various times after infection, but included days 0 and 12 after infection on all calves. Coagulation profiles (activated partial thromboplastin time, prothrombin time, fibrin degradation products, and fibrinogen) were performed using whole blood on days -2, 4, 10, and 12 of the experiment, according to standard procedure in the Clinical Pathology Section, Veterinary Teaching Hospital, Michigan State University.

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Isolation and purification of platelets for virus isolation and electron microscopy.

Whole blood was collected every other day from the jugular vein into plastic syringes containing 1.0 ml of 3.8% w/v sodium citrate for each 9.0 ml of blood. Platelet rich plasma was harvested by differential centrifugation (Steficek *et al.*, 1993). Purification of platelets was achieved through gel filtration. Briefly, 1 µl of 1 µM prostaglandin E_1[e] was added to 1 ml of platelet rich plasma prior to centrifugation at 800 x g for 15 minutes. The plasma was removed and the platelet pellet was resuspended in 1 ml Hank's Balanced Salt Solution (136 mM NaCl, 5.4 mM KCL, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, and 5.5 mM dextrose, pH 7.4). Platelets were then passed over a 10 ml Sepharose 4B column.[e] The flow-through fraction containing the platelets was collected and a manual platelet count was performed. The gel-filtered platelets were adjusted to a concentration of 200,000 platelets/µl in EMEM containing 10% FES, L-glutamine, and antibiotics, subjected to three freeze/thaw cycles, and centrifuged for 10 minutes at 1,500 x g. The supernatant was extracted and filtered through a sterile filter with an average pore diameter of 0.45 µm,[i] and stored at -80 C for later use in procedures to isolate BVDV.

For transmission electron microscopic examination of platelets, platelet rich plasma was harvested (Steficek *et al.*, 1993). Platelet rich plasma suspensions were divided into 0.5 ml aliquots in glass cuvettes, held at room temperature for 30 minutes, and then warmed for 5 minutes at 37 C. A teflon-coated magnetic stir bar was added to the sample, and the cuvette was then placed into an aggregometer.[j] The platelets were allowed to equilibrate in the aggregometer at a stir speed of 900 rpm for 30 seconds. At that time, 0.5 ml of 0.1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) was

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Pathologic examination

On day 12 after infection, all calves were euthanatized with a lethal injection of barbiturate.[k] A postmortem examination was performed and all gross postmortem lesions were recorded. Tissues collected for histopathologic examination included liver, spleen, lung, heart, small and large intestine, adrenal gland, kidney, thymus, tonsil, brain, spinal cord, mesenteric and bronchial lymph nodes, and bone marrow. All tissues were fixed in 10% neutral buffered formalin, with the exception of bone marrow, which was fixed in B-5 fixative.[l] Following fixation, the tissues were trimmed, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin (HE) according to standard procedure. To assess the numbers and morphology of the cell constituents, a detailed evaluation of the bone marrow was performed, including calculations of the numbers of megakaryocytes per high power field and the myeloid:erythroid series ratio (M:E ratio) (Harvey, 1984). Five different high power field areas were evaluated for each calf. Images in this dissertation are presented in color.

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Virologic and serologic examination

Serum and whole blood were collected daily for detection of BVDV. The whole blood was processed by hypotonic lysis of the red blood cells to yield the white blood cells (Carbrey *et al.*, 1972). The isolated white blood cells were then resuspended in EMEM containing 10% FES, L-glutamine, and antibiotics. Following three freeze/thaw cycles, the white blood cell preparations were centrifuged for 10 minutes at 1,500 x g. The supernatant was extracted and filtered through a sterile filter with an average pore diameter of 0.45 μm,[i] and stored at -80 C.

To determine the tissue distribution of the virus, virus isolation was performed on bone marrow, thymus, spleen, bronchial and mesenteric lymph nodes, tonsil, ileum, cerebellum, spinal cord, lung, esophagus, liver, and pulmonary artery collected postmortem. Approximately 0.5 gram of tissue was added to 4 ml of EMEM with 10% FES, L-glutamine, and antibiotics in a sterile mortar. Sterile sand was added and the tissue was homogenized by grinding with a pestle. The homogenized tissue sample was transferred into conical tubes and centrifuged for 30 minutes at 2,300 x g. The supernatant was removed and filtered through a filter with an average pore diameter of 0.45 μm.[i] Following processing, tissue preparations were stored at -80 C.

The serum, white blood cell preparations, gel-filtered platelet preparations, and tissue preparations were thawed, and 25 μl of each sample was inoculated in duplicate into wells on 96-well microtiter plates containing monolayers of bovine turbinate cells in EMEM with 10% FES, L-glutamine and antibiotics. After 3 days of incubation at 37 C

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Serology for BVDV was performed by a microtiter virus neutralization procedure (Carbrey *et al.*, 1972). Sera obtained on days 0 and 12 after infection from all experimental calves were tested for neutralizing antibodies to type II BVDV. Sera were inactivated for 30 minutes in a 56 C waterbath. Serial two-fold dilutions, ranging from 1:4 to 1:4,096, were made for each serum sample in a 96-well microtiter plate. The amount of virus used per test well was 500 $TCID_{50}$ of the type II cytopathic isolate MSU-AHDL #cp1080626.[m] Bovine turbinate cells were used as the indicator cells at a dilution of 15,000 cells per well. Each test included a back titration of the virus and a positive and negative serum control. The antibody titer was read as the highest dilution with complete inhibition of cytopathic effect.

Immunohistochemical and immunofluorescent antibody detection of BVDV antigen

Immunohistochemical staining for BVDV antigen was performed on paraffin-embedded sections of bone marrow, pulmonary artery, lung, and spleen using the monoclonal antibody, 15C5, and an avidin-biotin-complex immunoperoxidase technique as previously described (Haines *et al.*, 1992). The direct immunofluorescent antibody test for BVDV antigen was performed on fresh frozen tissue sections of bone marrow, pulmonary artery, lung, and spleen according to standard procedure in the Animal Health Diagnostic Laboratory, Michigan State University, using an anti-BVDV polyclonal antiserum conjugated to fluorescein isothiocyanate.[n]

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Statistical analysis

Differences in platelet and white blood cell counts, coagulation profile data, and rectal temperature measurements between control and infected calves were evaluated using the student's t-test. The virus isolation and virus neutralization data were analyzed using the Fisher's exact test. A P value < 0.05 was considered statistically significant.

Results

Physical examination findings

None of the calves met the criteria for euthanasia during the study period. In the infected calves, appetite remained normal, but diarrhea, characterized by blood and mucosal casts, was a consistent finding in all infected calves. Diarrhea was first noticed on day 5 after infection (2 calves), and by day 7 after infection, all infected calves developed diarrhea that continued throughout the study. Pyrexia (rectal temperature > 39.4 C) was observed in the infected calves from days 2 through 12 after infection. When compared to the control calves, there was a significant elevation in rectal temperatures of the infected calves on day 2 and days 4 through 12 after infection ($P \leq 0.05$). No clinical signs of disease were noted in the control calves.

Clinical pathologic findings

The platelet count decreased to less than 200,000/μl in all infected calves, and a platelet count of less than 100,000 platelets/μl was observed in 3 of 5 infected calves during the study. The manual platelet count reached the lowest point in the infected

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calves on day 11 after infection, with a mean platelet count of 139,300 platelets/μl (range: 98,000-178,000 platelets/μl). This compares to a mean platelet count of 801,125 platelets/μl (range: 642,000-825,000 platelets/μl) in the age-matched control calves (Figure 2.1). When compared to the age-matched control calves, a significantly decreased manual platelet count was observed from day 7 after infection to the end of study in the infected calves (Figure 2.1). The automated platelet counts closely followed the manual counts (Table 2.1). Platelet size, as measured by the mean platelet volume, was significantly reduced in the infected calves as the study proceeded over time (Table 2.1).

The white blood cell counts decreased in the infected calves, and a significant difference in daily manual white blood cell counts between infected and control calves was observed on day 6 and days 8 through 12 after infection. The white blood cell differential in infected calves demonstrated that the observed leukopenia was due primarily to a profound neutropenia (Table 2.1). The numbers of all the other cell types on the white blood cell differential were within normal limits and similar to the control calves.

No significant difference was noted between control and infected calves in the activated partial thromboplastin time, prothrombin time, fibrin degradation products, and fibrinogen of the coagulation profile (Table 2.1).

Transmission electron microscopic findings

Ultrastructural evaluation of platelets demonstrated a population of platelets that was heterogeneous in size. Compared to the control calves, there was an overall

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decreased platelet size in the infected calves, but several very large platelets were observed (Figure 2.2). Vesiculation of the platelet plasma membrane was also observed in platelets from calves infected with type II BVDV.

Virologic and serologic findings

Bovine viral diarrhea virus was not isolated from antemortem or postmortem samples collected from the control calves. In contrast, BVDV was isolated from white blood cells, platelets, and serum of all infected calves (Table 2.2). Bovine viral diarrhea virus was demonstrated earliest in white blood cells, followed by platelets, and then serum. Bovine viral diarrhea virus was isolated from all the tissues examined in all infected calves, with the exception of bone marrow (BVDV was isolated from 4 of 5 infected calves), and the liver, cerebellum, and spinal cord (BVDV was isolated in 3 of 5 infected calves).

All control and infected calves had BVDV-neutralizing antibody titers of ≤8 on the day of inoculation. The BVDV-neutralizing antibody titers remained ≤8 on day 12 after infection in all control and infected calves, with the exception of 1 infected calf, in which the antibody titer was 16 at day 12 after infection.

Pathologic findings

Petechial hemorrhages were present in all infected calves, with the hemorrhages being most prominent in the rumen, abomasum, and/or the endocardial surface of the heart. Oral erosions in the buccal mucosa were present in 3 of 5 infected calves. Abomasal edema, which was most prominent in the spiral folds of the fundus, was

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present in 4 of 5 infected calves. No significant lesions were present on gross postmortem examination of control calves.

Histologic evaluation of the bone marrow revealed differences between control and infected calves. Infected calves had more megakaryocytes per high power field (MK/hpf) than the control calves (Table 2.3). In the infected calves, many of the megakaryocytes did not have uniform nuclear size or chromatin pattern. Approximately half of the megakaryocytes in infected calves were either immature, or had undergone necrosis as evidenced by the loss of, or pyknosis of the nuclei (Figure 2.3B). Images in this dissertation are presented in color. The myeloid:erythroid series ratio (M:E ratio) was increased in the infected calves when compared to the control calves (Table 2.3).

Lymphoid depletion and reduced follicular size were present in the ileum and spleen in all infected calves. An occasional focus of necrosis of individual cells within the zona fasciculata of the adrenal gland was present in 4 of 5 infected calves and 1 control calf. Superficial erosions of the tongue with epithelial parakeratotic change, basal cell vacuolization with apoptosis, and mild submucosal inflammation was present in 4 of 5 infected calves. A mild angiopathy, characterized by deposits of eosinophilic protein on the surface of swollen endothelial cells, was observed in the cerebellum, cerebrum, brain stem, pulmonary artery, heart, and lung in 3 of 5 infected calves. In the lung, pulmonary edema fluid was present which distended the interlobular spaces. This lesion, which was present in all infected calves, was not associated with any significant inflammation.

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Immunohistochemistry and immunofluorescent antibody testing

In the control calves, there was no evidence of BVDV antigen as detected by immunohistochemical or immunofluorescent antibody staining. However, there was immunohistochemical staining for BVDV antigen in all paraffin-embedded tissue sections collected for evaluation from all infected calves. In 3 of 5 infected calves, immunohistochemical and immunofluorescent antibody staining of bone marrow revealed the presence of BVDV-specific antigen in megakaryocytes (Figures 2.3C and 2.3D). Immunohistochemical staining for BVDV was also present in the smooth muscle of blood vessels located in the spleen, lung, and the bone marrow. Mononuclear cells in the bone marrow, spleen, and lung and focal areas of lymphoid cells in the spleen also contained BVDV antigen.

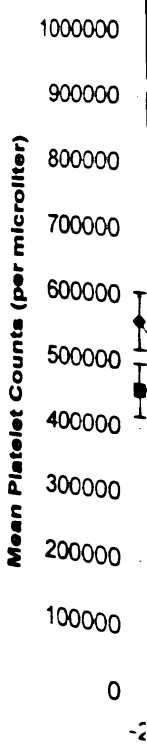
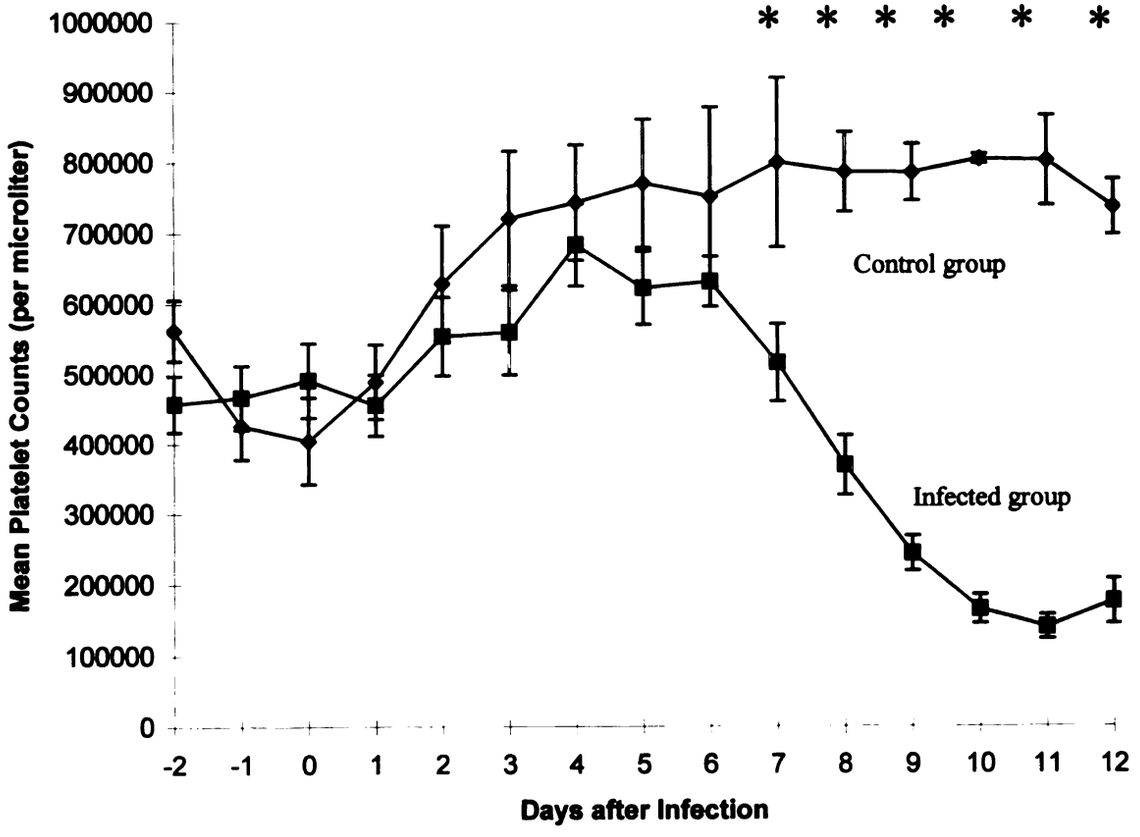


Figure 2.1. Mean whole blood platelet counts by manual determination in control and BVDV-infected calves. Calves were infected at day 0. Standard error bars are shown. A statistical difference ($p<0.05$, *t*-test) in platelet counts between the control and infected calves was detected from day 7 through day 12 after infection.

Figure 2.1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* * * * * *
Control group
Infected group

Table 2.1. Summary of clinical pathologic findings for 5 BVDV-infected and 4 uninfected control calves before infection and on day 12 after infection. Values are expressed as the mean ± standard error.

	control calves		infected calves	
Variable	*before infection†*	day 12 after infection	before infection	day 12 after infection

Table 2.1. Summary of clinical pathologic findings for 5 BVDV-infected and 4 uninfected control calves before infection and on day 12 after infection. Values are expressed as the mean ± standard error.

Variable	control calves		infected calves	
	before infection†	day 12 after infection	before infection	day 12 after infection
Manual platelet count	404,250±62,281	735,500±38,688	490,800±53,490	175,200±31,613 *
Automated platelet count	418,750±46,237	687,000±45,735	432,200±32,792	184,200±58,917 *
Mean platelet volume (fl)	5.95±0.19	5.95±0.25	5.38±0.15	4.42±0.31 *
Manual WBC count	9,166±985	12,955±1,416	9,179±1,401	4,511±451 *
Automated WBC count	9,893±1,534	14,840±2,494	13,666±1,287	4,810±399 *
WBC differential				
mature neutrophils	4,385±638	8,392±2,353	8,094±1,062	700±283 *
segmented neutr.	155±90	0	100±49	34±15
lymphocytes	3,873±470	6,148±378	4,636±1,029	3,930±348
monocytes	663±161	243±140	878±122	148±76
eosinophils	0	55±31	80±53	0
Coagulation profile‡				
PT (seconds)	23.58±1.95	21.78±1.67	21.94±0.92	22.20±1.03
APTT (seconds)	40.75±2.39	39.63±4.98	39.58±2.04	39.28±3.61
FDP (μg/ml)	<10	<10	<10	<10
fibrinogen (mg/dl)	188.25±32.20	404.25±97.40	203.20±15.16	347.00±48.93

* Denotes statistically significant differences between control and infected calves at day 12 after infection.

† Blood counts were performed on day 0 after infection (day of inoculation); coagulation profiles were performed 2 days prior to inoculation.

‡ PT=prothrombin time; APTT=activated partial thromboplastin time; FDP=fibrinogen degradation products.

Table 2.2. Sur

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Day 8
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Day 10
Day 11
Day 12

* numerator: # of c
+ ND—Not Done

Table 2.2. Summary of virus isolation results for BVDV on antemortem samples for 5 calves infected with type II BVDV.

Day after infection	Gel filtered platelets	White blood cells	Serum
Day 0	0/5*	0/5	0/5
Day 1	ND†	0/5	0/5
Day 2	0/5	0/5	0/5
Day 3	ND	1/5	0/5
Day 4	1/5	2/5	0/5
Day 5	ND	3/5	1/5
Day 6	4/5	5/5	3/5
Day 7	ND	5/5	4/5
Day 8	5/5	5/5	4/5
Day 9	ND	4/5	5/5
Day 10	5/5	4/5	3/5
Day 11	ND	4/5	3/5
Day 12	4/5	4/5	3/5

* numerator: # of calves which were virus positive; denominator: # of calves in infected group
† ND—Not Done

Table 2.3. My

field for 5 BVD

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* M:E=Myeloid:er

† MK hpf=megak

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Table 2.3. Myeloid:erythroid series ratio and numbers of megakaryocytes per high power field for 5 BVDV-infected and 4 uninfected control calves.

	control calves		infected calves	
	mean	range	mean	range
M:E series ratio*	0.5	0.39-0.80	0.68	0.40-0.93
MK/hpf†	5	1-10	10	4-21

* M:E=Myeloid:erythroid series ratio.Normal M:E ratio (Winqvist, 1954): mean: 0.46; range 0.40-0.52

† MK/hpf=megakaryocytes per high power field. Normal MK/hpf values have not been established for cattle.

Figure 2.2. Transmission electron micrograph of platelets from a control (left) and an infected (right) calf at day 12 after infection. In addition to observing a heterogeneity of size, most platelets from the infected calf were decreased in size. The surface membranes of platelets from infected calves are more frequently marked by adherent debris and membrane fragmentation. Bar = 1.0 μm.

Figure 2.2

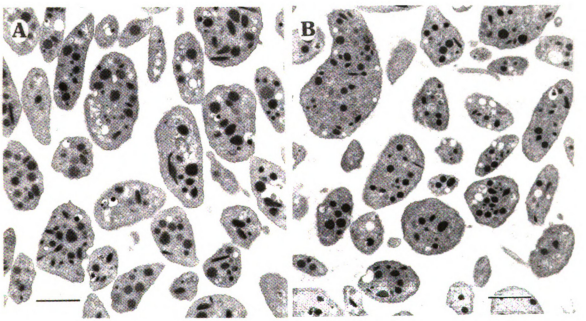
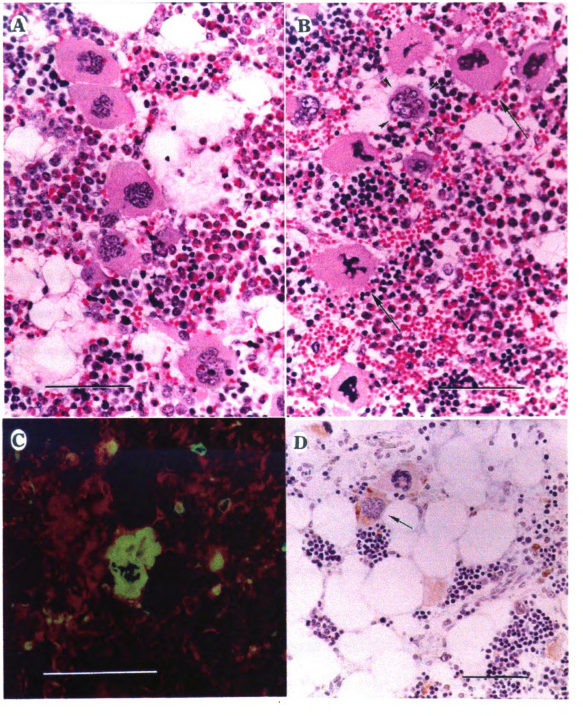


Figure 2.3. Histologic sections of bone marrow taken on day 12 after infection (postmortem examination). Bar = 100 μm. **A.** Histologic section of bone marrow from control calf. **B.** Histologic section of bone marrow from calf infected with type II BVDV. Note the degenerating megakaryocytes undergoing pyknosis (depicted by the arrows) and an immature megakaryocyte (depicted by the arrowheads). **C.** Positive immunofluorescent antibody staining for BVDV antigen in a megakaryocyte. **D.** Positive immunohistochemical staining for BVDV antigen in megakaryocytes (note reddish-brown staining depicted by arrow). Images in this dissertation are presented in color.

Figure 2.3



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Discussion

We have developed a reproducible model of the type II BVDV-induced thrombocytopenia using newborn male Holstein calves. The principal advantages of our model over previous studies on BVDV-induced thrombocytopenia are the demonstration of a decreased platelet count in all infected calves, the use of age-matched controls to demonstrate statistically significant differences in platelet counts between control and infected calves, an infection protocol which simulates natural infection, and a standardized infectious dose of virus. The use of a virus negative and BVDV antibody negative plasma transfusion should be considered an additional advantage of this model. Because a plasma donor was vaccinated for the common neonatal infectious diseases, the calves acquired adequate immunity against these agents, and thus, prevented the loss of calves from the study due to sepsis, respiratory disease, and enteritis. Therefore, we could study thrombocytopenia in neonatal calves without the confounding influence of other infectious diseases. This is particularly important as both sepsis and endotoxemia can cause thrombocytopenia in neonatal calves (Deldar *et al.*, 1984; Semrad & Dubielzig, 1993). An additional advantage in our model is that infection with BVDV 890 resulted in thrombocytopenia, but the infected calves did not become moribund and remained in the study until its completion.

A decline in the platelet count was the most prominent hematologic abnormality in the infected calves. Thrombocytopenia is defined as a reduction in the number of circulating platelets below the minimum normal level for the species (Jain, 1986). There is considerable variation in the literature concerning the platelet count in normal male

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Holstein calves, with one group of investigators reporting a range of 192,000-892,000 platelets\μl (Nelson *et al.*, 1974), and another reporting a range of 480,000-1,200,000 platelets\μl (Vaugher *et al.*, 1973). Therefore, defining thrombocytopenia based upon the literature is difficult. To circumvent these problems, we used age-, breed-, and sex-matched control calves in order to minimize the variation in the definition of thrombocytopenia, and to demonstrate statistically significant differences in the platelet count between calves infected with type II BVDV and their matched controls. Although previous studies have also demonstrated thrombocytopenia using different viral isolates (Bezek *et al.*, 1994; Corapi *et al.*, 1990; Corapi *et al.*, 1989), different routes of inoculation (Bezek *et al.*, 1994; Corapi *et al.*, 1990; Corapi *et al.*, 1989), and different ages and breeds of calves infected with BVDV (Bezek *et al.*, 1994; Bolin & Ridpath, 1992; Corapi *et al.*, 1990; Corapi *et al.*, 1989), they did not use matched controls. We have demonstrated both thrombocytopenia (platelet count less than 192,000 platelets\μl) and, more importantly, a significant difference in the platelet count between control and infected calves from days 7 through 12 after infection. Additional justification for the use of age-matched controls is the fact that there appears to be an age-related change in the platelet count in neonatal calves as demonstrated by the findings in this study. Since the platelet count in our age-matched control calves actually increased during the study, it is difficult to interpret a platelet count in an infected calf without the appropriate control. This change in platelet count has been alluded to in a previous study (Semrad & Dubielzig, 1993), in which the platelet count gradually increased from 212,050±73,945 platelets\μl at birth to 645,000±54,571 platelets\μl by 4 days after birth.

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In the present study, the mean platelet volume in infected calves was significantly decreased at day 12 after infection when compared to control calves. Ultrastructural evaluation of platelets by transmission electron microscopy also demonstrated that platelets were smaller in the infected calves. The mean platelet volume can be used to help classify thrombocytopenia (Bessman *et al.*, 1982). In general, platelets recently released into the bloodstream from the bone marrow are larger, and therefore, the mean platelet volume is elevated when the thrombocytopenia is due to increased destruction of platelets. Conversely, aged platelets are smaller and mean platelet volume is decreased when the thrombocytopenia is due to decreased marrow production (Bessman *et al.*, 1982). The low mean platelet volume in BVDV-induced thrombocytopenia suggests decreased production of platelets similar to that seen with human immunodeficiency virus infections and myelosuppressive disorders (Koenig *et al.*, 1991). The platelet volume has also been reported as decreased in pigs undergoing classical swine fever virus infection (hog cholera virus), which is closely related to BVDV; however, the significance of that finding was unknown at the time (Weiss *et al.*, 1973).

Other antemortem characteristics of this model of BVDV-induced thrombocytopenia which warrant discussion include the physical examination findings, the decreased white blood cell counts in the infected calves, and the normal coagulation profiles in infected calves. The physical examination findings of pyrexia and diarrhea are consistent with previous reports regarding type II BVDV infection in calves (Bezek *et al.*, 1994; Bolin & Ridpath, 1992; Corapi *et al.*, 1990; Corapi *et al.*, 1989). A decreased white blood cell count characterized by neutropenia has also been previously described with type II BVDV infection in immunocompetent calves (Corapi *et al.*, 1989; Ellis *et al.*, 1998).

Lymphopenia and anemia, observed by some investigators (Corapi *et al.*, 1989; Ellis *et al.*, 1998), were not seen in our model. The myeloid:erythroid series ratio was increased in our infected calves, which indicated the bone marrow responded by increasing the production of leukocytes. Coagulation profiles were within normal limits in our study, and normal findings were reported in a previous study on type II BVDV infection in calves (Corapi *et al.*, 1989). The latter findings and the results of this study support the hypothesis that type II BVDV-induced thrombocytopenia is not likely to be due to disseminated intravascular coagulation.

We have demonstrated an association of BVDV with platelets in the peripheral circulation through the isolation of BVDV in purified platelet preparations, and this virus-platelet association has been described by others using immunofluorescent antibody testing (Bezek *et al.*, 1994; Corapi *et al.*, 1990) and virus isolation (Bolin & Ridpath, 1992). It has been suggested that the platelets may serve as "carriers" of circulating virus (Corapi *et al.*, 1990). This virus-platelet association may be responsible for the higher level of virulence seen with some type II isolates of BVDV, as platelets may participate in a rapid dissemination of virus throughout the body. We have demonstrated megakaryocyte infection with BVDV, which may be the origin of the BVDV-platelet association rather than through an extra-medullary asssociation in the peripheral blood.

We have demonstrated BVDV antigen in megakaryocytes by immunohistochemistry. This confirms previous studies which used immunohistochemistry to define the tissue distribution of BVDV (Ellis *et al.*, 1998; Marshall *et al.*, 1996; Spagnuolo *et al.*, 1997). In addition to BVDV, several other viruses important in veterinary and human medicine can infect megakaryocytes (Axthelm & Krakowka, 1987; Ballem *et al.*, 1992; Gomez-

Villamandos *et al.*, 1998; Louache *et al.*, 1991; Perez *et al.*, 1997). Megakaryocyte infection is responsible, in part, for the virus-induced thrombocytopenia seen with human immunodeficiency virus infection (Ballem *et al.*, 1992; Louache *et al.*, 1991), canine distemper virus infection (Axthelm & Krakowka, 1987), and African swine fever virus infection (Gomez-Villamandos *et al.*, 1998; Perez *et al.*, 1997). The megakaryocyte is the hematopoietic precursor cell for platelets (Wright, 1906). Although megakaryocytes comprise only 0.05% of the nucleated cells in the bone marrow (Harker, 1968), they are responsible for maintaining the large circulating platelet population. Therefore, a decrease in the number of megakaryocytes during the early phase of type II BVDV infection, or disturbance in the maturation of megakaryocytes by a type II BVDV infection, could have a direct impact on the number of circulating platelets.

The contribution of megakaryocyte infection to the development of thrombocytopenia with type II BVDV infection is unknown. The virus could directly influence platelet production by infecting megakaryocytes or the effect could be indirect, via inhibitory cytokines or infection of cells which produce thrombopoietic stimulatory factors. Although viral antigen has been demonstrated in megakaryocytes, the number of megakaryocytes containing BVDV antigen were relatively few. In addition, we were only able to demonstrate BVDV antigen in megakaryocytes in 3 of 5 infected calves, yet all the calves became thrombocytopenic. This suggests that the thrombocytopenia may be primarily a result of indirect influences on the megakaryocyte caused by type II BVDV. However, we only examined the bone marrow and megakaryocytes at day 12 after infection, and this does not preclude the possibility that the majority of megakaryocytes become infected earlier in the course of the infection. BVDV-induced

thrombocytopenia may be due to both direct and indirect effects on the megakaryocytes. African swine fever virus causes thrombocytopenia through a combination of direct infection of megakaryocytes and indirect damage to megakaryocytes by inducing megakaryocyte apoptosis (Gomez-Villamandos *et al.*, 1998; Perez *et al.*, 1997).

Previous studies on the histologic examination of bone marrow from type II BVDV-thrombocytopenic infections have been contradictory. In calves naturally infected with BVDV, Scruggs et al. reported megakaryocyte necrosis (Scruggs *et al.*, 1995), while Rebhun et al. reported bone marrow necrosis or adequate to increased numbers of megakaryocytes (Rebhun *et al.*, 1989). Under experimental conditions, megakaryocyte hyperplasia has been reported 12 days after infection with a thrombocytopenia-inducing isolate of BVDV (Corapi *et al.*, 1989). Another experimental study on type II BVDV demonstrated bone marrow necrosis in 5 of 6 calves; and, in the remaining calf, megakaryocyte hyperplasia was observed in which immature megakaryocytes were present in relatively high numbers (Ellis *et al.*, 1998). We observed both megakaryocyte degeneration and megakaryocyte hyperplasia. The number of immature megakaryocytes was increased, likely in response to the thrombocytopenia, and yet there were also numerous megakaryocytes undergoing pyknosis and degeneration. We speculate that viral infection of the bone marrow occurs during the initial stages of viremia, leading to a transient suppression of platelet production by the megakaryocytes due to the combined direct result of viral infection and an undetermined indirect result of megakaryocyte degeneration.

Thrombocytopenia, regardless of the underlying etiology, results from only three basic mechanisms: decreased production, accelerated destruction, or abnormal

sequestration of platelets (Warkentin & Kelton, 1994). Viruses have been associated with thrombocytopenia in humans and animals (Kaplan *et al.*, 1992; Zucker-Franklin, 1994), but the underlying mechanisms are complex, multifactorial, and incompletely understood. Classical swine fever virus (hog cholera virus) induces thrombocytopenia and hemorrhage by multiple mechanisms including accelerated platelet destruction caused by direct damage to the platelets, virus-induced endothelial lesions, and disseminated intravascular coagulation (Weiss *et al.*, 1973); however, megakaryocyte necrosis and degeneration have also been reported (Hoffmann *et al.*, 1971). The mechanism of BVDV-induced thrombocytopenia may, like classical swine fever virus, involve a combination of two or more factors. However, in contrast to classical swine fever virus, we suspect that decreased production of platelets as a result of megakaryocyte infection may be the predominant mechanism.

Accelerated platelet destruction and abnormal sequestration of platelets as the cause of type II BVDV-induced thrombocytopenia are less likely based upon previous studies (Bezek *et al.*, 1994; Bolin & Ridpath, 1992; Corapi *et al.*, 1989), and the findings of this study. Coating of platelets with antibodies or deposition of viral antigen-antibody complexes on platelet membranes can trigger immune-mediated destruction of platelets (Shulman & Reid, 1994); however, a previous study has demonstrated that there was no evidence of platelet-associated antibody and complement in BVDV-induced thrombocytopenia (Corapi *et al.*, 1990). Disseminated intravascular coagulation also causes thrombocytopenia due to an accelerated destruction of platelets (Shulman & Reid, 1994). The presence of normal coagulation profiles in this study and a previous study

(Corapi *et al.*, 1990) suggests that disseminated intravascular coagulation is not involved in BVDV-induced thrombocytopenia.

In summary, we have developed a reproducible model of type II BVDV-induced thrombocytopenia. It appears likely that the thrombocytopenia associated with type II BVDV infection is due primarily to a decreased production of platelets. Our model of thrombocytopenia may prove useful for elucidating the pathophysiology of the hemorrhagic syndrome associated with type II BVDV infection and help us understand what makes some type II BVDV isolates more virulent than others in immunocompetent cattle.

Sources and Manufacturers

a. Butler Company, Dublin, OH

b. Schering-Plough Animal Health Corporation, Kenilworth, NJ

c. Dr. Steve Bolin, NADC, Ames, IA

d. JRH Biosciences, Lenexa, KS

e. Sigma Chemical Co., St. Louis, MO

f. Gibco BRL, Life Technologies, Grand Island, NY

g Becton Dickinson, Franklin, NJ

h. Technicon H-1 analyzer, Tehnicon Instrumental Corp., Tarrytown, NY

i. Millipore Corporation, Bedford, MA

j. Chronolog Corporation, Havertown, PA

k. Vortech Pharmaceuticals, Dearborn, MI

l. Columbia Diagnostics, Inc., Springfield, VA

m. Animal Health Diagnostic Laboratory, Virology Section, E. Lansing, MI

n. VMRD, Inc., Pullman, WA

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CHAPTER III:

JOURNAL ARTICLE: EFFECT OF EXPERIMENTALLY INDUCED TYPE II BOVINE VIRAL DIARRHEA VIRUS INFECTION ON PLATELET FUNCTION IN CALVES

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EFFECT OF EXPERIMENTALLY INDUCED TYPE II BOVINE VIRAL DIARRHEA VIRUS INFECTION ON PLATELET FUNCTION IN CALVES

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Abstract

Objective—To evaluate platelet aggregation responses in calves experimentally infected with a thrombocytopenia-inducing type II bovine viral diarrhea virus (BVDV) isolate (BVDV 890).

Animals—9 neonatal male Holstein calves.

Procedure—Five calves were inoculated with BVDV 890, and 4 were used as controls. Platelet aggregation studies and attempts to isolate BVDV from platelets were performed 2 days before, the day of, and every 2 days for 12 days after inoculation. Platelet function was assessed by means of optical aggregometry, using adenosine diphosphate and platelet activating factor as agonists. Bovine viral diarrhea virus was identified from purified platelet preparations by use of an immunoperoxidase monolayer assay.

Results—Maximum percentage aggregation and slope of the aggregation curve decreased over time in calves infected with BVDV. Bovine viral diarrhea virus was not isolated from platelets from control calves, but was isolated from infected calves from 4 through 12 days after inoculation.

Conclusions—Results suggest that platelet function may be depressed in calves infected with type II BVDV. Although the mechanism for altered platelet function was not determined, it likely involved an increase in the percentage of aged platelets in the circulation, a direct virus-platelet interaction, or an indirect virus-platelet interaction.

Clinical Relevance—Platelet dysfunction, in addition to thrombocytopenia, may contribute to the hemorrhagic syndrome associated with acute type II BVDV infection in calves.

Introduction

Bovine viral diarrhea virus (BVDV) infection is an economically important viral infection of cattle that can result in a wide spectrum of clinical manifestations, ranging from subclinical infections to severe fatal disease (Baker, 1995). The causative organism is an RNA virus and a member of the genus *Pestivirus* within the family Flaviviridae (Wengler, 1991). Isolates of BVDV can be classified into 2 genotypes, type I and type II, on the basis of differences in RNA sequences (Ridpath *et al.*, 1994). Infection of nonimmune cattle with some isolates of BVDV has been associated with a clinical disorder characterized by thrombocytopenia (Bolin & Ridpath, 1992; Corapi *et al.*, 1990; Rebhun *et al.*, 1989). To date, only infection with the type II genotype of BVDV has been associated with a hemorrhagic syndrome.[a]

Thrombocytopenia has been documented in adult cattle and calves with naturally occurring (Rebhun *et al.*, 1989) or experimentally induced BVDV infection (Bezek *et al.*, 1994; Bolin & Ridpath, 1992; Corapi *et al.*, 1990). Clinical signs reported for cattle with the hemorrhagic syndrome have included epistaxis, leukopenia, pyrexia, hemorrhage in multiple organ systems, bleeding from injection sites or insect bites, bloody diarrhea, and death (Bezek *et al.*, 1994; Bolin & Ridpath, 1992; Corapi *et al.*, 1990; Rebhun *et al.*, 1989). The cause of hemorrhage in cattle with acute BVDV infection has been attributed solely to thrombocytopenia (Corapi *et al.*, 1990; Rebhun *et al.*, 1989), even though the documented decrease in platelet number is often insufficient to result in spontaneous bleeding.

Platelets are small anucleate cells in the circulation that play a pivotal role in hemostasis and thrombosis (Day & Rao, 1986). Platelets circulate in a disc-like resting

state, but become activated following blood vessel injury. With injury to a blood vessel, platelets are exposed to subendothelial components, triggering a cascade of events that ultimately result in formation of a thrombus. Quantitative platelet disorders, in which circulating numbers of platelets are low, and qualitative platelet defects, in which platelets have altered functional characteristics, will result in an impaired hemostatic response, and hemorrhage may result (Jain, 1986).

Clinical signs of spontaneous hemorrhage occur when platelet counts are < 20,000 platelets/μl, and may develop if platelet counts are between 20,000 and 50,000 platelets/μl (Jain, 1986). Several investigations have determined that the bleeding tendency in cattle infected with type II BVDV only becomes clinically apparent when platelet counts in the circulation are less than these critical limits (Corapi *et al.*, 1990; Rebhun *et al.*, 1989). In a retrospective study of 15 cattle with acute BVDV-induced thrombocytopenia (Rebhun *et al.*, 1989), petechial and ecchymotic hemorrhages were reported in association with platelet counts ranging from 2,000 to 33,000 platelets/μl. In a separate study (Corapi *et al.*, 1990), calves with experimentally induced BVDV infection had clinical evidence of hemorrhage only in association with platelet counts < 5,000 platelets/μl. In contrast, experimentally induced type II BVDV infection resulted in hemorrhage and death in 3 of 7 calves in which platelet counts were not < 70,000 platelets/μl at the time of death (Bolin & Ridpath, 1992). This discrepancy between development of hemorrhage and platelet count has suggested that platelet function may be altered in cattle with type II BVDV infection. Because previous studies have not examined platelet function in infected cattle, the purpose of the study reported here was to evaluate platelet aggregation responses in calves experimentally infected with a

thrombocytopenia-inducing type II BVDV isolate (BVDV 890). The study was performed to determine whether altered platelet function, in addition to low platelet counts, may contribute to the hemorrhagic diathesis associated with acute type II BVDV infection in cattle.

Materials and Methods

Animals

Nine colostrum-deprived newborn male Holstein calves were obtained immediately after birth from local dairy farms. Calves were moved to an isolation facility, randomly allocated to control (n = 4) or treatment (5) groups, and individually housed in separate rooms. All calves were given a physical examination, and blood and serum were obtained for virus isolation procedures and determination of virus neutralization antibody titers to BVDV. Calves were then given 400 ml of plasma, IV. Plasma had been obtained from a donor cow that was negative for BVDV antibodies, as determined by means of virus neutralization, and for virus, as determined by means of virus isolation procedures (tests were performed on 3 serial samples obtained prior to collection of plasma). Calves were also given injections of vitamins A and D[b] and vitamin E and selenium,[c] as well as a commercial oral monoclonal antibody preparation against *Escherichia coli* K99 pilus antigen.[c] Calves were fed a nonmedicated milk replacer according to label directions at a rate of 12% of body weight twice daily throughout the study period.

Virus infection protocol

A noncytopathic type II BVDV isolate (BVDV 890) that has been shown to induce thrombocytopenia in calves (Bolin & Ridpath, 1992) was used for the experimental infection of calves in the treatment group. The virus was propagated in bovine turbinate cells in Eagle's minimum essential medium (EMEM)[d] containing 10% fetal equine serum (FES),[e] L-glutamine,[f] penicillin G,[e] and streptomycin.[e] The viral titer of the inoculum was determined according to described methods (Lennette & Schmidt, 1969).

After a 3-day adjustment period in the isolation facility, calves in the treatment group were inoculated by intranasal instillation with 10^7 median tissue culture infective doses of BVDV 890 in 5 ml of EMEM without serum (2.5 ml per nostril). Control calves were inoculated with a similar amount of an uninfected cell culture preparation. This dose of BVDV 890 was chosen because intranasal administration of 10^6 median tissue culture infective doses of BVDV 890 did not consistently induce thrombocytopenia in a previous study (Bolin & Ridpath, 1992).

Collection of platelets

Blood samples were collected for platelet aggregation studies and for isolation of BVDV from platelets 2 days before, the day of, and every 2 days for 12 days after inoculation of calves. Blood was collected by means of jugular venipuncture, using an 18-gauge needle, into plastic syringes containing 1 ml of 3.8% trisodium citrate for each 9 ml of blood. Platelet-rich plasma was obtained by means of 2 centrifugation steps at 1,324 × g for 60 seconds and 30 seconds, with platelet-rich plasma being removed after each centrifugation step. Platelet-poor plasma was obtained by means of centrifugation

of the remaining blood at 1,324 × g for 13 minutes. Platelets were manually counted, using a hemocytometer.[g]

Platelet aggregation responses

Aggregation studies were carried out in a dual-channel aggregometer.[h] Aggregation agonists used for this study were adenosine diphosphate (ADP),[e] at concentrations of 10 and 100 μ*M*, and platelet activating factor (PAF),[i] at concentrations of 0.1 and 1.0 μ*M*. Platelet-rich plasma was adjusted to 300,000 platelets/μl with homologous platelet-poor plasma. Adjusted platelet-rich plasma suspensions were then divided into 0.5 ml aliquots in glass cuvettes and held at room temperature (approx 20 C) for 30 minutes. The platelet-rich plasma suspensions were then warmed for 5 minutes at 37 C. A magnetic stir bar was added to the sample, and the cuvette was placed into the aggregometer. For each experiment, the aggregometer was calibrated using nonaggregated platelet-rich plasma to establish the 0% aggregation limit and platelet-poor plasma to establish the 100% aggregation limit. The sample was allowed to stir at 900 rpm for approximately 30 seconds, at which time 20 μl of agonist was added to the platelet suspensions, and change in light transmission recorded. Maximum percentage aggregation and slope of the aggregation curve were measured for each aggregation test. All aggregation studies were completed within 5 hours after collection of blood samples. All studies were done at least in duplicate, and the mean value was obtained.

Isolation of BVDV from platelets

Platelets were gel-filtered to remove plasma constituents. One microliter of 1 μ*M* prostaglandin E_1[e] was added per ml of platelet-rich plasma, and samples were centrifuged at 800 × g for 15 minutes. The supernatant was removed with a sterile pipette, and the platelet pellet was resuspended in 1 ml of Hank's balanced salt solution (136 m*M* NaCl, 5.4 m*M* KCl, 0.44 m*M* KH_2PO_4, 0.34 m*M* Na_2HPO_4, and 5.5 m*M* dextrose; pH 7.4). The platelet suspension was then transferred to a 10 ml polystyrene column containing Sepharose 4B.[e] The eluate was collected, and platelet count was determined manually. Gel-filtered platelet suspensions were adjusted to a count of 200,000 platelets/μl, using EMEM containing 10% FES, L-glutamine, and antibiotics. Suspensions were subjected to 3 freeze-thaw cycles and then centrifuged for 10 minutes at 1,500 × g. The supernatant was removed and filtered through a sterile filter with an average pore diameter of 0.45 μm.[j] Platelet preparations were stored at -80 C until used in virus isolation procedures.

To isolate BVDV from platelets, platelet preparations were thawed, and 25 μl of each sample was inoculated in duplicate into wells on 96-well microtitration plates containing monolayers of bovine turbinate cells in EMEM with 10% FES, L-glutamine, and antibiotics. After 3 days of incubation at 37 C in humidified air containing 5% CO_2, the bovine turbinate cells were stained for BVDV antigen by use of an immunoperoxidase monolayer assay (Meyling, 1988).

Statistical analysis

For each data collection period, maximum percentage aggregation and slope of the aggregation curve were compared between infected and control calves by use of

Student's *t*-test. Results of virus isolation from platelets were compared between groups by use of Fisher's exact test. A value of $P < 0.05$ was considered significant.

Results

Aggregation responses with ADP

Throughout the study, platelets from control calves displayed the expected shape change and aggregation in response to 10 μ*M* ADP (Figure 3.1). In BVDV-infected calves, the shape change appeared normal throughout the study, but maximum percentage aggregation and slope of the aggregation curve became progressively decreased over time (Figure 3.2). When 10 μ*M* ADP was used as the agonist, zero aggregation (ie, a failure to record an increase in light transmission, compared with baseline transmission, when the sample was allowed to stir in the aggregometer for 5 minutes after addition of the agonist) was observed for 2 of 5 infected calves on days 6 and 8 after inoculation, in all infected calves on day 10, and in 4 of 5 infected calves on day 12.

Throughout the study, 100 μ*M* ADP induced the expected shape change and aggregation responses in platelets from control calves (Figure 3.3). A shape change preceded the aggregation response in platelets from the BVDV-infected calves; however, maximum percentage aggregation and slope of the aggregation curve decreased over time in the infected calves (Figure 3.4). When 100 μ*M* ADP was used as the agonist, zero aggregation was also observed for 2 of 5 BVDV-infected calves on day 12 after inoculation.

Aggregation responses with PAF

Throughout the study, platelets from control calves displayed expected aggregation responses when mixed with 0.1 μ*M* PAF (Figure 3.5). For platelets from infected calves, maximum percentage aggregation and slope of the aggregation curve were progressively decreased over time (Figure 3.6). Similarly, 1.0 μ*M* PAF induced expected responses in platelets from control calves (Figure 3.7), but maximum percentage aggregation and slope of the aggregation curve were progressively decreased over time for platelets from infected calves (Figure 3.8).

Isolation of BVDV from platelets

Bovine viral diarrhea virus was not isolated from platelets from control calves, but was isolated from platelets from 1 BVDV-infected calf 4 days after inoculation, from 4 of 5 infected calves 6 and 12 days after inoculation, and from all infected calves 8 and 10 days after inoculation.

Figure 3.1. Aggregometer tracings of bovine platelet-rich plasma suspensions stimulated with adenosine diphosphate at a concentration of 10 μM. The top two tracings represent the aggregation response curves of bovine platelets obtained from the same control calf on days 0 and 12 after infection. The bottom two tracings represent the aggregation response curves of bovine platelets obtained from the same BVDV-infected calf on days 0 and day 12 after infection. In the infected calf at day 12 after infection, the lower percent change in light transmission, compared to the other aggregation tracings, represents a depressed aggregation response.

Figure 3.1

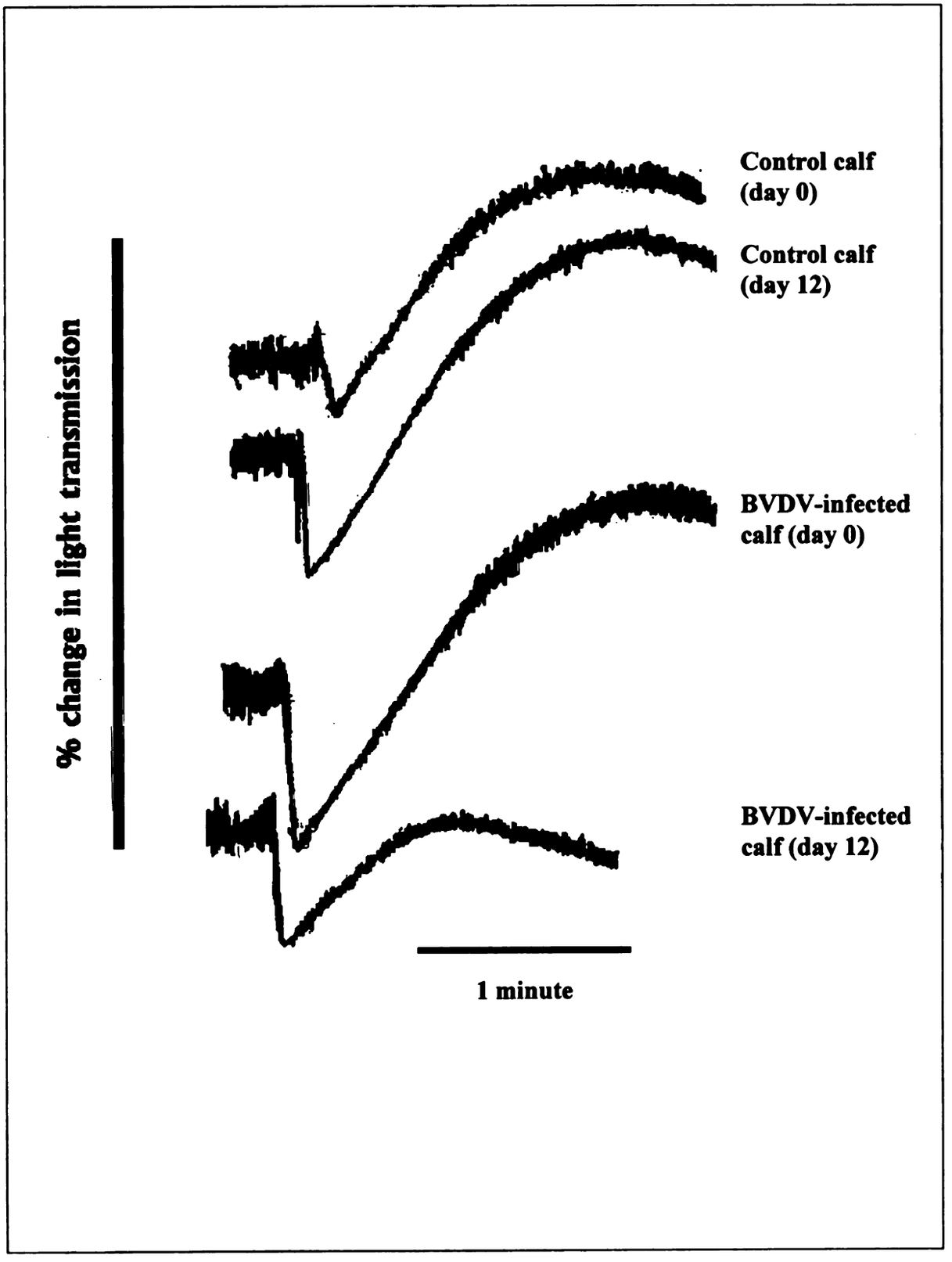

Control calf (day 0)
Control calf (day 12)
BVDV-infected calf (day 0)
BVDV-infected calf (day 12)
% change in light transmission
1 minute

Figure 3.2. Platelet aggregation responses (A: Maximum percent aggregation; B: Slope of the aggregation curve) induced by adenosine diphosphate at a concentration of 10μM for control (n=4) and BVDV-infected (n=5) calves during the experimental time period. Results are expressed as the mean ± standard error. * Indicates values that are significantly (*P<0.05, t*-test) different between control and BVDV-infected calves at the same collection time.

Figure 3.2

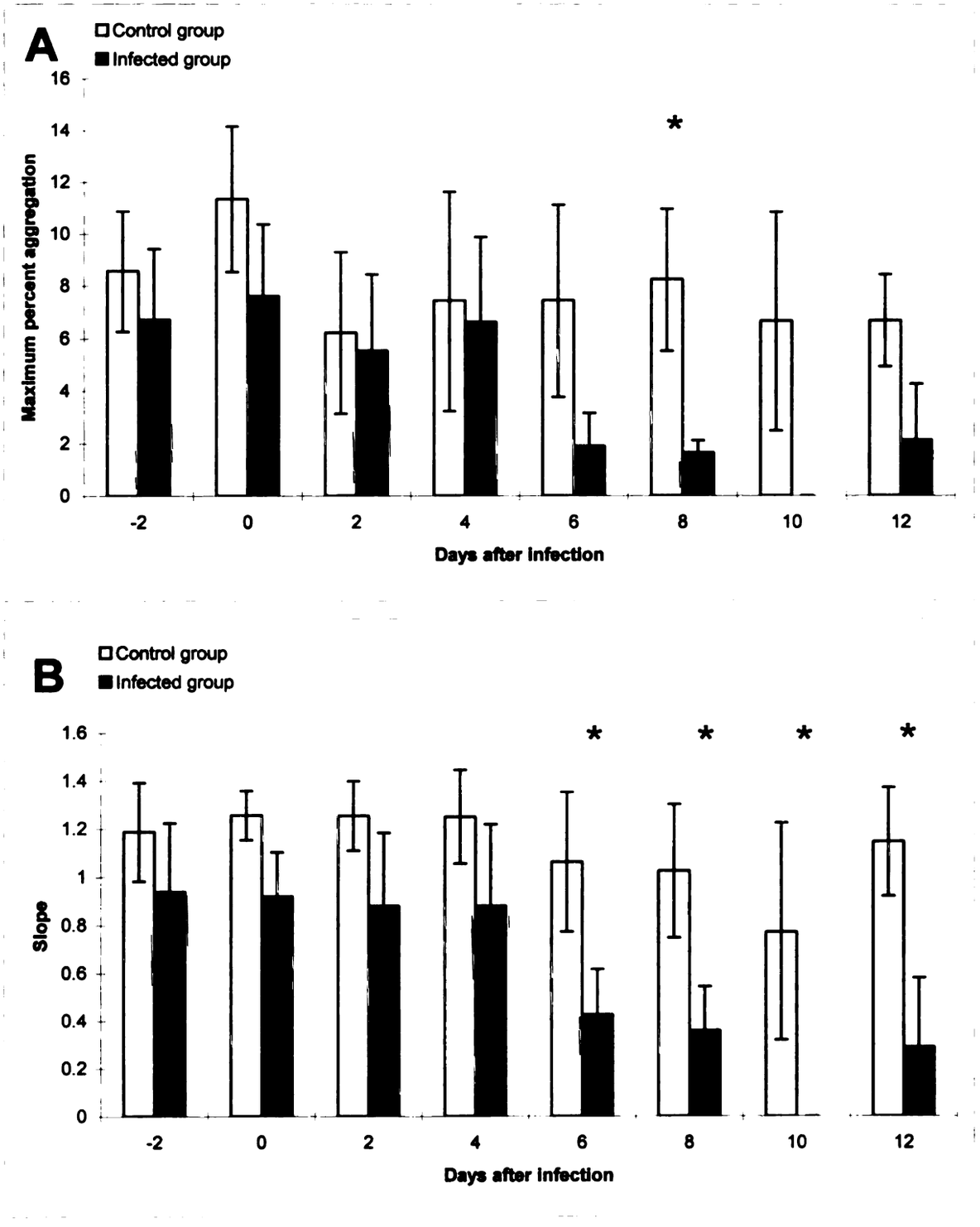

A
Control group
Infected group
Maximum percent aggregation
16
14
12
10
8
6
4
2
0
*
-2
0
2
4
6
8
10
12
Days after infection
B
Control group
Infected group
Slope
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
* * * *
-2
0
2
4
6
8
10
12
Days after infection

Figure 3.3. Aggregometer tracings of bovine platelet-rich plasma suspensions stimulated with adenosine diphosphate at a concentration of 100 μM. The top two tracings represent the aggregation response curves of bovine platelets obtained from the same control calf on days 0 and 12 after infection. The bottom two tracings represent the aggregation response curves of bovine platelets obtained from the same BVDV-infected calf on days 0 and 12 after infection. In the infected calf at day 12 after infection, the lower percent change in light transmission, compared to the other aggregation tracings, represents a depressed aggregation response.

Figure 3.3

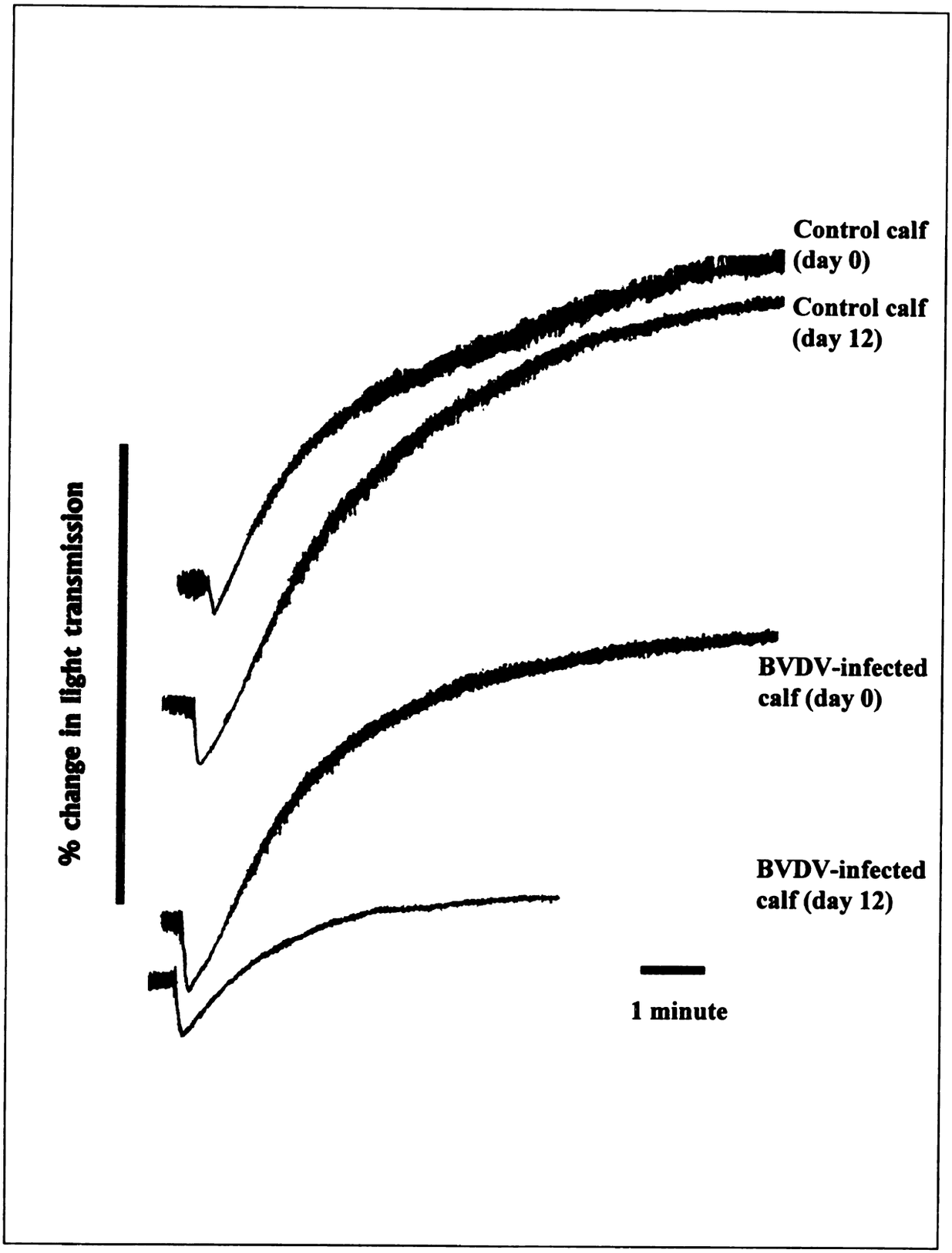

Control calf (day 0)
Control calf (day 12)
BVDV-infected calf (day 0)
BVDV-infected calf (day 12)
% change in light transmission
1 minute

Figure 3.4. Platelet aggregation responses (A: Maximum percent aggregation; B: Slope of the aggregation curve) induced by adenosine diphosphate at a concentration of 100μM for control (n=4) and BVDV-infected (n=5) calves during the experimental time period. Results are expressed as the mean ± standard error. * Indicates values that are significantly ($P<0.05$, *t*-test) different between control and BVDV-infected calves at the same collection time.

Figure 3.4

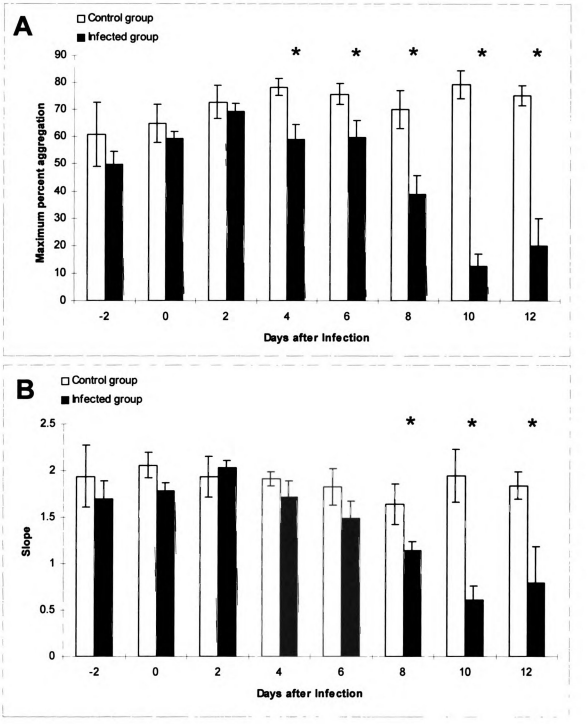

A
Control group
Infected group
Maximum percent aggregation
90
80
70
60
50
40
30
20
10
0
-2
0
2
4
6
8
10
12
Days after infection
B
Control group
Infected group
Slope
2.5
2
1.5
1
0.5
0
-2
0
2
4
6
8
10
12
Days after infection

Figure 3.5. Aggregometer tracings of bovine platelet-rich plasma suspensions stimulated with platelet activating factor at a concentration of 0.1 μM. The top two tracings represent the aggregation response curves of bovine platelets obtained from the same control calf on days 0 and 12 after infection. The bottom two tracings represent the aggregation response curves of bovine platelets obtained from the same BVDV-infected calf on days 0 and 12 after infection. In the infected calf at day 12 after infection, the lower percent change in light transmission, compared to the other aggregation tracings, represents a depressed aggregation response.

Figure 3.5

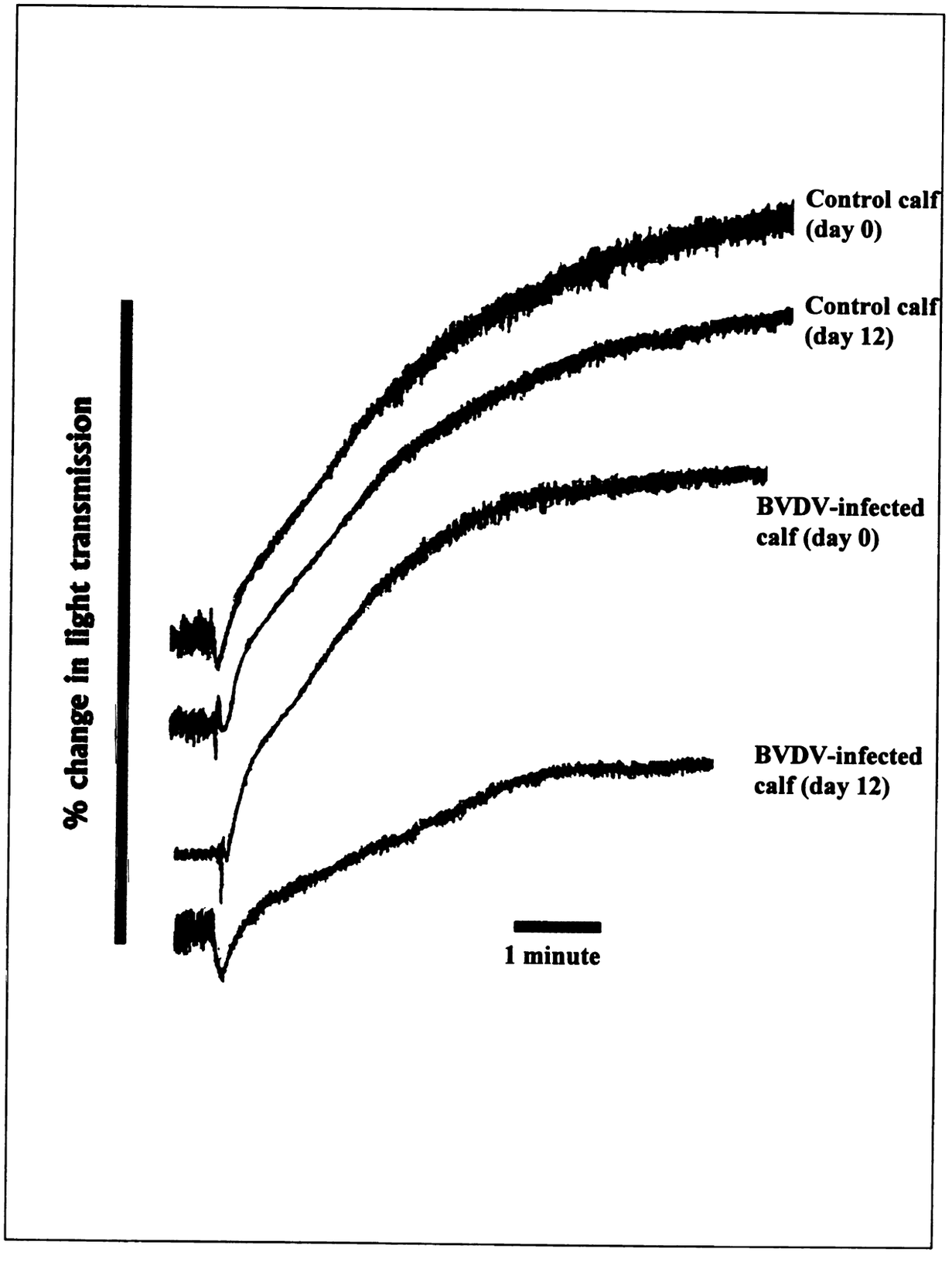

Control calf (day 0)
Control calf (day 12)
BVDV-infected calf (day 0)
BVDV-infected calf (day 12)
% change in light transmission
1 minute

Figure 3.6. Platelet aggregation responses (A: Maximum percent aggregation; B: Slope of the aggregation curve) induced by platelet activating factor at a concentration of 0.1μM for control (n=4) and BVDV-infected (n=5) calves during the experimental time period. Results are expressed as the mean ± standard error. * Indicates values that are significantly ($P<0.05$, *t*-test) different between control and BVDV-infected calves at the same collection time.

Figure 3.6

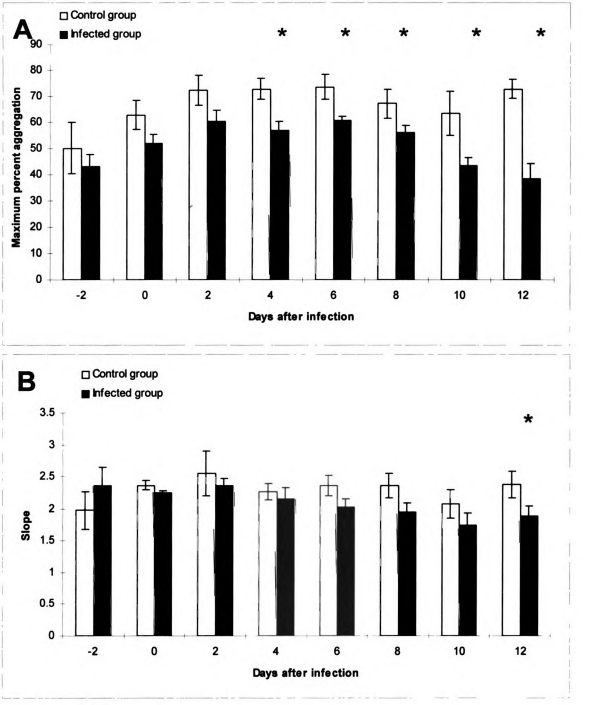

A
Control group
Infected group
Maximum percent aggregation
90
80
70
60
50
40
30
20
10
0
*
*
*
*
*
-2
0
2
4
6
8
10
12
Days after infection
B
Control group
Infected group
Slope
3.5
3
2.5
2
1.5
1
0.5
0
*
-2
0
2
4
6
8
10
12
Days after infection

Figure 3.7. Aggregometer tracings of bovine platelet-rich plasma suspensions stimulated with platelet activating factor at a concentration of 1.0 μM. The top two tracings represent the aggregation response curves of bovine platelets obtained from the same control calf on days 0 and 12 after infection. The bottom two tracings represent the aggregation response curves of bovine platelets obtained from the same BVDV-infected calf on days 0 and 12 after infection. In the infected calf at day 12 after infection, the lower percent change in light transmission, compared to the other aggregation tracings, represents a depressed aggregation response.

Figure 3.7

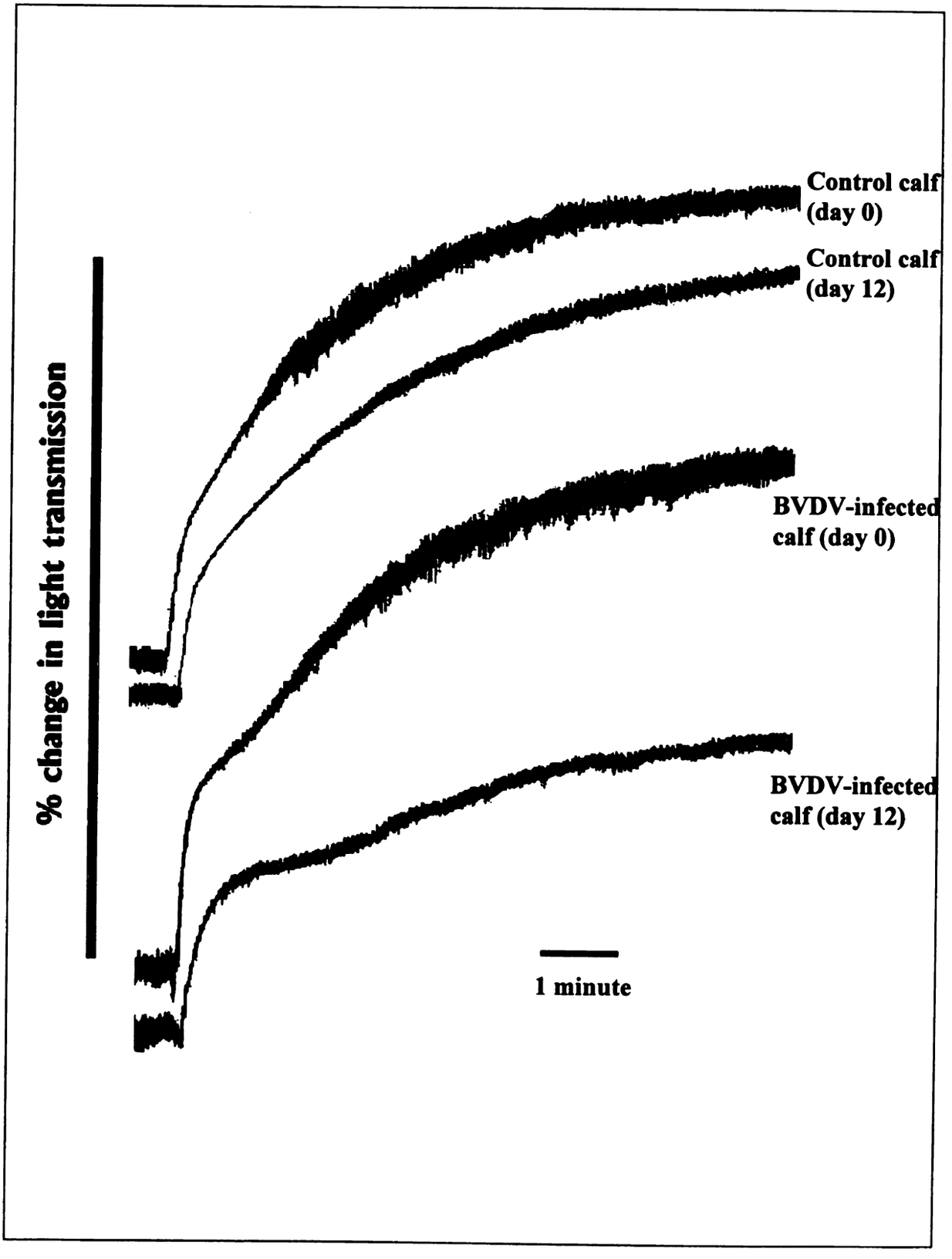
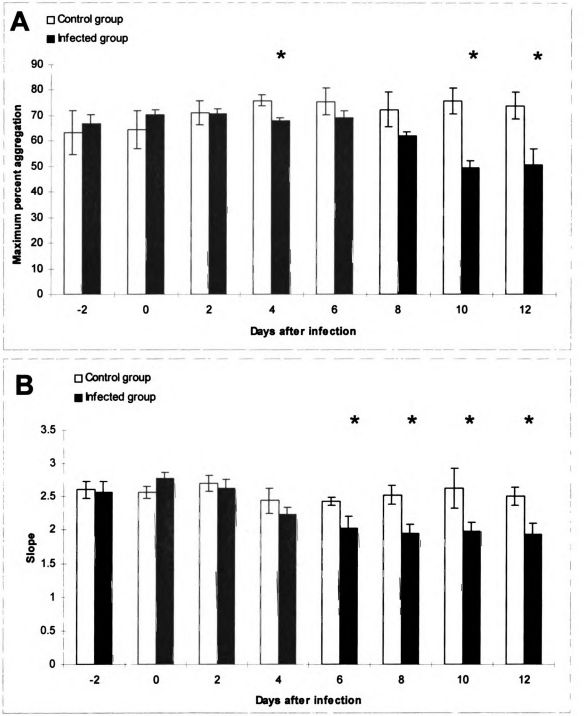

% change in light transmission
Control calf (day 0)
Control calf (day 12)
BVDV-infected calf (day 0)
BVDV-infected calf (day 12)
1 minute

Figure 3.8. Platelet aggregation responses (A: Maximum percent aggregation; B: Slope of the aggregation curve) induced by platelet activating factor at a concentration of 1.0μM for control (n=4) and BVDV-infected (n=5) calves during the experimental time period. Results are expressed as the mean ± standard error. * Indicates values that are significantly ($P<0.05$, *t*-test) different between control and BVDV-infected calves at the same collection time.

Figure 3.8


A
Control group
Infected group
Maximum percent aggregation
90
80
70
60
50
40
30
20
10
0
-2
0
2
4
6
8
10
12
Days after infection
*
*
*
B
Control group
Infected group
Slope
3.5
3
2.5
2
1.5
1
0.5
0
-2
0
2
4
6
8
10
12
Days after infection
*
*
*
*

Discussion

Platelets play an important role in hemostasis, and altered platelet function may lead to hemorrhage (Jain, 1986). Results of the present study indicate that platelets from calves infected with BVDV 890, a type II BVDV isolate, display abnormal aggregation responses. It has been reported that BVDV infection is associated with thrombocytopenia and hemorrhage (Bezek *et al.*, 1994; Bolin & Ridpath, 1992; Corapi *et al.*, 1990; Rebhun *et al.*, 1989), yet in some instances, the decrease in platelet count has been insufficient to be completely responsible for clinical evidence of hemorrhage. Findings of this study suggest that altered platelet function, in addition to thrombocytopenia, may contribute to the hemorrhage observed in some calves with type II BVDV infection.

Although the mechanism responsible for diminished platelet responses in association with BVDV 890 infection is unknown, it is likely to involve an alteration in the population of platelets in the circulation resulting in an increase in the percentage of aged platelets, a direct virus-platelet interaction, an indirect virus-platelet interaction involving intermediate factors, or some combination of these mechanisms.

Megakaryocytes are the precursor cells for platelets (Wright, 1906), and infection of megakaryocytes with BVDV has been demonstrated through immunohistochemical and immunofluorescent antibody techniques (Corapi *et al.*, 1989; Marshall *et al.*, 1996; Spagnuolo-Weaver *et al.*, 1997). Bovine viral diarrhea virus infection of megakaryocytes may decrease production of platelets, resulting in thrombocytopenia and increasing the percentage of older platelets in the circulation. In dogs, humans, and rats, platelets in the circulation become smaller and less sensitive to aggregatory stimuli as they age (Carty &

Gear, 1986; Peng *et al.*, 1994). Younger platelets are larger and substantially more sensitive than aged platelets to aggregation agonists, such as ADP and thrombin. In the present study, aggregation studies were performed on platelet-rich plasma suspensions, rather than blood, to control for changes in platelet count in calves infected with BVDV. Because platelet aggregation studies were performed on platelet-rich plasma suspensions adjusted to counts of 300,000 platelets/μl, decreased aggregation responses for platelets from infected calves could have been a result of an increase in the percentage of dysfunctional platelets, compared with control calves. These dysfunctional platelets may have been aged platelets in which aggregation responses were impaired as a result of decreased granule content of secretory factors, impaired signal transduction pathways, or alterations in the cell surface receptors for agonists.

In this study, we identified a direct virus-platelet interaction by isolating BVDV from purified platelet preparations. Previous studies have also identified an association between platelets and BVDV, using virus isolation procedures (Bolin & Ridpath, 1992) and immunofluorescent antibody testing (Bezek *et al.*, 1994; Corapi *et al.*, 1989). We attempted to isolate BVDV from purified platelet preparations at the same time aggregation studies were performed to determine whether a direct virus-platelet interaction could be responsible for platelet dysfunction in infected calves. We found that a significant decrease in maximum percentage aggregation in response to ADP or PAF only became apparent when virus could be isolated from platelets (ie, 4 through 12 days after inoculation). Therefore, it is possible that a direct virus-platelet interaction was responsible for the aggregation defects in infected calves. However, in a previous study (Bolin & Ridpath, 1992), the percentage of platelets from which BVDV could be isolated

was very low (approx 0.1%). Thus, it seems likely that direct virus-platelet interaction was not the sole cause of platelet dysfunction. In addition, it has been demonstrated that feline infectious peritonitis virus causes hyperaggregability, probably as a result of a direct virus-platelet interaction (Boudreaux *et al.*, 1990), not the hypoaggregability detected in the present study.

Prostacyclin (prostaglandin I_2), prostaglandin E, nitric oxide, circulating immune complexes, and a variety of other circulating substances have been demonstrated to inhibit platelet aggregation responses (Rao & Carvalho, 1994), and an increase in the concentration of 1 or more of these intermediate soluble factors in platelet-rich plasma suspensions could alter aggregation responses of platelets. Decreased aggregation responses have been demonstrated in human patients infected with Lassa fever virus, a hemorrhagic fever of humans (Cummins *et al.*, 1989). The platelet defect in Lassa fever virus infection has been determined to be a result of an inhibitory factor in plasma that does not appear to be a viral protein or virus antibody (Cummins *et al.*, 1989). In pigs infected with African swine fever virus, high plasma concentrations of the platelet aggregation inhibitor prostaglandin E_2 have been demonstrated (Anderson *et al.*, 1987). High concentrations of nitric oxide and prostaglandin E have been demonstrated in bone marrow macrophages (Adler *et al.*, 1994) and alveolar macrophages (Van Reeth & Adair, 1997), respectively, from cattle with BVDV infection, but what impact these high concentrations have on platelet function is not known.

Adenosine diphosphate and PAF were used as aggregation agonists in this study because of their predictable aggregation response in cattle (Bondy & Gentry, 1989) and ease in use. In addition, they use different receptors and signal transduction pathways to

induce platelet aggregation (Hawiger *et al.*, 1994). The shape changes induced by ADP throughout the study indicated that platelets had a functional ADP receptor. The observation of a shape change without aggregation (zero aggregation) in response to 10 μM and 100 μM ADP in some BVDV-infected calves suggested that although the receptor was functionally present, the enzyme pathway or secretion of additional ADP by platelets may have been impaired. When PAF was used, platelets from control and infected calves underwent a shape change and aggregated, but the aggregation response was diminished in platelets from the infected calves. Detection of functional ADP and PAF receptors suggests that the defect in function of platelets from infected calves reflects deficiencies in the signal transduction pathways, the release reaction of the granule constituents, or the secretory substances themselves.

In summary, hemorrhagic syndrome associated with BVDV 890 infection in neonatal calves can be attributable to thrombocytopenia and altered platelet function. At present, it is not known whether other type I or type II BVDV isolates also induce qualitative platelet defects. Further study is required to define the pathophysiologic mechanism of altered platelet function in infected calves and to identify the role that altered platelet function may have, particularly in relation to the increased virulence of some type II BVDV isolates.

Sources and Manufacturers

a. Bolin SR, National Animal Disease Center, Ames, IA: Personal communication, 1997.

b. Butler Company, Dublin, Ohio.

c. Schering-Plough Animal Health Corporation, Kenilworth, NJ.

d. JRH Biosciences, Lenexa, Kan.

e. Sigma Chemical Co, St. Louis, Mo.

f. Gibco BRL, Life Technologies, Grand Island, NY.

g. Becton Dickinson, Franklin, NJ.

h. Chronolog Corporation, Havertown, Pa.

i. Calbiochem Biochemicals, San Diego, Calif.

j. Millipore Corporation, Bedford, Mass.

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CHAPTER IV:

JOURNAL ARTICLE: PLATELET AGGREGATION RESPONSES AND VIRUS ISOLATION FROM PLATELETS IN CALVES EXPERIMENTALLY INFECTED WITH TYPE I OR TYPE II BOVINE VIRAL DIARRHEA VIRUS

CHAPTER IV: JOURNAL ARTICLE:

PLATELET AGGREGATION RESPONSES AND VIRUS ISOLATION FROM PLATELETS IN CALVES EXPERIMENTALLY INFECTED WITH TYPE I OR TYPE II BOVINE VIRAL DIARRHEA VIRUS

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Abstract

Altered platelet function has been reported in calves experimentally infected with type II bovine viral diarrhea virus (BVDV). The purpose of the present study was to further evaluate the ability of BVDV isolates to alter platelet function and to specifically determine if platelet dysfunction only occurs following challenge with type II BVDV. Colostrum-deprived Holstein calves were obtained immediately after birth, housed in isolation, and assigned to one of four groups (1 control and 3 treatment groups). Control calves (n=4) were sham inoculated, while calves in the infected groups (n=4 for each group) were inoculated by intranasal instillation with 10^7 $TCID_{50}$ of either BVDV 890 (type II), BVDV 7937 (type II), or BVDV TGAN (type I). Whole blood was collected prior to inoculation (day 0) and on days 4, 6, 8, 10, and 12 after inoculation for platelet function testing by optical aggregometry using the aggregation agonists adenosine diphosphate (10 and 100 μM) and platelet activating factor (0.1 and 1.0 μM). Virus isolation procedures from platelets were performed on the same days as platelet function with the exception of day 10 after inoculation. The maximum percentage aggregation and the slope of the aggregation curve decreased over time in BVDV-infected calves; however statistically significant differences (Freidman Repeated Measures ANOVA on Ranks, $p<0.05$) were only observed in calves infected with the type II BVDV isolates. Bovine viral diarrhea virus was not isolated from control calves, but was isolated from all calves infected with both type II BVDV isolates from days 4 through 12 after inoculation. In calves infected with type I BVDV, the virus was isolated from 1 of 4 calves on days 4 and 12 after inoculation and from all calves on days 6 and 8 after inoculation. Altered platelet function was observed in calves infected with both type II BVDV isolates, while

altered platelet function was not observed in calves infected with type I BVDV. Altered platelet function may be an important difference in virulence between type I and type II BVDV infections.

Introduction

Circulating platelets play an important role in hemostasis and thrombosis. Impairment of the hemostatic response may occur with quantitative platelet disorders (thrombocytopenia), in which circulating numbers of platelets are low, or qualitative platelet defects (thrombocytopathy), in which platelets have altered functional characteristics (Jain, 1986). Impairment of the hemostatic response can result in clinical signs of hemorrhage. Bovine viral diarrhea virus (BVDV) has been associated with outbreaks of severe peracute disease (Carman *et al.*, 1998) and a hemorrhagic syndrome characterized by thrombocytopenia, bleeding, and death (Bolin & Ridpath, 1992; Corapi *et al.*, 1989; Rebhun *et al.*, 1989).

Thrombocytopenia associated with type II BVDV infection has been reported in adult cattle and calves under natural conditions (Rebhun *et al.*, 1989), and has been experimentally reproduced (Bolin & Ridpath, 1992; Corapi *et al.*, 1990; Walz *et al.*, 1999a). Although most cases of BVDV-induced thrombocytopenia have been associated with type II BVDV, a recent study demonstrated that a type I isolate induced thrombocytopenia in experimentally infected calves (Marshall *et al.*, 1996).

In addition to thrombocytopenia, altered platelet function has been reported following experimental type II BVDV infection in calves (Walz *et al.*, 1999b); however, a single type II BVDV isolate was examined, and it is currently unknown if type I BVDV

isolates or other type II BVDV isolates are capable of altering platelet function. Altered platelet function may be an important contributing factor for the hemorrhagic syndrome and for the increased virulence observed with some type II BVDV isolates. Therefore, this study was designed to further evaluate the ability of BVDV isolates to alter platelet function, and to determine if platelet dysfunction occurs exclusively following challenge with type II BVDV.

Materials and Methods

Animals

Colostrum-deprived newborn male Holstein calves (n=16) were obtained immediately after birth from local dairy farms. Calves were moved to an isolation facility, allocated to one of four groups (1 control and 3 treatment groups), and individually housed in separate rooms. All calves were given a physical examination, and serum and whole blood were taken for virus isolation procedures and determination of antibody levels to BVDV by virus neutralization. The calves were administered an intravenous plasma transfusion, consisting of 400 ml of plasma obtained from a donor cow. The plasma donor was BVDV antibody negative by virus neutralization and virus negative by virus isolation procedures on three serial serum samples obtained prior to the collection of plasma. In addition, calves were given injections of vitamins A and D (Butler Company, Dublin, OH) and vitamin E and selenium (Schering-Plough Animal Health Corporation, Kenilworth, NJ), as well as a commercial oral monoclonal antibody preparation against *E. coli* K99 pilus antigen (Schering-Plough Animal Health

Corporation, Kenilworth, NJ). Calves were fed a nonmedicated milk replacer according to label directions at 12% of body weight twice daily throughout the study period. The experiment was performed with approval and under the guidelines of the Michigan State University All University Committee on Animal Use and Care.

Virus and infection protocol

The following three noncytopathic BVDV isolates were used for the experimental infection of calves: BVDV 890 (Dr. Steve Bolin, National Animal Disease Center, Ames, IA), BVDV 7937 (Dr. Clayton Kelling, University of Nebraska, Lincoln, NE), and BVDV TGAN (Dr. Steve Bolin, National Animal Disease Center, Ames, IA). The BVDV 890 is a type II isolate that has been previously shown to induce thrombocytopenia and platelet dysfunction in experimentally infected calves (Bolin & Ridpath, 1992; Walz *et al.*, 1999a; Walz *et al.*, 1999b). The BVDV 7937 (type II) and BVDV TGAN (type I) are BVDV isolates that have been reported not to induce thrombocytopenia (Bolin & Ridpath, 1992; Marshall *et al.*, 1996). The viral isolates were propagated in bovine turbinate cells in Eagle's minimum essential medium (EMEM; JRH Biosciences, Lenexa, KS) containing 10% fetal equine serum (FES; Sigma Chemical Co., St. Louis, MO), L-glutamine (Gibco BRL, Life Technologies, Grand Island, NY), penicillin G (Sigma Chemical Co.), and streptomycin (Sigma Chemical Co.). The viral titer of the inoculum was determined according to previously described methods (Lennette & Schmidt, 1969).

After a three-day adjustment period in the isolation facility, all calves were inoculated. Calves in the 3 infected groups (n=4 for each group) were inoculated by

intranasal instillation with 10^7 $TCID_{50}$ of the respective viral isolate in 5 ml of EMEM without serum (2.5 ml per nostril). Control calves (n=4) were inoculated by intranasal instillation with a sham inoculum, consisting of a BVDV-uninfected cell culture preparation.

Collection of platelets

Whole blood was collected for platelet aggregation studies on the day of inoculation (pre-inoculation), and then on days 4, 6, 8, 10, and 12 after inoculation. In addition, whole blood was collected for virus isolation from platelets on the day of inoculation, and then on days 4, 6, 8, and 12 after inoculation. Blood was collected by jugular venipuncture through an 18-gauge needle into plastic syringes containing 1.0 ml of 3.8% trisodium citrate for each 9.0 ml of blood. Platelet-rich plasma was obtained by two centrifugation steps at 1,324 x g for 60 seconds and 30 seconds, with the platelet-rich plasma being removed after each centrifugation. Platelet-poor plasma was obtained by centrifugation of the remaining blood at 1,324 x g for 13 minutes. Platelets were manually counted using a white blood cell microcollection system (Becton Dickinson, Franklin, NJ) and a hemocytometer.

Platelet aggregation responses

Aggregation studies were performed in an aggregometer (Chronolog Corporation, Havertown, PA) by optical aggregometry. Aggregation agonists used for this study were adenosine diphosphate (ADP; Sigma Chemical Co.), at concentrations of 10 and 100 μM, and platelet activating factor (PAF; Calbiochem Biochemicals, San Diego, CA), at

concentrations of 0.1 and 1.0 μM. Platelet-rich plasma was adjusted to 300,000 platelets/μl with homologous platelet-poor plasma. Adjusted platelet-rich plasma suspensions were then divided into 0.5 ml aliquots in glass cuvettes and held at room temperature for 30 minutes. The platelet-rich plasma suspensions were warmed for 5 minutes at 37°C. A magnetic stir bar was added to the sample, and the cuvette was then placed into the aggregometer. For each experiment, the aggregometer was calibrated using nonaggregated platelet-rich plasma to establish the 0% aggregation limit and platelet-poor plasma to establish the 100% aggregation limit. The sample was allowed to stir at 900 rpm for approximately 30 seconds, at which time 20 μl of agonist was added to the platelet suspensions, and the changes in light transmission recorded. The maximum percentage aggregation and the slope of the aggregation curve were measured for each aggregation run. All aggregation studies were completed within 5 hours of collection of blood samples. All studies were done in duplicate, and the mean value was obtained.

Virus isolation from platelets

Platelets were gel-filtered and washed to remove plasma constituents. Platelet-rich plasma was collected as described above and 1 μl of 1 μM prostaglandin E_1 (Sigma Chemical Co.) was added per 1 ml platelet-rich plasma prior to centrifugation at 800 x g for 15 minutes. Plasma supernatant was removed with a sterile pipette, and the platelet pellet was resuspended in 1 ml of Hank's Balanced Salt Solution (136 mM NaCl, 5.4 mM KCL, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, and 5.5 mM dextrose, pH 7.4). The platelet suspension was transferred to a 10 ml polystyrene column containing Sepharose 4B (Sigma Chemical Co.). The eluate was collected, washed three times in 10 ml of

Hank's Balanced Salt Solution, resuspended in Hank's Balanced Salt Solution, and counted manually. Approximately 10^7 platelets were inoculated into wells on 96-well microtiter plates containing monolayers of bovine turbinate cells in EMEM with 10% FES, L-glutamine and antibiotics. After 5 days of incubation at 37°C in humidified air containing 5% CO_2, 50 µl of supernatant was transferred to 96-well microtiter plates freshly seeded with bovine turbinate cells. After an additional 5 days of incubation at 37°C in humidified air containing 5% CO_2, the bovine turbinate cells were stained for BVDV antigen by an immunoperoxidase monolayer assay (Meyling, 1988).

Statistical analysis

Comparisons of the maximum percentage aggregation and the slope of the aggregation curve among groups were made using the Freidman repeated measures analysis of variance on ranks. When significant ($P<0.05$) differences were determined, post-hoc comparisons were made between the day 0 sample period (pre-inoculation) to days 4, 6, 8, 10, and 12 sample periods (after inoculation) by use of Dunn's multiple comparisons test.

Results

Aggregation responses with adenosine diphosphate (ADP)

The aggregation results for the control and BVDV-infected groups of calves in response to 10 µM ADP are presented in Figure 4.1. Throughout the study, platelets from control calves displayed an expected shape change and aggregation response to 10 µM ADP. A normal shape change was also observed in the BVDV-infected calves in

response to 10 μM ADP. However, platelet function was depressed in the two groups of calves inoculated with type II BVDV, with the greatest depression occurring in the calves inoculated with the type II isolate BVDV 890 (Figure 4.1). Zero aggregation, which is defined as a failure to record an increase in light transmission above the zero baseline after the addition of agonist, was observed using 10 μM ADP in all calves infected with the type II isolate BVDV 890 on day 10 after infection. In calves infected with the type I isolate BVDV TGAN, a difference in platelet function was not observed between pre- and after inoculation samples in response to 10 μM ADP.

The aggregation results for the control and infected groups of calves in response to 100 μM ADP are presented in Figure 4.2. In the control calves, ADP at a concentration of 100 μM induced the expected shape change and aggregation response throughout the study. A shape change also preceded the aggregation response in the BVDV-infected calves; however, a decrease in the maximum percentage aggregation and the slope of the aggregation curve was observed in calves infected with both type II BVDV isolates as the experiment proceeded over time (Figure 4.2). The greatest depression in platelet function occurred on day 10 after inoculation, and aggregation tracings from a representative calf in each of the 4 groups are presented in Figure 4.3. Zero aggregation, as previously defined, was observed in response to 100 μM ADP in 2 of 5 calves infected with the type II isolate BVDV 890 on day 10 after inoculation. Although a statistically significant difference was not detected between pre- and post-inoculation sample periods in calves infected with the type I isolate BVDV TGAN, there was a mild depression in platelet function as the experiment proceeded over time (Figures 4.2 and 4.3).

Aggregation responses with platelet activating factor (PAF)

The aggregation results for the control and infected groups of calves in response to 0.1 μM PAF are presented in Figure 4.4. The platelets from control calves displayed a normal aggregation response to 0.1 μM PAF throughout the study period, while platelets from the calves infected with the type II isolate BVDV 890 exhibited a significantly depressed maximum percentage aggregation and slope on day 10 after inoculation. Although a statistically significant depression in platelet function was not detected between pre- and after inoculation samples in calves infected with the type II isolate BVDV 7937, there was a depression in the maximum percentage aggregation and the slope of the aggregation curve as the experiment proceeded over time. The greatest depression in platelet function in the type II BVDV-infected calves occurred on day 10 after inoculation, and aggregation tracings from a representative calf in each of the 4 groups are presented in Figure 4.5. A statistically significant difference was not detected between pre- and post-inoculation samples in calves infected with the type I isolate BVDV TGAN, but there was a mild depression in the maximum percentage aggregation and the slope of the aggregation curve as the experiment proceeded over time (Figures 4.4 and 4.5).

The aggregation results for the control and infected groups of calves in response to 1.0 μM PAF are presented in Figure 4.6. The maximum percentage aggregation and slope of the aggregation curve in calves infected with the type II isolate BVDV 890 were significantly depressed on day 10 and days 8, 10, and 12 after inoculation, respectively, when compared to day 0 in response to 1.0 μM PAF. The trend of a depressed maximum percentage aggregation and slope of the aggregation curve was also observed in calves

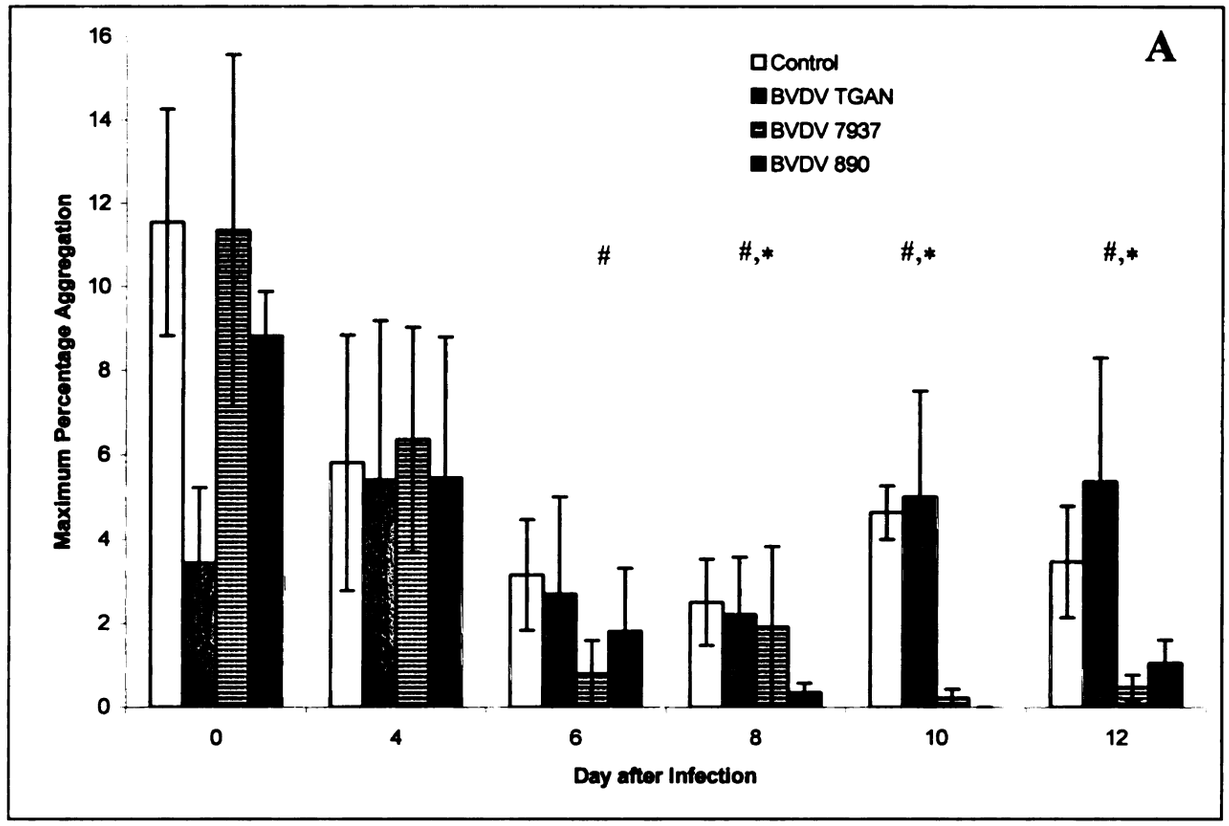
infected with the type II isolate BVDV 7937 as the experiment proceeded over time, but statistically significant differences were only observed with respect to the slope of the aggregation curve. There was a mild depression in the maximum percentage aggregation in calves infected with the type I isolate BVDV TGAN, but the differences were not statistically significant when compared to the day 0 sample.

Isolation of BVDV from platelets

Bovine viral diarrhea virus was not isolated from platelet preparations from control calves. Bovine viral diarrhea virus was isolated from platelets from all post-inoculation time periods in calves that were infected with the type II isolates (Table 4.1). Bovine viral diarrhea virus was also isolated from all calves infected with the type I isolate BVDV TGAN, but was not isolated from all the calves at all post-inoculation time periods (Table 4.1).

Figure 4.1. Platelet aggregation responses (A: Maximum percent aggregation; B: Slope of the aggregation curve) induced by adenosine diphosphate at a concentration of 10μM for uninfected control calves, calves infected with the type I isolate BVDV TGAN, calves infected with the type II isolate BVDV 7937, and calves infected with the type II isolate BVDV 890. Calves were infected at day 0. Results are expressed as the mean ± standard error. Statistically significant differences (Freidman RM ANOVA on ranks, $p < 0.05$, Dunn's test) are denoted as follows: * = BVDV 890 after inoculation vs. before inoculation (day 0); # = BVDV 7937 after inoculation vs. before inoculation (day 0).

Figure 4.1


A
Control
BVDV TGAN
BVDV 7937
BVDV 890
Maximum Percentage Aggregation
16
14
12
10
8
6
4
2
0
#
#,*
#,*
#,*
0
4
6
8
10
12
Day after Infection

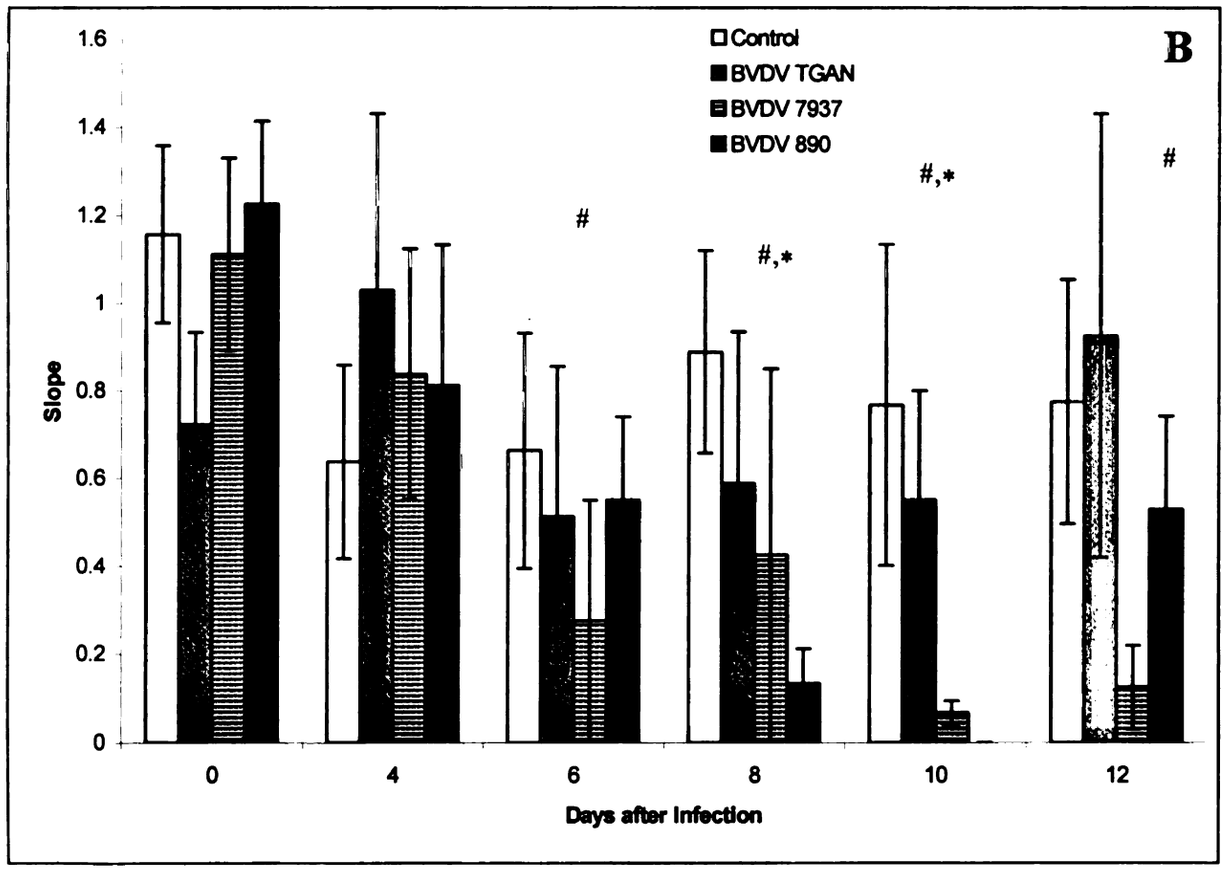

B
Control
BVDV TGAN
BVDV 7937
BVDV 890
Slope
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
#
#,*
#,*
#
0
4
6
8
10
12
Days after Infection

Figure 4.2. Platelet aggregation responses (A: Maximum percent aggregation; B: Slope of the aggregation curve) induced by adenosine diphosphate at a concentration of 100μM for uninfected control calves, calves infected with the type I isolate BVDV TGAN, calves infected with the type II isolate BVDV 7937, and calves infected with the type II isolate BVDV 890. Calves were infected at day 0. Results are expressed as the mean ± standard error. Statistically significant differences (Freidman RM ANOVA on ranks, $p < 0.05$, Dunn's test) are denoted as follows: * = BVDV 890 after inoculation vs. before inoculation (day 0); # = BVDV 7937 after inoculation vs. before inoculation (day 0).

Figure 4.2

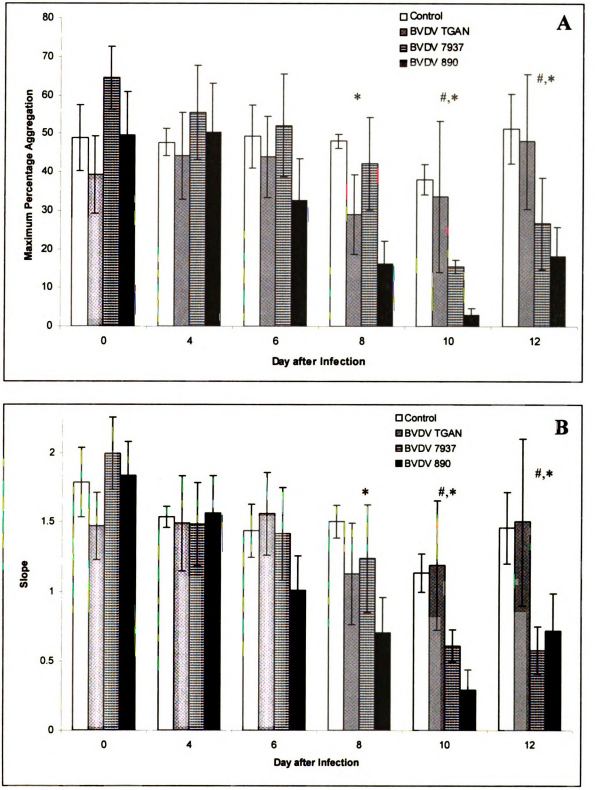

A
Control
BVDV TGAN
BVDV 7937
BVDV 890
Maximum Percentage Aggregation
80
70
60
50
40
30
20
10
0
*
#,*
#,*
0
4
6
8
10
12
Day after Infection
B
Control
BVDV TGAN
BVDV 7937
BVDV 890
Slope
2
1.5
1
0.5
0
*
#,*
#,*
0
4
6
8
10
12
Day after Infection

Figure 4.3. Aggregometer tracings of bovine platelet-rich plasma suspensions stimulated with adenosine diphosphate at a concentration of 100 μM. The four tracings represent the aggregation response curves of bovine platelets obtained from a calf in each group on day 10 after inoculation. In the BVDV-infected calves, the lower percent change in light transmission, compared to the other aggregation tracing from the control calf, represents a depressed aggregation response.

Figure 4.3

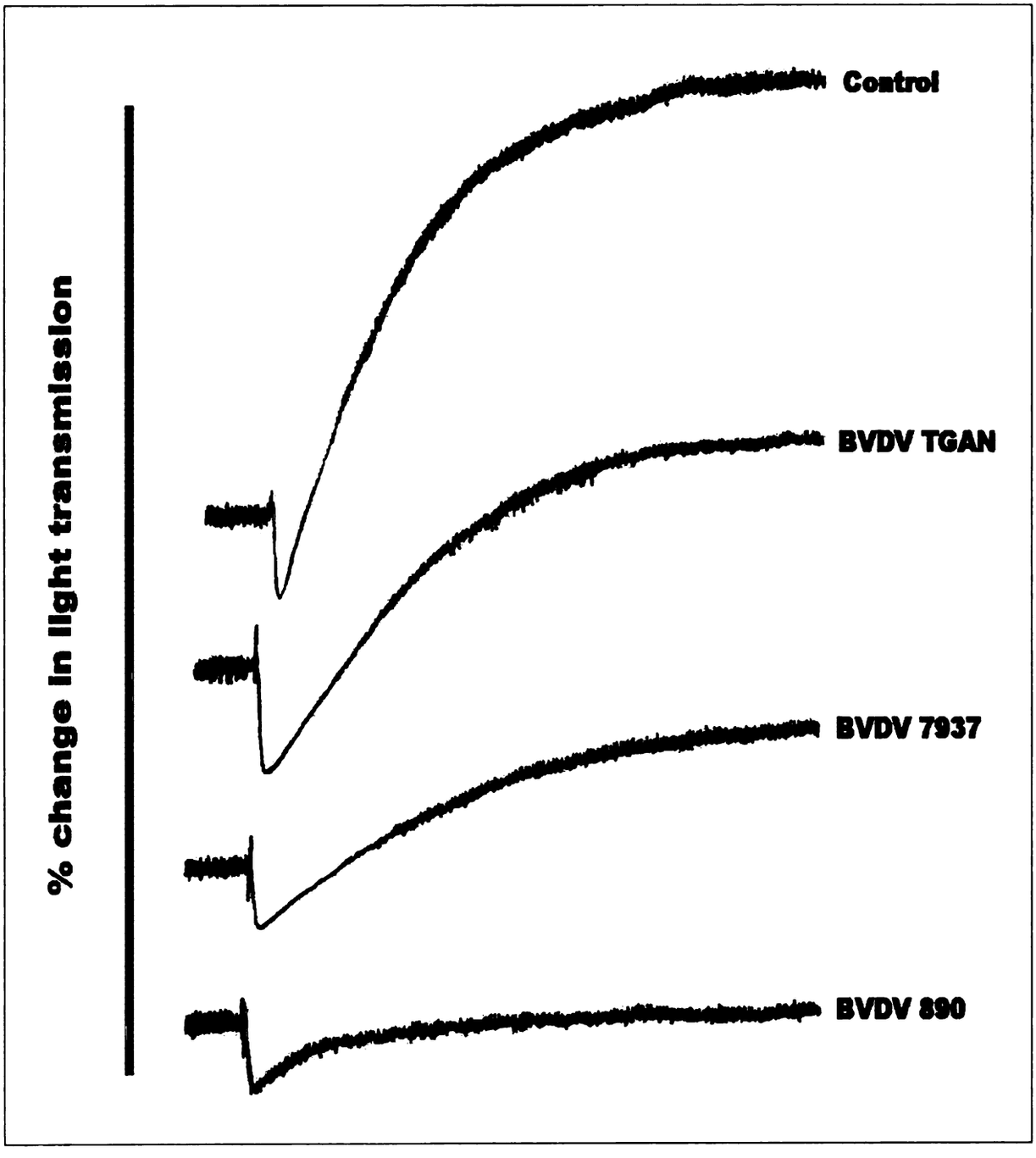
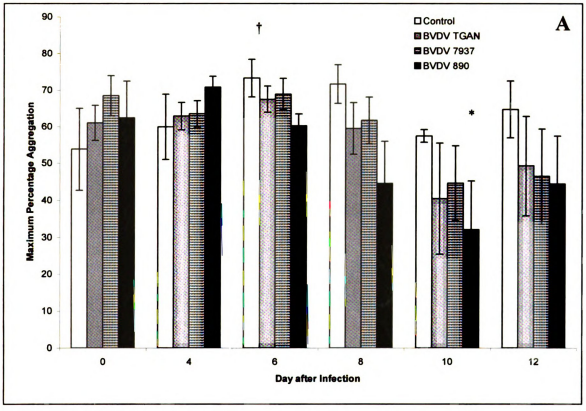

% change in light transmission
Control
BVDV TGAN
BVDV 7937
BVDV 890

Figure 4.4. Platelet aggregation responses (A: Maximum percentage aggregation; B: Slope of the aggregation curve) induced by platelet activating factor at a concentration of 0.1μM for uninfected control calves, calves infected with the type I isolate BVDV TGAN, calves infected with the type II isolate BVDV 7937, and calves infected with the type II isolate BVDV 890. Calves were infected at day 0. Results are expressed as the mean ± standard error. Statistically significant differences (Freidman RM ANOVA on ranks, $p < 0.05$, Dunn's test) are denoted as follows: * = BVDV 890 after inoculation vs. before inoculation (day 0); † = Control post-sham inoculation vs. pre-sham inoculation (day 0).

Figure 4.4


A
Control
BVDV TGAN
BVDV 7937
BVDV 890
Maximum Percentage Aggregation
Day after Infection
†
*
0
4
6
8
10
12
0
10
20
30
40
50
60
70
80
90

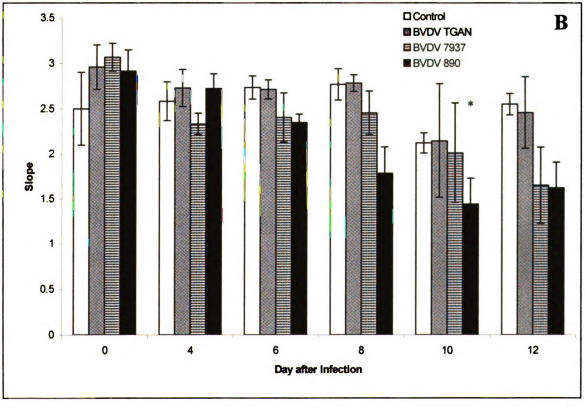

B
Control
BVDV TGAN
BVDV 7937
BVDV 890
Slope
Day after Infection
*
0
4
6
8
10
12
0
0.5
1
1.5
2
2.5
3
3.5

Figure 4.5. Aggregometer tracings of bovine platelet-rich plasma suspensions stimulated with platelet activating factor at a concentration of 0.1μM. The four tracings represent the aggregation response curves of bovine platelets obtained from a calf in each group on day 10 after inoculation. In the BVDV-infected calves, the lower percent change in light transmission, compared to the other aggregation tracing from the control calf, represents a depressed aggregation response.

Figure 4.5

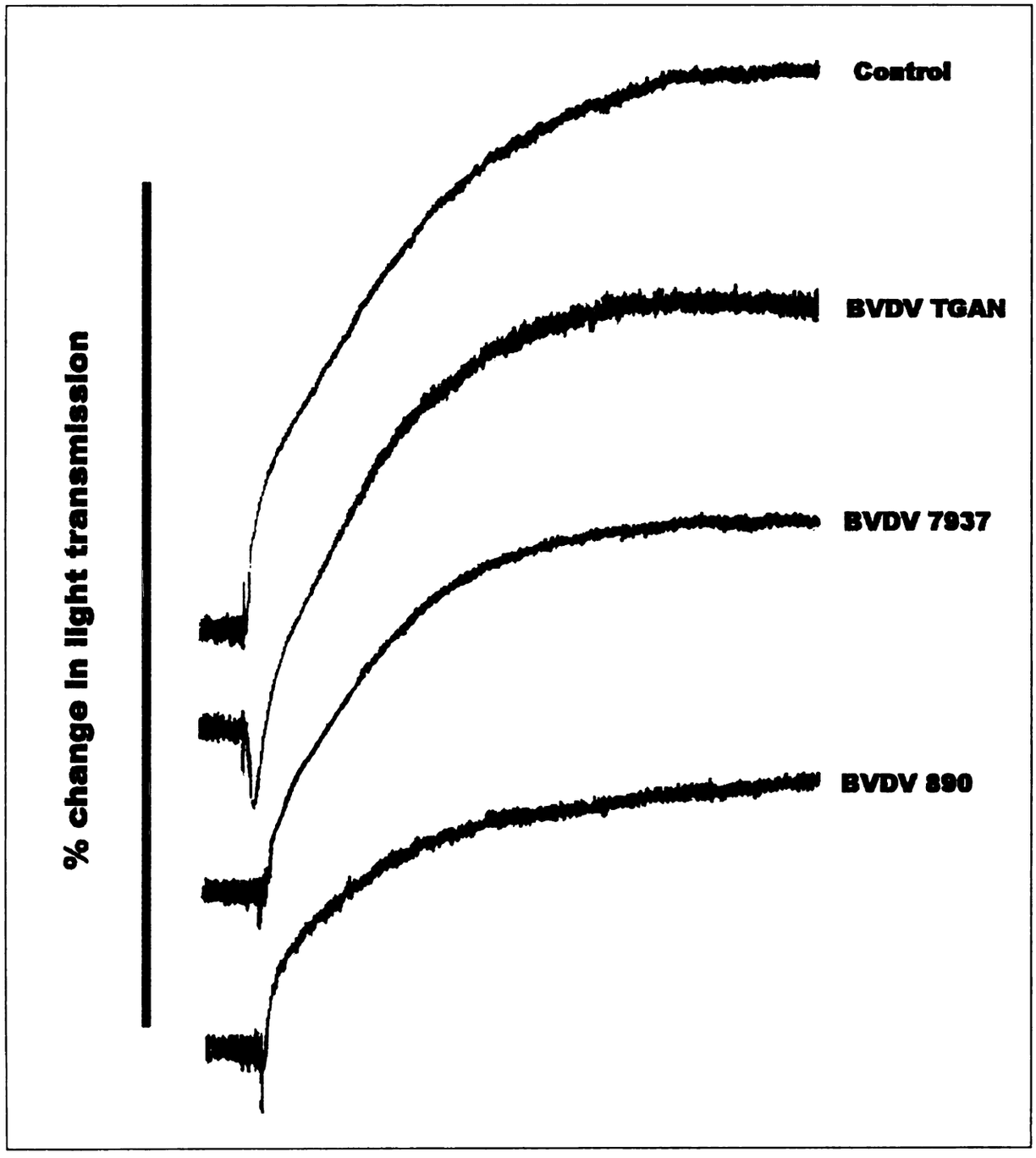
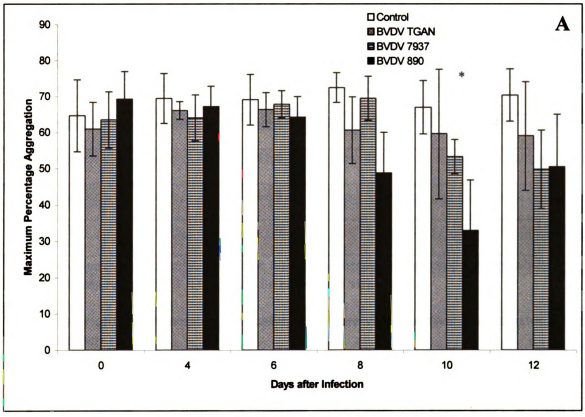

% change in light transmission
Control
BVDV TGAN
BVDV 7937
BVDV 890

Figure 4.6. Platelet aggregation responses (A: Maximum percentage aggregation; B: Slope of the aggregation curve) induced by platelet activating factor at a concentration of 1.0μM for uninfected control calves, calves infected with the type I isolate BVDV TGAN, calves infected with the type II isolate BVDV 7937, and calves infected with the type II isolate BVDV 890. Calves were infected at day 0. Results are expressed as the mean ± standard error. Statistically significant differences (Freidman RM ANOVA on ranks, $p < 0.05$, Dunn's test) are denoted as follows: * = BVDV 890 after inoculation vs. before inoculation (day 0); # = BVDV 7937 after inoculation vs. before inoculation (day 0).

Figure 4.6


A
Control
BVDV TGAN
BVDV 7937
BVDV 890
Maximum Percentage Aggregation
90
80
70
60
50
40
30
20
10
0
*
0
4
6
8
10
12
Days after Infection

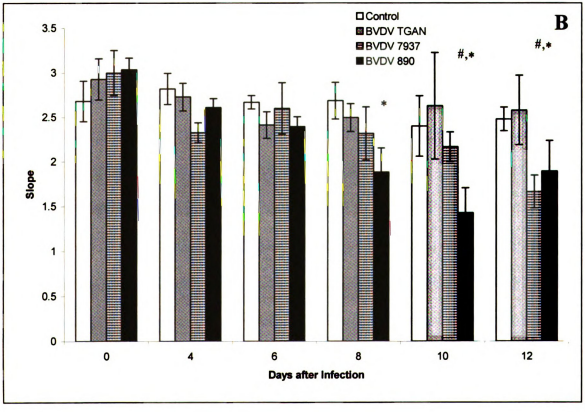

B
Control
BVDV TGAN
BVDV 7937
BVDV 890
Slope
3.5
3
2.5
2
1.5
1
0.5
0
#,*
#,*
*
0
4
6
8
10
12
Days after Infection

Table 4.1. Virus isolation results for purified platelet samples from calves experimentally infected with type I or type II BVDV [a]

Day after infection	Control (Uninfected)	BVDV TGAN (Type I)	BVDV 7937 (Type II)	BVDV 890 (Type II)
Day 0	0 / 4[b]	0 / 4	0 / 4	0 / 4
Day 4	0 / 4	1 / 4	4 / 4	4 / 4
Day 6	0 / 4	4 / 4	4 / 4	4 / 4
Day 8	0 / 4	4 / 4	4 / 4	4 / 4
Day 12	0 / 4	1 / 4	4 / 4	4 / 4

[a] Control calves were inoculated with an uninfected tissue culture preparation, while infected calves received 10^7 $TCID_{50}$ of the respective viral isolate

[b] Cells under groups: numerator: # of calves that were virus positive; denominator: # of calves in group

Discussion

Platelets are essential for normal hemostasis, and alterations in their function may lead to hemorrhage (Jain, 1986). Depression of platelet function has been previously demonstrated in calves experimentally infected with the type II isolate BVDV 890 (Walz *et al.*, 1999b), and this altered platelet function likely contributes to the pathogenesis of the hemorrhagic syndrome, as clinical signs of hemorrhage were observed in the infected calves (Walz *et al.*, 1999a). The findings of this study further support the ability of the type II isolate BVDV 890 to alter platelet function in experimentally infected calves. In comparison to the previous BVDV 890 infection study (Walz *et al.*, 1999b), the mean values for maximum percentage aggregation and slope of the aggregation curve in response to both ADP and PAF were slightly lower, thus indicating a greater depression in platelet function observed in this study.

An additional type II BVDV isolate was tested in this study to determine if altered platelet function occurs only in calves infected with the type II isolate BVDV 890. The type II isolate BVDV 7937 was utilized in this study because it was an isolate that has been reported to not induce thrombocytopenia (Marshall *et al.*, 1996), and as such, would allow a comparison between two type II isolates that do and do not induce thrombocytopenia. Platelet function was depressed in calves infected with the type II isolate BVDV 7937, although the depression observed was less than that observed in calves infected with the type II isolate BVDV 890. Type II BVDV infection results in altered platelet function. In addition, type II BVDV isolates that also induce

thrombocytopenia may be more adept at depressing platelet function than type II BVDV isolates that do not induce thrombocytopenia, as observed by the results in this study.

Prior to the report describing thrombocytopenia in calves experimentally infected with a type I BVDV isolate, it was believed that only type II isolates were capable of inducing thrombocytopenia. We examined the type I isolate BVDV TGAN because it was reported to not induce thrombocytopenia (Bolin & Ridpath, 1992). This would allow a comparison between a thrombocytopenia-inducing type II BVDV isolate (BVDV 890) with a type I isolate that does not induce thrombocytopenia. Our study, which examined a single type I isolate that does not induce thrombocytopenia, also demonstrates that this type I isolate did not induce platelet dysfunction. This does not rule out the potential for other type I BVDV isolates to alter platelet function. Our studies were initiated prior to the report describing the ability of the type I BVDV isolate NY-1 to induce thrombocytopenia (Marshall *et al.*, 1996). Platelet function testing in calves infected with the type I isolate BVDV NY-1 may further characterize type I BVDV infections and alterations in platelet function.

Variability in platelet aggregation responses, as evidenced by the large standard error, and a mild depression in platelet function was observed in control calves in response to ADP 10μM, as the calves aged. A similar depression in platelet function in response to ADP 10μM was observed in an earlier study as well (Walz *et al.*, 1999b). This may be an age-related effect that is obscured in the BVDV-infected calves as a result of infection. In addition to hypoaggregability in response to ADP 10 μM in the control calves, a relative thrombocytosis peaking at approximately 7 days of age was also observed in a previous study (Walz *et al.*, 1999a). These results reinforce the importance

of age-matched control calves in studying BVDV infection and platelet disorders in neonatal calves. Another observation that requires discussion is the depression in platelet function in response to ADP 10 μM observed prior to inoculation in the calves infected with BVDV TGAN. No explanation can be afforded for this depression, although analysis of variance testing revealed no difference between any of the groups at the day 0 sample period.

Although the exact mechanism responsible for the diminished platelet response associated with type II BVDV infection is unknown, the isolation of BVDV from platelets may be an important factor. The association of platelets and BVDV is supported by previous studies that have also demonstrated an association using virus isolation procedures (Bolin & Ridpath, 1992) and immunofluorescent antibody testing (Bezek *et al.*, 1994; Corapi *et al.*, 1989). Platelets are small, anucleate cytoplasmic fragments that are derived from precursor cells in the bone marrow, the megakaryocytes (Wright, 1906). Bovine viral diarrhea virus infection of megakaryocytes has been demonstrated through immunohistochemical and immunofluorescent antibody techniques identifying BVDV antigen (Corapi *et al.*, 1989; Ellis *et al.*, 1998; Marshall *et al.*, 1996; Spagnuolo-Weaver *et al.*, 1997). The infection of megakaryocytes with BVDV may result in BVDV association with platelets, but more importantly, the virus infection of megakaryocytes may be responsible for altered platelet function. Bovine viral diarrhea virus infection in megakaryocytes may alter thrombopoiesis and/or megakaryocytopoiesis, thus resulting in thrombocytopenia and a population of predominantly older platelets in the peripheral circulation. As platelets age in the peripheral circulation, they become less sensitive to aggregatory stimuli (Carty & Gear, 1986; Peng *et al.*, 1994). In contrast, younger

platelets are significantly more sensitive than aged platelets to the aggregation agonists, ADP and thrombin (Carty & Gear, 1986). A population of predominantly aged platelets in the peripheral circulation may explain our observations of platelet dysfunction in calves experimentally infected with type II BVDV. Further studies are required to associate the roles of aged platelets, BVDV association with platelets, and type II BVDV infection of megakaryocytes in the mechanism of altered platelet function associated with type II BVDV infection.

In summary, we have demonstrated altered platelet function using two different type II BVDV isolates. Altered platelet function was not observed in the single type I BVDV isolate examined. Circulating platelets participate in many diverse physiologic and pathologic processes, including inflammation, coagulation, maintenance of vascular integrity, and vascular repair. Alteration of platelet function in cattle infected with type II BVDV likely contributes to the pathogenesis of the hemorrhagic syndrome, but type II BVDV infection may also be important for other platelet-related functions. In addition, altered platelet function may be an important factor for differences in virulence between type I and type II BVDV infections.

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CHAPTER V:

JOURNAL ARTICLE: RELATIONSHIP BETWEEN LEVEL OF VIREMIA AND DISEASE IN CALVES EXPERIMENTALLY INFECTED WITH BOVINE VIRAL DIARRHEA VIRUS

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RELATIONSHIP BETWEEN LEVEL OF VIREMIA AND DISEASE IN CALVES EXPERIMENTALLY INFECTED WITH BOVINE VIRAL DIARRHEA VIRUS

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Lana Kaiser, Roger K. Maes, and John C. Baker

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Abstract

Objective—To compare levels of viremia and clinical, hematologic, virologic, immunohistochemical, and pathologic findings during type I and type II bovine viral diarrhea virus (BVDV) infection in calves.

Animals —16 neonatal male Holstein calves.

Procedure—Colostrum-deprived calves were obtained immediately after birth, housed in isolation, and assigned to one of four groups (1 control and 3 treatment groups). Control calves (n=4) were sham inoculated, while calves in the infected groups (n=4 for each group) were inoculated by intranasal instillation with 10^7 $TCID_{50}$ of either BVDV 890 (type II), BVDV 7937 (type II), or BVDV TGAN (type I). Blood cell counts and virus isolation from serum and leukocytes were performed daily, while levels of viremia (serum viral titer and percentages of lymphocytes and platelet associated with BVDV) were determined on day 0 (before inoculation) and days 4, 6, 8, and 12 after inoculation. All experimental calves were euthanatized on day 12 after inoculation, and pathologic, virologic, and immunohistochemical examinations were performed.

Results—Type II BVDV 890 induced the highest level of viremia, followed by type II BVDV 7937, and then type I BVDV TGAN. In addition, BVDV was isolated from serum and leukocytes more frequently and for a longer duration in calves infected with BVDV 890, followed by BVDV 7937, and then BVDV TGAN. A parallel relationship between the level of viremia and rectal temperatures and an inverse relationship between the level of viremia and blood cell counts was observed, that is the greatest elevations in rectal temperature and greatest depressions in platelet and leukocyte counts were observed in calves infected with BVDV 890. Pathologic and immunohistochemical

examinations revealed more pronounced lesions and more extensive viral antigen distribution in the type II BVDV-infected calves as compared to the type I BVDV-infected calves and control calves.

Conclusions—The level of viremia induced during BVDV infection is positively correlated to the development of disease in calves experimentally infected with type I or type II BVDV. In addition, the level of viremia induced during infection may explain the differences in virulence between isolates of BVDV.

Clinical Relevance—Isolates of BVDV, which induce higher levels of viremia, may be more capable of inducing clinical signs of disease. Severe peracute BVD and the hemorrhagic syndrome may occur as a result of infection with isolates of BVDV capable of inducing high levels of viremia. Strategies such as vaccination that reduce viremia may be adequate to control clinical signs associated with acute infection with BVDV.

Introduction

Bovine viral diarrhea virus (BVDV) is an important infectious disease in cattle, resulting in economic losses throughout the world (Baker, 1995). Bovine viral diarrhea virus is an RNA virus and a member of the genus *Pestivirus* within the family Flaviviridae (Wengler, 1991). Isolates of BVDV can be classified into two biotypes, noncytopathic and cytopathic, and two genotypes, type I and type II (Ridpath *et al.*, 1994). Bovine viral diarrhea virus infection may result in a wide spectrum of clinical manifestations, with the majority of BVDV infections occurring without clinical signs of infection (Baker, 1995). However, outbreaks of severe peracute disease in cattle have been reported in association with type II BVDV (Carman *et al.*, 1998; Pellerin *et al.*,

1994). In addition, infection with some type II BVDV isolates has been associated with a hemorrhagic disorder (Bolin & Ridpath, 1992; Corapi *et al.*, 1990; Corapi *et al.*, 1989; Rebhun *et al.*, 1989). These infections are characterized by thrombocytopenia, bloody diarrhea, epistaxis, petechial and ecchymotic hemorrhages on mucosal membranes, hyphema, bleeding from injection sites, pyrexia, leukopenia, and death (Bolin & Ridpath, 1992; Corapi *et al.*, 1990; Rebhun *et al.*, 1989). Thrombocytopenia associated with BVDV has been reported from outbreaks in adult cattle and calves, and has also been experimentally reproduced (Bezek *et al.*, 1994; Bolin & Ridpath, 1992; Corapi *et al.*, 1990; Ellis *et al.*, 1998; Rebhun *et al.*, 1989; Walz *et al.*, 1999a).

The differences in virulence between type I and type II BVDV and the mechanisms by which some type II BVDV isolates cause severe peracute disease and the hemorrhagic syndrome are unknown. A difference in the level of viremia induced during infection has been demonstrated between a type I and a type II BVDV isolate (Bolin & Ridpath, 1992), and this higher level of viral replication with type II BVDV may be important in the pathogenesis of severe peracute BVDV and the hemorrhagic syndrome. The purpose of the present study was to characterize the level of viremia induced during BVDV infection, and correlate levels of viremia with severity of clinical disease.

Materials and Methods

Animals

Colostrum-deprived newborn male Holstein calves (n=16) were obtained immediately after birth from local dairy farms. Calves were moved to an isolation

facility, allocated to one of four groups (1 control and 3 treatment groups), and individually housed in separate rooms. All calves were given a physical examination, and serum and whole blood were obtained for virus isolation procedures and determination of antibody levels to BVDV by virus neutralization. Calves were then administered 400 ml of plasma obtained from a donor cow that was BVDV antibody negative by virus neutralization and virus negative by virus isolation procedures on three serial serum samples obtained prior to collection of plasma. In addition, calves were given injections of vitamins A and D[a] and vitamin E and selenium,[b] as well as an oral monoclonal antibody preparation against *E. coli* K99 pilus antigen.[b] Calves were fed a nonmedicated milk replacer according to label directions. Physical examinations, including rectal temperature measurements, were made on all calves twice daily throughout the study period. Prior to initiation of the study, criteria for euthanasia were established. Calves demonstrating any one or a combination of the following were euthanatized: complete anorexia, recumbency with inability to rise, dehydration in excess of 12% of body weight, and hemorrhagic diathesis.

Viruses and infection protocol

Three noncytopathic BVDV isolates were used for the experimental infection of calves: BVDV 890,[c] BVDV 7937,[d] and BVDV TGAN.[c] The BVDV 890 is a type II isolate that has been previously shown to induce thrombocytopenia and platelet dysfunction in experimentally infected calves (Bolin & Ridpath, 1992; Walz *et al.*, 1999b). The BVDV 7937 (type II) and BVDV TGAN (type I) are BVDV isolates that have not been associated with thrombocytopenia (Bolin & Ridpath, 1992; Marshall *et al.*,

1996). The viral isolates were propagated in bovine turbinate cells in Eagle's minimum essential medium[e] (EMEM) containing 10% fetal equine serum[f] (FES), L-glutamine,[g] penicillin G,[f] and streptomycin.[f] After a three-day adjustment period in the isolation facility, calves in the 3 infected groups (n=4 for each group) were inoculated by intranasal instillation with 10^7 $TCID_{50}$ of the respective viral isolate in 5 ml of EMEM without serum (2.5 ml per nostril). Control calves (n=4) were inoculated by intranasal instillation with an uninfected cell culture supernate.

Clinical pathologic examination

Manual platelet and leukocyte counts were performed daily on whole blood, using a white blood cell microcollection system[h] and a hemocytometer. Automated blood cell counts, which included the mean platelet volume, were performed on day 0 (before inoculation) and day 12 after inoculation using an electronic particle counter.[i]

Determination of the levels of viremia

Serum virus titer and the percentages of lymphocytes and platelets carrying virus were used to assess the level of viremia (Bolin & Ridpath, 1992). The titer of virus in serum was determined by making serial 5-fold dilutions of serum in EMEM. The serial dilutions were inoculated in 96-well microtiter plates containing monolayers of bovine turbinate cells in EMEM with 10% FES, L-glutamine, and antibiotics. After 3 days of incubation at 37°C in humidified air containing 5% CO_2, the bovine turbinate cells were stained for BVDV antigen by an immunoperoxidase monolayer assay and the viral titer calculated (Meyling, 1988).

A limiting dilution method was used for estimating the frequency of association of BVDV with platelets and lymphocytes (Bolin & Ridpath, 1992). Whole blood was collected for virus isolation from platelets and lymphocytes on the day of challenge, and then on days 4, 6, 8, and 12 after challenge. Blood was collected by jugular venipuncture through an 18-gauge needle into plastic syringes containing 1.0 ml of 3.8% trisodium citrate for each 9.0 ml of blood. Platelet-rich plasma was obtained by two centrifugation steps at 1,324 x g for 60 seconds and 30 seconds, with the platelet-rich plasma being removed after each centrifugation. Prostaglandin E_1[f] (1 µl of 1 µM prostaglandin per ml of platelet-rich plasma) was added to platelet-rich plasma prior to centrifugation at 800 x g for 15 minutes. The plasma supernatant was removed with a sterile pipette, and the platelet pellet was resuspended in 1 ml of Hank's Balanced Salt Solution (HBSS; 136 mM NaCl, 5.4 mM KCL, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, and 5.5 mM dextrose, pH 7.4). The platelet suspension was transferred to a 10 ml polystyrene column containing Sepharose 4B.[f] The eluate was collected, washed three times in 10 ml of HBSS, and a manual platelet count was determined. Approximately 10^7 platelets were inoculated into the top left-hand well of a 96-well microtiter plate containing EMEM. Serial twofold dilutions were made down the left-hand column of the plate, then serial twofold dilutions were made across the plate, beginning at the left-hand column of wells. Bovine turbinate cells (9 x 10^4 cells/ml) suspended in EMEM containing 10% FES, L-glutamine, and antibiotics were added to each well. After 5 days incubation at 37°C in humidified air containing 5% CO_2, 50µl of supernatant from each well was transferred to a new 96-well microtiter plate. Bovine turbinate cells suspended in EMEM containing 10% FES, L-glutamine, and antibiotics were added to each well. After another 5 days of

incubation at 37°C in humidified air containing 5% CO_2, the bovine turbinate cells were stained for BVDV antigen by an immunoperoxidase monolayer assay (Meyling, 1988), and the percentage of platelets associated with BVDV was calculated.

For virus isolation from lymphocytes, citrated whole blood that had the platelet-rich plasma previously removed was diluted in HBSS, and the suspension was layered on Ficoll-sodium diatrizoate medium.[j] After centrifugation at 600 x g for 40 minutes at 18°C, the lymphocytes were harvested and resuspended in 10 ml HBSS. Contaminating red blood cells were removed by hypotonic lysis to yield lymphocytes and residual platelets (Carbrey *et al.*, 1972). In order to remove contaminating platelets, centrifugation at 600 x g for 40 minutes at 18°C in a 15% sucrose gradient layered over Ficoll-sodium diatrizoate medium was used to purify lymphocytes. This procedure successfully removed contaminating platelets, and lymphocytes were collected below the sucrose at the interface of the Ficoll sodium diatrizoate medium, washed three times in HBSS, and manually counted. Approximately 10^6 lymphocytes were inoculated into the top left-hand well of a 96-well microtiter plate containing EMEM. Serial twofold dilutions were made as described for platelets. Detection of virus association with lymphocytes was performed as described for platelets.

Virologic and serologic examination

Serum and whole blood were collected daily for detection of BVDV to determine the frequency of virus isolation over the experimental time period. Whole blood was processed by hypotonic lysis of the red blood cells to yield the white blood cells (Carbrey *et al.*, 1972). Isolated white blood cells were then resuspended in EMEM containing

10% FES, L-glutamine, and antibiotics. Following three freeze/thaw cycles, white blood cell preparations were centrifuged for 10 minutes at 1,500 x g. The supernatant was extracted and stored at –80°C.

Virus isolation was also performed on bone marrow, thymus, spleen, bronchial and mesenteric lymph nodes, and ileum collected postmortem. Approximately 0.5 gram of tissue was added to 4 ml of EMEM with 10% FES, L-glutamine, and antibiotics in a sterile mortar. Sterile sand was added and the tissue was homogenized by grinding with a pestle. The homogenized tissue sample was transferred into conical tubes and centrifuged for 30 minutes at 2,300 x g. The supernatant was removed and then stored at –80°C.

The serum, white blood cell preparations, and tissue preparations were thawed, and 25 µl of each sample was inoculated in duplicate into wells on 96-well microtiter plates containing monolayers of bovine turbinate cells in EMEM with 10% FES, L-glutamine, and antibiotics. After 3 days of incubation at 37°C in humidified air containing 5% CO_2, the bovine turbinate cells were stained for BVDV antigen by an immunoperoxidase monolayer assay (Meyling, 1988).

Serology for BVDV was performed by a microtiter virus neutralization procedure (Carbrey *et al.*, 1972). Sera obtained on days 0 and 12 after infection from all experimental calves were tested for neutralizing antibodies to type I and type II BVDV. Serial two-fold dilutions, ranging from 1:4 to 1:4,096, were made for each serum sample in a 96-well microtiter plate. The amount of virus used per test well was 500 $TCID_{50}$ of the type II cytopathic isolate 125[k] or the type I cytopathic isolate Singer.[k] Bovine turbinate cells were used as the indicator cells at a concentration of 15,000 cells per well.

Each test included a back titration of the virus and a positive and negative serum control. The antibody titer was read as the reverse of the highest dilution with complete inhibition of cytopathic effect.

Pathologic examination

On day 12 after infection, all calves were euthanatized with a lethal injection of barbiturate.[l] A postmortem examination was performed and all gross postmortem lesions were recorded. Tissues collected for histopathologic examination included liver, spleen, lung, heart, small and large intestine, adrenal gland, kidney, thymus, tonsil, brain, spinal cord, mesenteric and bronchial lymph nodes, and bone marrow. All tissues were fixed in 10% neutral buffered formalin, with the exception of bone marrow, which was also fixed in B-5 fixative.[m] Following fixation, the tissues were trimmed, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin (HE) according to standard procedure. Numbers of megakaryocytes per high power field were calculated by averaging the number of megakaryocytes under ten high power fields for each calf. Images in this dissertation are presented in color.

Immunohistochemical examination for BVDV antigen

Immunohistochemical staining for BVDV antigen was performed on paraffin-embedded sections of bone marrow, lung, thymus, lymph node, ileum, and spleen, as previously described (Grooms *et al.*, 1996). Tissue sections were cut at 5 μm on silane-coated slides and dried. Sections were then deparaffinized and rehydrated by sequential immersion of the slides in a limonene-based solvent,[n] followed by graded concentrations

of ethanol, then tap water. Inactivation of tissue peroxidases, digestion with protease, and blocking of nonspecific antibody binding were performed prior to the application of the primary monoclonal antibody, 15C5.[o] Following incubation with primary antibody, BVDV antigen was detected using an indirect avidin-biotin-peroxidase detection system,[p] and the chromagen, 3-amino-9-ethyl carbazole.[f] After immunohistochemical staining, the slides were counterstained with Mayer's hematoxylin and coverslipped using an aqueous mounting medium. To detect nonspecific binding, the primary antibody was replaced with a similar concentration of an antibody of the same isotype directed against infectious bursal disease virus.[q] Tissue sections from a persistently infected calf and a calf not infected with BVDV as determined by virus isolation and serology were used as positive and negative controls, respectively.

Statistical analyses

Comparisons of rectal temperatures and manual and automated platelet and leukocyte counts were performed by one-way ANOVA. When differences in group means were statistically significant ($P < 0.05$), the Bonferroni t-test was used for pairwise comparisons of control versus infected group means. Mean platelet volume measurements and data describing the levels of viremia (serum viral titer and virus association with lymphocytes and platelets) were analyzed using the Kruskal-Wallis one-way ANOVA on ranks. When differences in mean platelet volume values were statistically significant ($P < 0.05$), the Dunn's method was used for comparisons of control versus infected group means. When differences in the means in the data

describing the level of viremia were statistically significant ($P < 0.05$), the Student-Newman-Keuls method was used for all pairwise comparisons of group means.

Results

Physical examination findings

None of the calves met the predetermined criteria for euthanasia during the study period. Appetite remained normal in all control and infected calves throughout the study. All calves infected with type II BVDV developed diarrhea, while 3 of 4 calves infected with type I BVDV developed diarrhea during the study. In calves infected with type II BVDV 890, the diarrhea was longer in duration than calves infected with the other type II isolate or the type I isolate, and was characterized by hemorrhage and mucosal casts. Pyrexia (rectal temperature > 39.4°C) was only observed in BVDV-infected calves. Statistically significant differences in the mean rectal temperature measurements were observed between control calves and BVDV-infected calves from days 6 through 12 after inoculation, with the longest duration of an elevated temperature being observed in calves infected with the type II isolate BVDV 890 (Figure 5.1).

Clinical pathologic findings

The greatest depression in platelet count occurred in calves infected with the type II isolate BVDV 890 (Figure 5.2). The platelet count decreased to less than 200,000/μl in 2 of 4 calves infected with BVDV 890 and 1 of 4 calves infected with BVDV 7937. The platelet count did not decrease to less than 100,000 platelets/μl in any calves, and did not decrease to less than 200,000/μl in any control calves or calves infected with BVDV

TGAN. When compared to control calves, a significantly decreased manual platelet count was observed from day 7 after inoculation to the end of study in the BVDV-infected calves (Figure 5.2). The automated platelet counts closely followed the manual counts (Table 5.1). Platelet size, as measured by the mean platelet volume, was significantly reduced in the BVDV-infected calves on day 12 after inoculation when compared to the control calves (Figure 5.3).

The automated white blood cell counts decreased in the infected calves, but statistically significant differences were only observed in calves infected with type II BVDV, when compared to control calves (Table 5.1). With respect to manual white blood cell counts, statistically significant differences were observed between the control group and all BVDV-infected groups. The only statistically significant difference observed between calves infected with BVDV TGAN and control calves occurred on day 3 after inoculation, and a significant difference was only observed on days 3 and 12 after inoculation in the BVDV 7937-infected calves. A statistically significant difference in the manual white blood cell count was observed on days 3-5, 7, and 10-12 after inoculation between control calves and calves infected with BVDV 890.

Levels of viremia

A clear difference in the level of viremia induced during infection was observed among the 3 groups of BVDV-infected calves, with the highest serum viral titer and percentages of lymphocytes and platelets associated with BVDV being observed in the calves infected with BVDV 890 (Tables 5.2, 5.3, 5.4). The highest serum viral titer in calves infected with BVDV 890 was observed on day 12 after inoculation (Table 5.2,

mean log titer: 5.4771; range:4.1021-6.1021). In contrast, the viral titer was highest on day 8 after inoculation in the BVDV 7937-infected calves (mean log titer: 3.5187; range: 2.7687-4.2687) and the BVDV TGAN-infected calves (mean log titer: 1.9771; range: 1.4354-3.4354). With respect to the association of BVDV with lymphocytes, a similar trend was observed in the 3 groups of calves, with the percentage association highest on day 12 after inoculation in the BVDV 890-infected calves (Table 5.3, mean maximal percentage association: 7.2648; range: 3.2258-12.50). The highest percentage association in the BVDV 7937-infected calves (mean maximal percentage association: 2.2814; range: 0.0498-6.667) and the BVDV TGAN-infected calves (mean maximal percentage association: 0.1098; range: 0.0004-0.4098) was observed on day 8 after inoculation. Finally, the percentage of platelets associated with BVDV was highest on day 8 after inoculation in all BVDV-infected groups of calves, with the BVDV 890-infected calves highest (Table 5.4, mean maximal percentage association: 0.2457; range: 0.1638-0.3277). The percentage of platelets associated with BVDV was lower for the BVDV 7937-infected calves (mean maximal percentage association: 0.0320; range: 0.0051-0.0820), and the BVDV TGAN-infected calves (mean maximal percentage association: 0.0030; range: 0.0008-0.0103).

Virologic and serologic findings

Bovine viral diarrhea virus was not isolated from antemortem or postmortem samples collected from the control calves. In the infected groups of calves, BVDV was isolated from serum and white blood cells more frequently and for a longer duration in calves infected with BVDV 890, followed by BVDV 7937, and then BVDV TGAN

(Table 5.5). Bovine viral diarrhea virus was isolated from all tissues from all calves infected with either BVDV 890 or BVDV 7937, as compared to calves infected with BVDV TGAN, in which the thymus was the only tissue that BVDV was isolated in all calves (Table 5.5).

All control and BVDV-infected calves had BVDV-neutralizing antibody titers of ≤8 on day 0 (before inoculation) and day 12 after inoculation.

Pathologic findings

Petechial hemorrhages were present on gross postmortem examination in all calves infected with BVDV 890 and BVDV 7937, and in 2 of 4 calves infected with BVDV TGAN. The hemorrhages were most prominent in calves infected with BVDV 890, and were located primarily on the mucosal surfaces of the urinary bladder, ileum, colon, and rumen. Hemorrhages were only observed on the colonic mucosa in the calves infected with BVDV TGAN. Abomasal edema, which was most prominent in the spiral folds of the fundus, was present in all calves infected with BVDV 890, in 3 of 4 calves infected with BVDV 7937, and in 1 of 4 calves infected with BVDV TGAN. No significant lesions were present on gross postmortem examination of control calves.

Histologic evaluation of the bone marrow revealed differences between the 4 groups of calves (Figure 5.4A-D). Images in this dissertation are presented in color. Sections of bone marrow in calves infected with BVDV 890 were hypercellular, when compared to the other groups of calves. In addition, calves infected with BVDV 890 had the most megakaryocytes per high power field (MK/hpf; mean: 8.85 MK/hpf; range: 7.62-10.45), followed by calves infected with BVDV 7937 (mean: 5.98 MK/hpf; range: 5.09-6.76),

calves infected with BVDV TGAN (mean: 5.24 MK/hpf; range: 3.62-6.56), then the control calves (mean: 3.60 MK/hpf; range: 1.84-5.32). In the calves infected with BVDV 890, many of the megakaryocytes did not have uniform nuclear size or chromatin pattern. Approximately 30% of the megakaryocytes in BVDV 890-infected calves were immature, as characterized by a small nuclear:cytoplasmic ratio and nonsegmented nucleus. Immature megakaryocytes were also observed in the other groups, but less frequently (calves infected with BVDV 7937: 18%; calves infected with BVDV TGAN: 5%; and controls: 3%). Approximately 7.4% of megakaryocytes in the BVDV 890-infected calves had undergone necrosis, as evidenced by the loss of, or pyknosis of the nuclei. However, more pyknotic megakaryocytes were observed in calves infected with BVDV 7937 (approximately 9.5%) and calves infected with BVDV TGAN (approximately 9.3%), when compared to calves infected with BVDV 890 and control calves (approximately 3.9%).

Lymphoid depletion and reduced follicular size were present in the thymus (Figure 5.5), ileum and spleen in all BVDV-infected calves. Images in this dissertation are presented in color. The greatest lymphoid depletion was observed in calves infected with BVDV 890 (Figure 5.5).

Immunohistochemistry findings

In the control calves, there was no evidence of BVDV antigen as detected by immunohistochemical antibody staining. However, there was immunohistochemical staining for BVDV antigen in all paraffin-embedded tissue sections collected for evaluation from all BVDV-infected calves, but viral antigen was more prevalent in the calves infected with type II BVDV than calves infected with BVDV TGAN (Table 5.6).

In 3 of 4 calves infected with BVDV 7937 and in 2 of 4 calves infected with BVDV 890, immunohistochemical staining of bone marrow revealed the presence of BVDV-specific antigen in megakaryocytes (Figure 5.4D and 5E). In addition, BVDV-specific antigen was present in myeloid precursors in the bone marrow (Figure 5.4D). Immunohistochemical staining for BVDV was also present in the muscular trabeculae and smooth muscle of blood vessels located in the spleen of the type II BVDV-infected calves. Mononuclear cells in the medullary sinus of the lymph node, bone marrow, red pulp of the spleen, and ileum also contained BVDV antigen. In the ileum, viral antigen was present in lymphoid cells in Peyer's patch follicles and in perifollicular connective tissue, and viral antigen was also present in lymphoid and mononuclear cells in the cortex and medulla of the thymus.

Figure 5.1. Mean rectal temperature measurements for uninfected control calves, calves infected with the type I isolate BVDV TGAN, calves infected with the type II isolate BVDV 7937, and calves infected with the type II isolate BVDV 890. Calves were infected at day 0. Standard error bars are shown. Statistically significant differences (ANOVA, $p < 0.05$, Bonferroni t-test) are denoted as follows: * = BVDV 890 vs. Control; + = BVDV 7937 vs. Control; # = BVDV TGAN vs. Control.

Figure 5.1

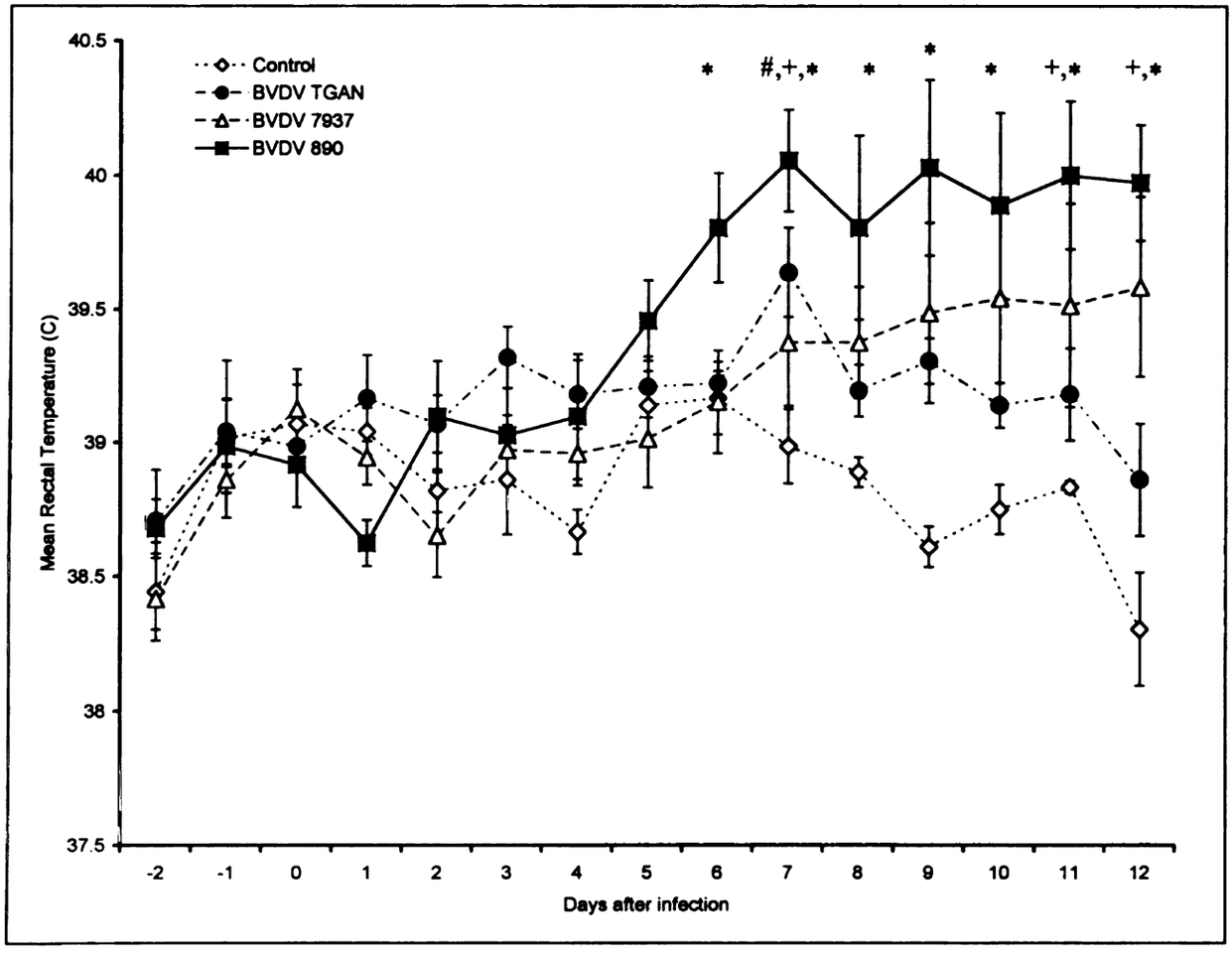
Control
BVDV TGAN
BVDV 7937
BVDV 890
Mean Rectal Temperature (C)
40.5
40
39.5
39
38.5
38
37.5
-2
-1
0
1
2
3
4
5
6
7
8
9
10
11
12
Days after infection
*
#,+,*
*
*
*
+,*
+,*

Figure 5.2. Mean whole blood platelet counts by manual determination in uninfected control calves, calves infected with the type I isolate BVDV TGAN, calves infected with the type II isolate BVDV 7937, and calves infected with the type II isolate BVDV 890. Calves were infected at day 0. Standard error bars are shown. Statistically significant differences (ANOVA, $p < 0.05$, Bonferroni t-test) are denoted as follows: * = BVDV 890 vs. Control; + = BVDV 7937 vs. Control; # = BVDV TGAN vs. Control.

Figure 5.2

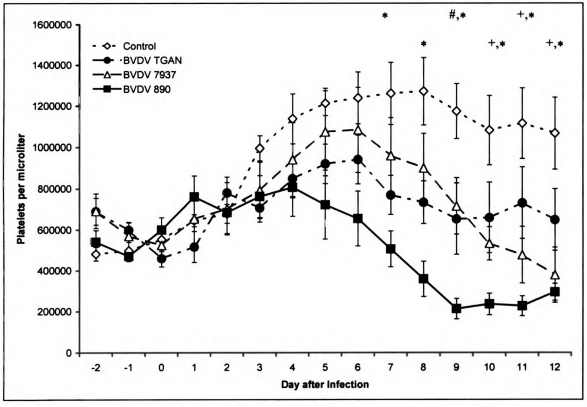
Control
BVDV TGAN
BVDV 7937
BVDV 890
Platelets per microliter
Day after Infection
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
-2
-1
0
1
2
3
4
5
6
7
8
9
10
11
12
*
#,*
+,*
*
+,*
+,*

Figure 5.3. Mean platelet volume for uninfected control calves, calves infected with the type I isolate BVDV TGAN, calves infected with the type II isolate BVDV 7937, and calves infected with the type II isolate BVDV 890. Calves were infected at day 0. Standard error bars are shown. Statistically significant differences (Kruskal-Wallis ANOVA on Ranks, $p < 0.05$, Dunn's test) are denoted as follows: * = BVDV 890 vs. Control; + = BVDV 7937 vs. Control; # = BVDV TGAN vs. Control.

Figure 5.3

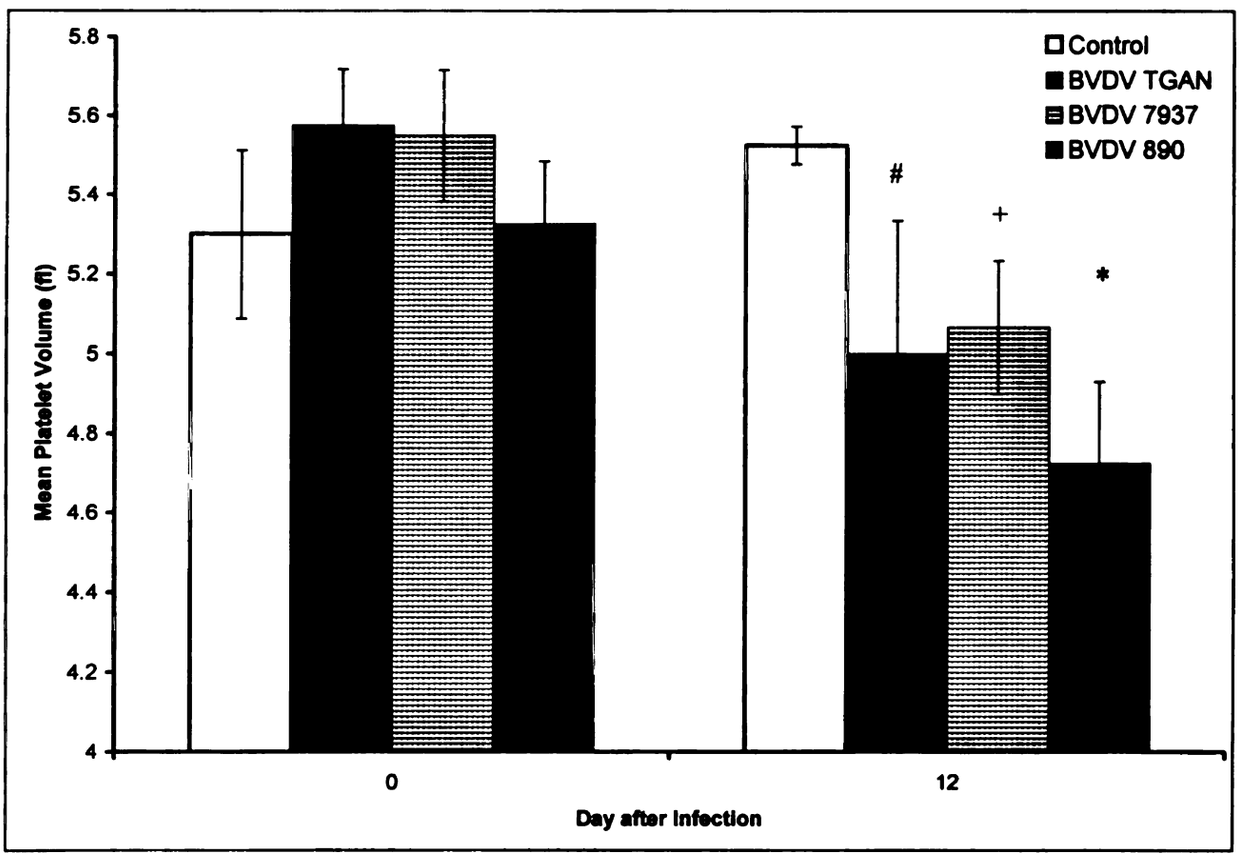
Control
BVDV TGAN
BVDV 7937
BVDV 890
Mean Platelet Volume (fl)
5.8
5.6
5.4
5.2
5
4.8
4.6
4.4
4.2
4
0
12
Day after infection
#
+
*

Table 5.1. Manual and automated hematology results for control and BVDV-infected calves before inoculation (day 0) and on day 12 after inoculation. Values are expressed as the mean ± standard error.

	control calves		Calves infected with type I isolate BVDV TGAN	
Variable	before inoculation†	day 12 after inoculation	before infection	day 12 after inoculation
Manual platelet count	554,000 ± 47,922	1,067,250 ± 174,004	461,125 ± 41,667	649,500 ± 150,142
Automated platelet count	486,750 ± 28,179	843,250 ± 182,698	426,000 ± 50,917	591,500 ± 144,043
Manual WBC count	8,931 ± 1,322	8,972 ± 882	10,361 ± 1,319	8,806 ± 266
Automated WBC count	9,508 ± 1,098	9,538 ± 724	10,773 ± 2,093	8,543 ± 378
	Calves infected with type II isolate BVDV 7937		Calves infected with type II isolate BVDV 890	
	before infection	day 12 after inoculation	before infection	day 12 after inoculation
Manual platelet count	525,750 ± 30,444	379,375 ± 136,119 +	600,625 ± 59,523	295,500 ± 41,724 *
Automated platelet count	744,500 ± 278,113	388,333 ± 182,648 +	540,500 ± 63,121	312,500 ± 54,785 *
Manual WBC count	9,820 ± 2,928	5,222 ± 552 +	8,875 ± 902	2,833 ± 523 *
Automated WBC count	12,450 ± 3,199	6,350 ± 1,195 +	9,268 ± 1,978	3,283 ± 656 *

† Blood counts were performed on day 0 (day of inoculation) and on day 12 after inoculation

Statistically significant differences (ANOVA, $p < 0.05$, Bonferroni t-test) are denoted as follows: * = BVDV 890 vs. Control; + = BVDV 7937 vs. Control

Table 5.2. Serum viral titer (level of viremia) results for uninfected control calves, calves infected with the type I isolate BVDV TGAN, calves infected with the type II isolate BVDV 7937, and calves infected with the type II isolate BVDV 890. Values are expressed as the mean with the range in parentheses. Statistically significant differences (Kruskal-Wallis ANOVA on Ranks, $p < 0.05$, Student-Newman-Keuls test) are denoted as follows: * = BVDV 890 vs. Control; ** = BVDV 890 vs. Control and BVDV TGAN; *** = BVDV 890 vs. Control, BVDV TGAN, and BVDV 7937; + = BVDV 7937 vs. Control; ++ = BVDV 7937 vs. Control and BVDV TGAN; # = BVDV TGAN vs. Control.

	Serum viral titer			
Day after inoculation	Control	BVDV TGAN	BVDV 7937	BVDV 890
Day 0	0	0	0	0
Day 4	0	0	0.3589 (0 – 1.4354)	1.24321 (0 – 1.9354)
Day 6	0	0.3172 (0 – 1.2687)	2.0766 (0 – 4.9354)	4.4771 ** (3.9354-4.7687)
Day 8	0	1.9771 # (1.4354 – 3.4354)	3.5187 ++ (2.7687 – 4.2687)	4.8105 *** (4.4359 – 5.1021)
Day 12	0	0	1.5604 ++ (1.4354 – 1.7687)	5.4771 *** (4.1021 – 6.1021)

Table 5.3. Percentage of lymphocytes associated with BVDV (level of viremia) for uninfected control calves, calves infected with the type I isolate BVDV TGAN, calves infected with the type II isolate BVDV 7937, and calves infected with the type II isolate BVDV 890. Values are expressed as the mean with the range in parentheses. Statistically significant differences (Kruskal-Wallis ANOVA on Ranks, $p < 0.05$, Student-Newman-Keuls test) are denoted as follows: * = BVDV 890 vs. Control; ** = BVDV 890 vs. Control and BVDV TGAN; *** = BVDV 890 vs. Control, BVDV TGAN, and BVDV 7937; + = BVDV 7937 vs. Control; ++ = BVDV 7937 vs. Control and BVDV TGAN; # = BVDV TGAN vs. Control.

Percentage of lymphocytes associated with BVDV				
Day after inoculation	Control	BVDV TGAN	BVDV 7937	BVDV 890
Day 0	0	0	0	0
Day 4	0	0.0072 # (0.0016 - 0.01278)	0.0416 + (0.0064 - 0.1024)	0.0640 ** (0.0512 - 0.1024)
Day 6	0	0.0576 # (0.0064 - 0.2049)	0.3842 ++ (0.1024 – 0.8197)	1.3320 ** (0.4098 – 1.6393)
Day 8	0	0.1098 # (0.0004 – 0.4098)	2.2814 ++ (0.4098 – 1.6393)	6.8682 ** (1.6393 – 12.50)
Day 12	0	0.0128 (0 – 0.0512)	0.8452 ++ (0.0013 – 3.2258)	7.2648 *** (3.2258 – 12.50)

Table 5.4. Percentage of platelets associated with BVDV (level of viremia) for uninfected control calves, calves infected with the type I isolate BVDV TGAN, calves infected with the type II isolate BVDV 7937, and calves infected with the type II isolate BVDV 890. Values are expressed as the mean with the range in parentheses. Statistically significant differences (Kruskal-Wallis ANOVA on Ranks, $p < 0.05$, Student-Newman-Keuls test) are denoted as follows: * = BVDV 890 vs. Control; ** = BVDV 890 vs. Control and BVDV TGAN; *** = BVDV 890 vs. Control, BVDV TGAN, and BVDV 7937; + = BVDV 7937 vs. Control; ++ = BVDV 7937 vs. Control and BVDV TGAN; # = BVDV TGAN vs. Control.

Percentage of platelets associated with BVDV				
Day after inoculation	Control	BVDV TGAN	BVDV 7937	BVDV 890
Day 0	0	0	0	0
Day 4	0	0.00004 (0 – 0.0002)	0.00014 (0.0001 – 0.0002)	0.00042 * (0.0001 - 0.0013)
Day 6	0	0.0015 # (0.0001 – 0.0051)	0.0022 + (0.0006 – 0.0051)	0.0467 *** (0.00256 – 0.1638)
Day 8	0	0.0030 # (0.001 – 0.0103)	0.03201 ++ (0.0051 – 0.0820)	0.2457 *** (0.1638 – 0.3277)
Day 12	0	0.00008 (0 – 0.0003)	0.0070 ++ (0.0013 – 0.0205)	0.0461 *** (0.0205 – 0.0820)

Table 5.5 Summary of virus isolation results for calves infected with the type I isolate BVDV TGAN, calves infected with the type II isolate BVDV 7937, and calves infected with type II isolate BVDV 890. Calves were infected at day 0. Virus isolation from tissues was performed on day 12 post-inoculation.

	BVDV TGAN (type I)		BVDV 7937 (type II)		BVDV 890 (type II)	
Day after inoculation	Serum	White blood cells	Serum	White blood cells	Serum	White blood cells
Day 3	0 / 4*	0 / 4	0 / 4	1 / 4	0 / 4	1 / 4
Day 4	0 / 4	1 / 4	0 / 4	1 / 4	1 / 4	2 / 4
Day 5	0 / 4	2 / 4	0 / 4	1 / 4	3 / 4	4 / 4
Day 6	0 / 4	2 / 4	1 / 4	2 / 4	4 / 4	4 / 4
Day 7	3 / 4	3 / 4	3 / 4	3 / 4	4 / 4	4 / 4
Day 8	2 / 4	1 / 4	3 / 4	4 / 4	4 / 4	4 / 4
Day 9	0 / 4	0 / 4	4 / 4	4 / 4	4 / 4	4 / 4
Day 10	0 / 4	0 / 4	3 / 4	4 / 4	4 / 4	4 / 4
Day 11	0 / 4	0 / 4	3 / 4	4 / 4	4 / 4	4 / 4
Day 12	0 / 4	0 / 4	1 / 4	4 / 4	4 / 4	4 / 4

Tissue	BVDV TGAN (type I)	BVDV 7937 (type II)	BVDV 890 (type II)
Thymus	4 / 4	4 / 4	4 / 4
Spleen	0 / 4	4 / 4	4 / 4
Ileum	2 / 4	4 / 4	4 / 4
Mesenteric LN	2 / 4	4 / 4	4 / 4
Lung	2 / 4	4 / 4	4 / 4
Bronchial LN	1 / 4	4 / 4	4 / 4
Bone marrow	0 / 4	4 / 4	4 / 4

* numerator: # of calves which were virus positive; denominator: # of calves in infected group
BVDV was not isolated from any control calves, nor was it isolated prior to day 3 after inoculation in any infected calves

Figure 5.4. Histologic sections of bone marrow taken on day 12 after infection (postmortem examination). Bar = 100μm. A. Histologic section of bone marrow from control calf. B. Histologic section of bone marrow from calf infected with BVDV TGAN (type I). C. Histologic section of bone marrow from calf infected with BVDV 7937 (type II). D. Histologic section of bone marrow from calf infected with BVDV 890 (type II). E. Positive immunohistochemical staining for BVDV antigen in megakaryocytes and myeloid and erythroid precursors from calf infected with BVDV 7937 (note reddish-brown staining depicted by arrow). F. Positive immunohistochemical staining for BVDV antigen in a megakaryocyte from calf infected with BVDV 890. Images in this dissertation are presented in color.

Figure 5.4

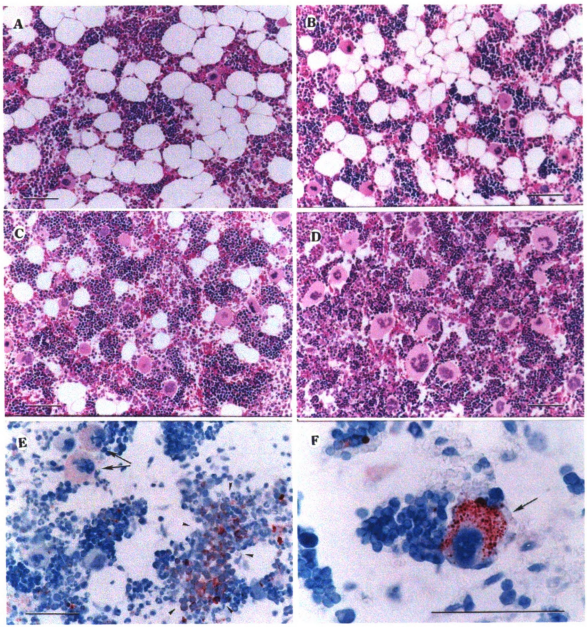

Figure 5.5 Histologic sections of thymus taken on day 12 after infection (postmortem examination). Bar = 100μm. A. Histologic section of thymus from control calf. B. Histologic section of thymus from calf infected with BVDV TGAN (type I). C. Histologic section of thymus from calf infected with BVDV 7937 (type II). D. Histologic section of thymus from calf infected with BVDV 890 (type II). Images in this dissertation are presented in color.

Figure 5.5

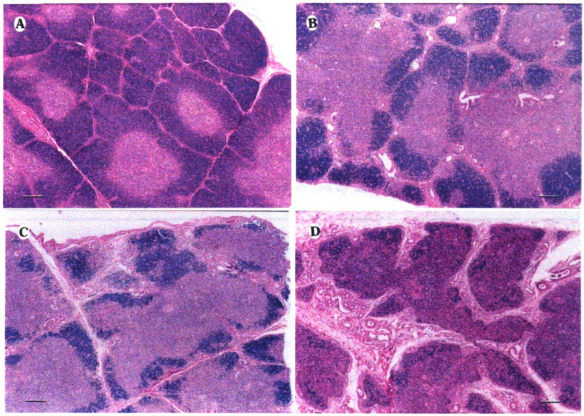

Table 5.6. Summary of immunohistochemistry results for uninfected control calves, calves infected with the type I isolate BVDV TGAN, calves infected with the type II isolate BVDV 7937, and calves infected with the type II isolate BVDV 890. Tissues were collected at day 12 after inoculation.

Tissue	Control	BVDV TGAN (type I)	BVDV 7937 (type II)	BVDV 890 (type II)
Thymus	0 / 4*	2 / 4	4 / 4	4 / 4
Spleen	0 / 4	1 / 4	2 / 4	4 / 4
Bone marrow	0 / 4	1 / 4	4 / 4	4 / 4
(+ megakaryocytes)	(0 / 4)	(0 / 4)	(3 / 4)	(2 / 4)
Mesenteric Lymph Node	0 / 4	3 / 4	4 / 4	4 / 4
Ileum	0 / 4	4 / 4	3 / 4	4 / 4
Lung	0 / 4	0 / 4	1 / 4	3 / 4

* numerator: # of calves that were positive for viral antigen by immunohistochemistry; denominator: # of calves in infected group

Discussion

A clear difference in the level of viremia induced during infection was demonstrated in the 3 groups of BVDV-infected calves in this study. In addition, a parallel relationship between the level of viremia and rectal temperatures, and an inverse relationship between the level of viremia and blood counts was observed, with the greatest elevations in rectal temperature and greatest depressions in platelet and leukocyte counts being observed in calves infected with BVDV 890. The results of this study, indicating that the level of viremia is correlated to disease manifestations, support the hypothesis that the level of viremia induced during BVDV infection is an important virulence factor, and may be a defining factor for differences in virulence observed between BVDV isolates.

The three BVDV isolates utilized in this study were chosen because they would allow a comparison among BVDV isolates of predetermined and different virulence. The type II isolate BVDV 890 was considered the most virulent, as it had been previously demonstrated to induce pyrexia, diarrhea, thrombocytopenia, leukopenia, altered platelet function, and death in experimentally infected calves (Bolin & Ridpath, 1992; Walz *et al.*, 1999a; Walz *et al.*, 1999b). The other type II isolate, BVDV 7937, was considered less virulent than BVDV 890, as it had previously been shown to induce pyrexia and a depression in the platelet count, but did not induce diarrhea, leukopenia, thrombocytopenia, or death in experimentally infected calves (Marshall *et al.*, 1996). The BVDV TGAN was chosen because it was a type I isolate that did induce pyrexia, but did not induce leukopenia, thrombocytopenia, or death in experimentally infected calves (Bolin & Ridpath, 1992). The ability of these 3 isolates to induce different disease manifestations allowed for comparisons with the level of viremia. The clinical signs

associated with BVDV infection in this study are similar to previous reports for each of the 3 isolates, with BVDV 890 inducing the more severe clinical signs of disease when compared to BVDV 7937 and BVDV TGAN. Pyrexia (rectal temperature > 39.4 C) and diarrhea observed in the BVDV 890-infected calves are similar to previous reports (Bolin & Ridpath, 1992; Walz *et al.*, 1999a), and are typical of a virulent type II BVDV infection. Pyrexia was also observed in the BVDV 7937-infected calves, but in contrast to a previous study (Marshall *et al.*, 1996), diarrhea was observed in 3 of 4 calves in this study. The reason for this difference is unknown, but may be due to the use of younger calves in the present study. Similar to a previous report (Bolin & Ridpath, 1992), pyrexia was observed in all BVDV TGAN-infected calves.

A statistically significant difference in platelet counts was observed between the control calves and all BVDV-infected groups of calves, including calves infected with the type I isolate BVDV TGAN; however, thrombocytopenia was only observed in 2 of 4 calves infected with BVDV 890. Thrombocytopenia is defined as a reduction in the number of circulating platelets below the minimum normal level for the species (Jain, 1986), but defining thombocytopenia is difficult because there is considerable variation in the literature concerning the platelet count in normal male Holstein calves (Nelson *et al.*, 1974; Vaugher *et al.*, 1973). We used age-, breed-, and sex-matched control calves in order to demonstrate statistically significant differences in the platelet count between control calves and BVDV-infected calves. This was important, because our results and the results from previous studies (Semrad & Dubielzig, 1993; Walz *et al.*, 1999b) demonstrate a relative thrombocytosis peaking at approximately 8 days of age in newborn Holstein calves. In this study, the age-related relative thrombocytosis is likely obscured

in the BVDV-infected calves as a result of infection, and may explain the inability to induce thrombocytopenia in all BVDV 890-infected calves. In addition, the absence of the relative thrombocytosis in the BVDV TGAN-infected calves may be the result of infection, and may indicate that greater depressions in the platelet count following infection with BVDV TGAN might have been observed if older calves had been used in our experimental infection. These results reinforce the importance of age-matched control calves in studying BVDV infection and platelet disorders in neonatal calves.

In the present study, the mean platelet volume was significantly decreased at day 12 after infection in all BVDV-infected groups of calves when compared to control calves. The mean platelet volume can be used to help classify the mechanism of thrombocytopenia (Bessman *et al.*, 1982). Platelets recently released into the bloodstream from the bone marrow are larger and, consequently, the mean platelet volume is elevated when thrombocytopenia is due to increased destruction of platelets in the peripheral circulation. Alternatively, older platelets are smaller and the mean platelet volume is decreased when the thrombocytopenia is due to decreased marrow production (Bessman *et al.*, 1982). Platelet size, as measured by the mean platelet volume, was smallest in the BVDV 890-infected calves, which supports a previous study demonstrating low mean platelet volume in BVDV 890-infected calves (Walz *et al.*, 1999a). In addition, the concept that decreased production of platelets is the cause of BVDV-induced thrombocytopenia is supported by these findings. Interestingly, although the mean platelet volume was smaller in the BVDV TGAN-infected calves than in calves infected with the other type II isolate, BVDV 7937, the platelet count was lower in the BVDV 7937-infected calves. The reason for this discrepancy is unknown, but

megakaryocyte degeneration was demonstrated in the BVDV TGAN-infected calves, and a lack of platelet production by the bone marrow in these calves cannot be ruled out.

An increased cellularity of the bone marrow and megakaryocyte hyperplasia were observed in the BVDV 890-infected calves in this study and a previous study (Walz *et al.*, 1999a), and likely occurs in response to the decreased peripheral platelet and leukocyte counts, respectively. Previous studies evaluating the bone marrow histology from type II BVDV-thrombocytopenic infections have reported both megakaryocyte necrosis (Ellis *et al.*, 1998; Scruggs *et al.*, 1995), and adequate to increased numbers of megakaryocytes (Corapi *et al.*, 1989; Ellis *et al.*, 1998; Rebhun *et al.*, 1989). Similar to those previous reports examining different BVDV isolates, we observed both megakaryocyte degeneration and megakaryocyte hyperplasia. Megakaryocyte hyperplasia was greatest in the BVDV 890-infected calves. Megakaryocyte degeneration was also observed in the BVDV 890-infected calves, but the numbers of megakaryocytes undergoing degeneration, as evidenced by the loss of, or pyknosis of the nucleus were greater in calves infected with BVDV 7937 and BVDV TGAN. These differences may be due to different levels of viremia induced during infection or may be related to different infection kinetics with BVDV 890, BVDV 7937, and BVDV TGAN. We speculate that viral infection of the bone marrow in the BVDV 890-infected calves occurs earlier in the course of infection and to a greater extent than in calves infected with BVDV 7937 or BVDV TGAN. This is supported by our virus isolation data from antemortem samples and the level of viremia data that demonstrate earlier isolation of virus from blood components and higher levels of viremia, respectively. In turn, this earlier and higher level of bone marrow infection may lead to suppression of platelet

production by the megakaryocytes, and greater depression in the peripheral platelet count. At day 12 after inoculation, bone marrow megakaryocyte hyperplasia was observed in response to the thrombocytopenia in the BVDV 890-infected calves. Conversely, bone marrow infection may occur later and to a lower level in the BVDV TGAN-infected calves. At day 12 after inoculation, extensive megakaryocyte hyperplasia is not observed in the BVDV TGAN-infected calves because thrombocytopenia is not observed. In addition, we speculate that megakaryocyte degeneration is observed in the BVDV TGAN-infected calves as a result of bone marrow suppression occurring later during the course of BVDV infection.

Virus isolation from tissues and immunohistochemical staining for BVDV antigen in tissues were performed to compare the virus distribution among the 3 BVDV-infected groups. Identification of BVDV antigen and isolation of BVDV in the thymus, lymph node, Peyer's patches of the ileum, bone marrow, and spleen are similar to results from previous studies (Ellis *et al.*, 1998; Marshall *et al.*, 1996; Spagnuolo-Weaver *et al.*, 1997). However, the results of this study demonstrate considerable differences in the ability to isolate BVDV and demonstrate BVDV antigen by immunohistochemistry between calves experimentally infected with type I or type II BVDV. Isolation of BVDV from tissues and demonstration of viral antigen in tissues was more frequent in the type II BVDV-infected calves, as compared to BVDV TGAN-infected calves. This observation is compatible with the level of viremia results demonstrating higher levels of viremia in the type II BVDV-infected calves. In addition, the implications from this finding may be significant from a diagnostic standpoint, as it may be difficult to successfully identify calves acutely infected with type I BVDV.

The mechanism allowing for the type II isolates to achieve higher levels of viremia than the type I isolate BVDV TGAN is unknown, but may be the result of differences in RNA sequence. Comparison of nucleic acid sequences from type I and type II BVDV revealed a homology of less than 70% (Ridpath *et al.*, 1994). This dissimilarity is concentrated in the regions of the BVDV genome encoding for the structural glycoprotein E2 and the 5'untranslated region. The E2 glycoprotein is responsible for the viral attachment to cells (Xue & Minocha, 1993), while the 5'untranslated region contains the internal ribosome entry site element, which is responsible for the initiation of viral RNA translation (Poole *et al.*, 1995). Differences between type I and type II BVDV in the RNA sequence coding for the E2 glycoprotein may enable type II isolates to possess increased ability for cell attachment. Differences in the RNA sequence corresponding to the 5'untranslated region may give type II isolates an advantage in viral RNA translation. A higher level of viremia may be observed with either situation.

In summary, we have demonstrated a positive correlation between levels of viremia and manifestations of disease in cattle experimentally infected with type I and type II BVDV. In addition, severe peracute BVDV and the hemorrhagic syndrome may occur as a result of infection with isolates of BVDV capable of inducing high levels of viremia.

Sources and Manufacturers

a. Butler Company, Dublin, OH

b. Schering-Plough Animal Health Corporation, Kenilworth, NJ

c. Dr. Steve Bolin, National Animal Disease Center, Ames, IA

d. Dr. Clayton Kelling, University of Nebraska, Lincoln, NE

e. JRH Biosciences, Lenexa, KS

f. Sigma Chemical Co., St. Louis, MO

g. Gibco BRL, Life Technologies, Grand Island, NY

h. Becton Dickinson, Franklin, NJ

i. Technicon H-1 analyzer, Technicon Instrumental Corp., Tarrytown, NY

j. Amersham Pharmacia Biotech USA, Piscataway, NJ

k. National Veterinary Services Laboratory, Ames IA

l. Vortech Pharmaceuticals, Dearborn, MI

m. Columbia Diagnostics, Inc., Springfield, VA

n. Fischer Scientific, Pittsburgh, PA.

o. Dr. Ed Dubovi, Cornell University, Ithaca, NY

p. Vector Laboratories, San Francisco, CA

q. Dr. Y. M. Saif, Ohio Agricultural Research and Development Center, Wooster, OH.

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CHAPTER VI:

SUMMARY AND CONCLUSIONS

Some isolates of BVDV have been associated with severe clinical disease and a hemorrhagic syndrome characterized by thrombocytopenia. The experimental studies described in this dissertation were designed and conducted to provide insight into the mechanism of BVDV-induced thrombocytopenia and the pathogenesis of the hemorrhagic syndrome associated with BVDV infection.

Thrombocytopenia associated with type II BVDV infection has been reported under natural (Rebhun *et al.*, 1989) and experimental conditions (Bezek *et al.*, 1994; Bolin & Ridpath, 1992; Corapi *et al.*, 1990; Corapi *et al.*, 1989; Ellis *et al.*, 1998). Previous experimental studies on BVDV-induced thrombocytopenia considered principally the clinical and pathologic findings associated with BVDV-induced thrombocytopenia, but these previous studies failed to define the following:

1. What is/are the mechanism(s) of type II BVDV-induced thrombocytopenia?
2. Why are hemorrhages observed in experimentally infected calves, yet the platelet count is not decreased to a magnitude consistent with the occurrence of spontaneous hemorrhage?
3. Why are thrombocytopenia and severe clinical disease most often associated with infection of cattle with type II BVDV, and not type I BVDV. That is, what are the differences between a type I and type II BVDV that are responsible for the increased virulence observed during type II BVDV infections in cattle?

The focus of our first experimental study was to address the need for a reproducible model of BVDV-induced thrombocytopenia. This was important, as our subsequent pathogenesis studies needed to be based upon a reproducible model of type II BVDV-

induced thrombocytopenia. Previous studies failed to generate a reproducible experimental model because of several limitations. Several of the previous studies on BVDV-induced thrombocytopenia used an infection protocol that did not allow quantitation of the infectious dose of virus, which makes reproduction of their study difficult (Bezek *et al.*, 1994; Corapi *et al.*, 1990; Corapi *et al.*, 1989). Furthermore, several studies failed to use controls or failed to use age matched controls (Bolin & Ridpath, 1992; Corapi *et al.*, 1990; Corapi *et al.*, 1989). Control calves are critical in the evaluation of thrombocytopenia because of the variation in the normal platelet count (Nelson *et al.*, 1974; Semrad & Dubielzig, 1993); and age-matching is important as the platelet count varies between calves of different ages (Nelson *et al.*, 1974; Semrad & Dubielzig, 1993; Vaugher *et al.*, 1973). Finally, some investigations used type II BVDV isolates that failed to consistently induce thrombocytopenia in all infected calves (Corapi *et al.*, 1989; Ellis *et al.*, 1998).

In addition to developing a model, we also wanted to characterize the clinical, hematologic, virologic, and pathologic features of the thrombocytopenic BVDV infection in neonatal calves. Characterization of the above parameters in an experimental thrombocytopenic BVDV infection would provide information into the mechanism of BVDV-induced thrombocytopenia. Decreased mean platelet volume, which was reported for the first time in BVDV-infected calves in this study, and the presence of viral antigen in megakaryocytes provide support to the hypothesis that BVDV-induced thrombocytopenia is a result of decreased platelet production. In addition, normal coagulation profiles, which rules out disseminated intravascular coagulation, and an early induction of thrombocytopenia prior to the presence of neutralizing antibodies provide

evidence that BVDV-induced thrombocytopenia is not due to increased destruction of platelets in the peripheral circulation by an immune-mediated mechanism. Sequestration of platelets as the cause of BVDV-induced thrombocytopenia is not likely because splenomegaly and hepatomegaly were not observed on postmortem or histologic examination in BVDV-infected calves. Further study evaluating platelet turnover in the peripheral circulation and/or reticulated platelet assays are necessary to fully evaluate the decreased platelet production hypothesis.

The discrepancy between development of hemorrhage and platelet count suggested that platelet function might be altered in cattle infected with type II BVDV. Evaluation of platelet function in calves experimentally infected with the type II isolate BVDV 890 was the focus of the second experiment. Platelet function was depressed in calves experimentally infected with the type II isolate BVDV 890, when compared to sham-infected control calves. This was the first report describing altered platelet function in calves infected with BVDV. In addition, the depressed platelet function likely contributed to hemorrhaging, as bloody diarrhea was observed antemortem and hemorrhages were observed on postmortem examination in those BVDV 890-infected calves whose platelet function was assessed.

The hemorrhagic syndrome and outbreaks of severe disease have been associated with type II isolates of BVDV, and not type I BVDV. Altered platelet function may be an important contributing factor for the hemorrhagic syndrome and for the increased virulence observed with some type II BVDV isolates. The third experiment of this dissertation was designed to further evaluate the ability of BVDV isolates to alter platelet function, and to determine if platelet dysfunction occurs exclusively following challenge

with type II BVDV, as compared to type I BVDV. Platelet dysfunction was only observed in the type II BVDV-infected calves. Another important observation from this third experiment was that the effect of BVDV infection on platelet function was reproducible. Like the second experiment, similar depressions in platelet function were observed in this study utilizing a different group of BVDV 890-infected calves. Depressed platelet function may be an important factor for differences in virulence between type I and type II BVDV; however, further study evaluating other type I and type II isolates is necessary to determine if this observation remains valid.

Differences in virulence between type I and type II BVDV may be the result of differences in the level of viral replication. A difference in the level of viremia has been previously demonstrated between a type I and a type II BVDV infection (Bolin & Ridpath, 1992). This higher level of viral replication with type II BVDV may be important in the pathogenesis of severe peracute BVDV and the hemorrhagic syndrome. The purpose of the fourth and final experiment was to characterize the level of viremia induced during BVDV infection, and correlate levels of viremia with severity of clinical disease. A clear difference in the level of viremia induced during infection was demonstrated in calves infected with type I or type II BVDV. The level of viremia was also correlated to severity of clinical disease and pathology. The type I isolate BVDV TGAN induced the lowest level of viremia, and clinical signs of disease were less severe and depressions in platelet and leukocyte counts were milder when compared to the type II isolates, BVDV 7937 and BVDV 890. In addition, a parallel relationship between the level of viremia and rectal temperatures, and an inverse relationship between the level of viremia and platelet and leukocyte counts was observed, with the greatest elevations in

rectal temperature and greatest depressions in platelet and leukocyte counts being observed in calves infected with BVDV 890. The results of this study, indicating the level of viremia is correlated to disease manifestations, support the hypothesis that the level of viremia induced during BVDV infection is an important factor for differences in virulence among BVDV isolates. Isolates of BVDV that induce higher levels of viremia may be more capable of inducing clinical signs of disease. Severe peracute BVD and the hemorrhagic syndrome may occur as a result of infection with isolates of BVDV capable of inducing high levels of viremia.

Future studies in the area of BVDV-induced thrombocytopenia may now be initiated as a result of the findings described in this dissertation. Platelet turnover and quantification of reticulated platelets may determine the exact mechanism of type II BVDV-induced thrombocytopenia. The mechanism of altered platelet function in calves infected with type II BVDV may be determined through further evaluation of aggregation responses utilizing other agonists, or evaluating platelet secretion.

Future studies can also be directed at other related aspects of BVDV infection. Uncovering the molecular basis for the differences in the level of viremia between type I and type II BVDV isolates is an important future study. In addition, evaluating strategies, such as vaccination, that reduce viremia are essential, and these same strategies may be important in reducing clinical signs associated with BVDV infection. Moreover, limiting the level of viremia during acute BVDV infection may limit reproductive consequences of BVDV infection. This is critical because strategies designed to limit the level of viremia may also be important for preventing fetal infection with BVDV, and thus, eliminating the birth of persistently infected calves.

In summary, the studies encompassed by this dissertation contribute information on the pathogenesis of acute BVDV infections in general and BVDV-induced thrombocytopenia specifically. Three major findings have emerged from the studies described in this dissertation. First, decreased production of platelets may be the primary mechanism associated with BVDV-induced thrombocytopenia. Second, altered platelet function is important in the pathogenesis of the hemorrhagic syndrome. Finally, the ability to induce a high level of viremia is an important factor for differences in virulence between type I and type II BVDV.

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